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Investigating Xanthine Oxidase Toxicity Models in Cultured Cerebellar Granule Neurons

by

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Abstract

In the last few decades, evidence has been accumulating for a role for xanthine oxidoreductase (XOR)-generated toxic reactive oxygen species (ROS) in a variety of pathological conditions that affect different organ systems. This enzyme in mammals exists in two inter-convertible forms: xanthine dehydrogenase (XDH) (the predominant intracellular form under physiological conditions) and xanthine oxidase (XO). A combination of XO and its oxidizable substrate xanthine (X) (or hypoxanthine (HX)) is widely used as a model to produce ROS and to study their effects in a variety of cell culture studies. However, the effect of the combination of XOR and the reduced nicotinamide adenine dinucleotide (NADH) in cell cultures is much less studied. NADH is another oxidizable substrate for XOR that binds to a different site on the enzyme from that of X binding.

The aim of this project was to investigate some aspects of the in vitro toxicity of XOR, which might provide more insights into its in vivo toxicity. The main investigation was a comparison between the well studied X / XO and the much less studied NADH / XO toxicity models. Also, secondary studies were undertaken to investigate those aspects of X / XO toxicity where there are uncertainties about them.

These studies were performed using primary cell cultures. Cell cultures are now widely used to study different diseases, and although they have their drawbacks, they have their advantages over the in vivo studies. For this project, primary cultures of cerebellar granule neurons (CGNs) were used. In the beginning, some problems were encountered with CGNs. The main problem was the immediate damage induced to the neurons (including those in the control groups) at the intervention/experiments day (i.e. day 8 or 9 after plating) by manipulating the cultures (i.e. aspirating the culture medium, adding treatment and control vehicles, and adding the restoration medium).

After several months of investigation, it was serendipitously discovered that the immediate damage seen in the neurons (including those in the control groups) when they are manipulated at the experiments/intervention day was due to glutamate excitotoxicity (through activating its N-methyl-D-aspartate (NMDA) receptors). The source of glutamate was the fresh serum which is present at 10% V/V in the fresh

culture medium that is added to the cultures at that day. After solving this problem, it was possible to conduct reliable experiments to investigate XO toxicity models.

Regarding investigating XO toxicity, it was found that both of the X / XO and NADH / XO combinations were toxic to cultures of CGNs. However, the concentration of NADH needed to cause the toxicity was much higher than that of the other substrate, X, which is in agreement with previous cell-free experiments that showed that NADH is a much weaker substrate than X for the bovine milk XO used here. Blocking the site of X binding on XO prevented X / XO toxicity, but did not prevent NADH / XO toxicity. On the other hand, blocking the site of NADH binding prevented both X / XO and NADH /XO toxicities. Another difference between the two systems was that deactivating either superoxide or hydrogen peroxide (both are ROS) generated by XO prevented NADH / XO toxicity, whereas although deactivating hydrogen peroxide prevented X / XO toxicity, deactivating superoxide generated from this combination did not. In the NADH / XO system, an extracellular metal contaminant (likely contaminating XO powder/preparation) seemed to be involved in the toxicity. The two toxicity models were similar in the mediation of toxicity by intracellular iron ion. In X / XO toxicity, although superoxide generated extracellularly from the combination has no role in the toxicity, intracellularly produced superoxide seemed to play a role.

Conclusions:

1. Culturing/experimental conditions have been optimised for viability studies in CGNs cultures.
2. The combination of NADH and XO induces damage to CGNs, where although blocking the NADH binding site prevents this damage, blocking the X binding site does not. It is feasible that the oxidation of NADH by some forms of XOR (other than the one used here) that are known to be very efficient in oxidizing NADH might produce in vivo toxicity.
3. A possibility raised by this study is that a metal (like the metal contaminant proposed to play a role in NADH / XO toxicity in this study) might contribute to XOR toxicity in vivo.
4. Intracellular superoxide often mediates XOR toxicity.
5. The results add support to many previous studies which suggested that intracellular hydroxyl radical (or a similar species) is involved in XOR toxicity.

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Author's declaration

I, Majed A AL-Gonaiah, declare that this thesis was composed by myself, and also that the experiments described therein were performed by myself, except where referenced.

Majed A AL-Gonaiah

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Abbreviations	
AIF	Apoptosis-inducing factor
ALS	Amyotrophic lateral sclerosis
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
Cat.	Catalase
CGNs	Cerebellar granule neurons
cm^3	Cubic centimetre
CNS	Central nervous system
CO_2	Carbon dioxide
Cu,Zn-SOD	Copper,Zinc-Superoxide dismutase
Cyt c	Cytochrome c
DCF	2′,7′-dichlorofluorescein
DCFDA	2′,7′-dichlorodihydrofluorescein diacetate
DCFH	2′,7′-dichlorodihydrofluorescein
DDC	Diethyldithiocarbamate
deferox.	Deferoxamine
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate-Buffered Saline
DPI	Diphenyleneiodonium
EDTA	Ethylenediaminetetraacetate
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
Fe^{2+}	Ferrous (reduced iron)
Fe^{3+}	Ferric (oxidized iron)
Fig.	Figure
HCO_3-	Bicarbonate
HEPES	N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
3-HK	3-Hydroxykynurenine
H_2O_2	Hydrogen peroxide
hr	Hour

HX	Hypoxanthine
K^+	Potassium
KGDHC	α-ketoglutarate dehydrogenase
LDH	Lactate dehydrogenase
L-NAME	NG-nitro-L-arginine methyl ester
MEM	Minimum Essential Medium
mg	Milligram
µg	Microgram
ml	Millilitre
µl	Microlitre
mm^3	Cubic millimetre
µm	Micometre
mM	Millimolar
µM	Micromolar
mmHg	Millimetre of mercury
Mn-SOD	Manganese-Superoxide dismutase
MnTBAP	Manganese (III) Tetrakis (4-Benzoic Acid) Porphyrin chloride
Mo	Molybdenum
MPT	Mitochondrial permeability transition
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
Na^+	Sodium
NAD^+	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
nm	Nanometre
nM	Nanomolar
NMDA	N-methyl-D-aspartate
NOS	Nitric oxide synthase
NSAIDs	Non-steroidal anti-inflammatory drugs
$O_2^{\cdot-}$	Superoxide
OD	Optical density
$OH^{\cdot}$	Hyroxyl radical

PCD	Programmed cell death
POBN	α-(4-Pyridyl N-oxide)-N-tert-butylnitrone
ROS	Reactive oxygen species
RPM	Rounds per minute
S.E.M	Standard error of mean
SHR	Spontaneously hypertensive rats
SNAP	S-Nitroso-N-acetyl-DL-penicillamine
SOD	Superoxide dismutase
SOD-1	Superoxide dismutase-1 (Copper,Zinc-Superoxide dismutase)
Tiron	4,5-Dihydroxy-1,3-benzenedisulfonic acid
TRP	Transient receptor potential
V/V	Volume/Volume
X	Xanthine
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
XOR	Xanthine oxidoreductase
XTT	2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt

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1 Introduction

1.1 Reactive oxygen species and disease

Oxygen consumption by the body is safely handled to produce useful products, mainly energy in the form of adenosine triphosphate (ATP). Un-needed extra-products e.g. excess carbon dioxide can be detoxified. Also, the consumption of oxygen produces potentially toxic metabolites called reactive oxygen species (ROS). These ROS are kept under low levels (by naturally occurring antioxidants) in normal situations and some of these ROS do even exert physiological roles. Examples of ROS include hydrogen peroxide, superoxide, hydroxyl radical, nitric oxide, and peroxynitrite. When there is an overproduction of ROS and/or deficiency in antioxidant mechanisms, a damaging event called oxidative stress ensues [*Turrens, 2003, Halliwell, 2006, Halliwell and Whiteman, 2004, Fatokun et al., 2008a*].

ROS can be generated in vivo by many sources. Superoxide is generated in the mitochondria, at more than one site in the respiratory chain, and as a product of some other mitochondrial enzymes e.g. the enzyme complex alpha-ketoglutarate dehydrogenase [*Turrens, 2003, Starkov et al., 2004*]. Other cellular sources of superoxide include cytochrome P450-dependent oxygenases [*Turrens, 2003*]. Another important source of superoxide is the enzyme NADPH-oxidase [*Turrens, 2003*], which is expressed largely in some immune system cells (e.g. macrophages), where although ROS released by these immune cells are meant to kill invading microorganisms, they may also insult nearby host tissues. Also, another important in vivo source of superoxide is the enzyme xanthine oxidoreductase (XOR) [*Harrison, 2002*]. Hydrogen peroxide can be produced by the dismutation of superoxide, where this dismutation can be either spontaneous or through the action of the antioxidant enzyme superoxide dismutase (SOD) [*Turrens, 2003, Starkov et al., 2004, Fridovich, 2004*]. Hydrogen peroxide can also be produced directly by some enzymes e.g. XOR [*Harrison, 2002, Fridovich, 1970*]. Hydrogen peroxide can be converted, through interaction with a reduced metal ion e.g. iron or copper, to the very reactive and toxic hydroxyl radical [*Fridovich, 1998, Turrens, 2003*]. Nitric oxide is produced by nitric oxide synthases (NOSs), where the powerful oxidant

molecule peroxynitrite is produced by reaction of nitric oxide with superoxide [*Guzik et al., 2003, Turrens, 2003*].

Many ROS are free radicals i.e. have an unpaired electron, so they seek to attract an electron from (or donate their unpaired electron to) a non-radical biological molecule (e.g. a fatty acid, a protein, or a DNA molecule) to be chemically stable, rendering the attacked molecule with an unpaired electron (i.e. a new free radical) which can in turn attack another non-radical biological molecule and so on, which can lead to the destruction of cellular components [*Halliwell, 2006*]. Not all ROS are free radicals, and some important ROS that are not free radicals include hydrogen peroxide and peroxynitrite, which can exert their toxic effects either directly or through their conversion to free radicals.

There are numerous antioxidant mechanisms which deactivate ROS in vivo. These include antioxidant molecules (small and large) e.g. glutathione, thioredoxin, alpha-tocopherol (vitamin E), ascorbic acid (vitamin C), and coenzyme Q. Antioxidants also include enzymes e.g. SOD (which deactivates superoxide by converting two molecules of it into one molecule of hydrogen peroxide plus oxygen), catalase (which deactivates hydrogen peroxide by converting it into oxygen and water), and glutathione peroxidase (which also deactivates hydrogen peroxide) [*Halliwell, 2006, Fridovich, 1998, Turrens, 2003*]. Antioxidants may also include proteins e.g. albumin which can work as antioxidants in the circulation [*Roche et al., 2008*] and neuroglubin which may work as antioxidant in the brain [*Garry and Mammen, 2003, Wang et al., 2008*].

Body tissues differ in their vulnerability to oxidative stress. For example, brain is particularly vulnerable to oxidative stress. It contains an elevated amount of iron (see later the role of metals in oxidative stress), and consumes a high amount of oxygen. Also, the brain contains a high amount of unsaturated fatty acids which can be easily attacked by free radicals. The major excitatory neurotransmitter in the brain, glutamate, when present in excessive amounts at the synaptic cleft, can elicit damage through stimulating postsynaptic intracellular generation of ROS [*Halliwell, 2006, Patel et al., 1996*]. Moreover, mature neurons do not divide, and hence neuronal tissues may not be able to undergo repair/regeneration when damaged by ROS.

Countless number of pre-clinical studies, both in vivo and in vitro, has shown that antioxidant interventions do attenuate the damage observed in models of some of those human diseases where signs of oxidative stress are observed in the patients affected [*Behl et al., 1994, Carney and Floyd, 1991, Cherny et al., 2001, Przedborski et al., 1992, Pong et al., 2000, Eliasson et al., 1999, Ayata et al., 1997, van der Worp et al., 1999, Hantraye et al., 1996, Moussaoui et al., 2000*] (see also the section on XOR). On the other hand, clinical trials of antioxidants in the treatment of the relevant diseases were much less successful than the pre-clinical studies [*Willcox et al., 2008, Canter et al., 2007, Halliwell, 2006*].

So, why have many clinical studies failed? We can only speculate, and only some explanations will be discussed here. There are many differences between humans and animals in the patho-physiology of the relevant diseases. However, it is unlikely that oxidative stress plays a central role in some diseases like brain or heart ischemic diseases in animals while it has no role at all in those diseases in humans, for many reasons. One reason is that not all clinical trials have failed, where many clinical trials, small and large, with antioxidants in the treatment of the relevant diseases showed positive results [*Behr et al., 1997, Demedts et al., 2005, Tomioka et al., 2005, Stephens et al., 1996, Angstwurm et al., 2007, Boaz et al., 2000, Fang et al., 2002, Thies et al., 1998, Weigand et al.,2001, Cerwenka et al., 1999, Murray et al., 2008, Milman et al., 2008, Hager et al., 2007, Sanders et al., 2007, Di Prospero et al., 2007, Plantinga et al., 2007, Yamaguchi et al., 1998*] (see also the section on XOR). Also, oxidative stress is not alone in the failure in clinical trials. For examples, although some other damaging events (e.g. glutamate receptor activation and disruption of calcium homeostasis) were very evidently shown to play a major role in acute ischemic brain diseases in animal models, many clinical trials directed against these damaging events have failed [*Ginsberg, 2008*].

Also, in animals, a closer look at (or manipulation of) oxidative stress (almost direct intervention) can be achieved e.g. in animals it is possible to knock out or over-express an antioxidant or a pro-oxidant gene, but this is not possible in humans. Also, many clinical trials have tried direct free radical scavengers, where although this is perhaps the most feasible way in the clinical situation, it is not the best way to treat oxidative stress. The reason is that a free radical is generally non selective in its reactions, and to scavenge it, a scavenger needs to be applied in a very high concentration in order to outcompete the many biological vulnerable targets (i.e. scavengers) of the free radical. For example, dimethyl sulfoxide (DMSO), a classical hydroxyl radical scavenger, when

applied in a cell-free system at 1 mM in the presence of only one competing hydroxyl radical scavenger (mannitol, 10 mM), can scavenge only 50% of the hydroxyl radicals that it can scavenge in the absence of mannitol [*Babbs and Griffin, 1989*] (though the case with other ROS may not be as bad as the case with hydroxyl radical). A better way is to prevent the generation of ROS.

Moreover, we like to mention the possibility that some researchers tend not to publish their results if they were negative in the pre-clinical studies. Unfortunately, this in our opinion is possibly due to a less appreciated environment in the academic journals of negative results compared to positive results. We were enlightened to know that some others in the scientific community share our opinion [*Knight, 2003, Rockwell et al., 2006*]. On the other hand, in clinical trials, although there is evidence for bias against negative results [*Rockwell et al., 2006*], we believe that (we might be wrong) it is not as bad as with pre-clinical studies. A possible reason for the appreciation of negative results in clinical studies is that large trials are announced/registered from the outset of the study, and hence the results have to be announced as well. This might give too great an impression that clinical trials have failed more than pre-clinical studies. A lot is unknown about bias against negative results, which demands a systematic investigation, which has already been started in clinical studies literature, but rarely done in pre-clinical studies literature.

Finally, some authors have discussed the possibility that many previous clinical trials with antioxidants were not well designed e.g. lower than optimal dose with no dose response curve, short duration of the study (or inappropriate timing of intervention), no carful selection of the antioxidant intervention, or no careful selection of the study population [*Willcox et al., 2008, Ginsberg, 2008*]. Anyway, the future will indeed bring the true explanation(s) to the light.

1.2 Interplay between oxidative stress and other damaging events

The damage observed in pathologic conditions where signs of oxidative stress are observed usually involves damage cascades, where many damaging events including oxidative stress can trigger each other. These include: energy depletion, mitochondrial

dysfunction, disruption of calcium homeostasis, metal accumulation, inflammation, oxidative stress, and (in the brain) glutamate receptor-mediated excitation (see later). In this section, some examples of the toxic interplay between oxidative stress and the other damaging events will be discussed. More examples will also be encountered in the section on XOR.

1.2.1 Mitochondrial dysfunction

Mitochondrial dysfunction is thought to be an early event in the damage observed in acute ischemic models of many diseases e.g. myocardial infarction and stroke, and also of many chronic neurodegenerative diseases [*Chinopoulos and Adam-Vizi, 2006, Starkov et al., 2004, Halliwell, 2006, Turrens, 2003, Keating, 2008, Moro et al., 2005, Lesnefsky et al., 2001*]. In acute ischemic disease models, the ischemic phase is thought to induce defects in the mitochondria which can lead to, upon reperfusion (i.e. re-delivery of oxygen to mitochondria), ROS generation at more than one site in the respiratory chain, and also at other sites in the mitochondria e.g. the enzyme complex: α-ketoglutarate dehydrogenase complex (KGDHC) [*Chinopoulos and Adam-Vizi, 2006, Starkov et al., 2004, Lesnefsky et al., 2001*]. The KGDHC complex is also a vulnerable target for ROS, whose deactivation will deactivate the Krebs cycle. Interestingly, deactivated KGDHC is a common feature of many neurodegenerative diseases [*Halliwell, 2006*].

1.2.2 Glutamate

In the brain, the major excitatory neurotransmitter, glutamate, when present in excess amount at the synaptic cleft (as in stroke), can elicit damage through stimulating its postsynaptic receptors leading to intracellular generation of toxic ROS [*Reynolds and Hastings, 1995, Dawson et al., 1993, Araújo et al., 2004, Carriedo et al., 1998, Dawson et al., 1996, Patel et al., 1996, Lafon-Cazal et al., 1993*]. ROS can exacerbate the damage by blocking glutamate uptake into the cells (i.e. blocking its clearance from the synaptic cleft) [*Trotti et al., 1996*]. Also it was shown in neuronal cultures that glutamate, when present extracellularly (≥ 300 μM), can induce intracellular oxidative stress through a glutamate receptor-independent mechanism, which is the inhibition of the uptake of cystine, a precursor involved in the synthesis of the universal antioxidant glutathione [*Murphy et al., 1989, Murphy et al., 1990*].

1.2.3 Disruption of Ca^{2+} homeostasis

Disruption of Ca^{2+} homeostasis is a central player in the damage cascade observed in many pathological conditions that affect different organ systems. This disruption in Ca^{2+} homeostasis can be induced by disruption of the ATP-dependent plasma membrane Na^+/K^+ ATPase pump or by other mechanisms [*Inserte et al., 2005*]. In the brain, this can also be induced by glutamate-receptor activation. Increased intracellular levels of Ca^{2+} can activate some ROS generating enzymes e.g. NOS. Also, ROS can activate a lethal atypical type of Ca^{2+} and cation currents across the cell membrane called non-selective cation currents. These currents are likely mediated by a member(s) of atypical cation channels permeable to Ca^{2+} on the cell membrane called transient receptor potential (TRP) cation channels [*Chinopoulos and Adam-Vizi, 2006, Aarts et al., 2003*]. In a neuronal culture study that used prolonged oxygen-glucose deprivation as a toxicity model, it was shown that Ca^{2+} influx and the subsequent cell damage was not blocked by treatment with typical calcium channels blockers e.g. glutamate ionotropic receptor blockers or an L-type Ca^{2+} channel blocker, but was blocked by treatment with either some ROS suppressors or by blocking the above mentioned atypical cation channels [*Aarts et al., 2003*]. Another example of the harmful augmentation between Ca^{2+} and ROS is that Ca^{2+} is shown to accumulate inside the mitochondria under an ischemic insult [*Babcock et al., 1997, Herrington et al., 1996, Zaidan and Sims, 1994*], and participates with ROS in the opening of the so-called mitochondrial permeability transition (MPT) pore, initiating many damaging events [*Chinopoulos and Adam-Vizi, 2006*].

1.2.4 Inflammation

Toxic interplay is observed between ROS and some components of inflammation in many pathological conditions in different organ systems, including the brain. For example, A-beta (a peptide whose aggregation is observed in patients with Alzheimer's disease) stimulated the microglia (which are considered resident macrophages in the brain) in vitro to produce nitric oxide [*Ii et al., 1996*]. In the same study, the pro-inflammatory molecule, interferon-gamma, augmented A-beta in activating the microglia to produce nitric oxide, where A-beta alone or in combination with interferon-gamma, in the presence of microglia, caused toxicity to co-cultured neurons that was inhibited by a NOS inhibitor. Interestingly also in the same study, the production of nitric oxide by microglia activated by A-beta (and interferon-gamma) was inhibited by

aspirin and indomethacin (members of the non-steroidal anti-inflammatory drugs (NSAIDs)). A-beta used at a lower concentration than that used in the above study, caused toxicity in a mixed culture of neurons and microglia that was mediated by superoxide produced by NADPH-oxidase located in the microglia [*Qin et al., 2002*]. In a cell culture model of Parkinson's disease, it was also observed that the presence of microglia in a neuronal culture, again through producing superoxide by the NADPH-oxidase located in the microglia, very significantly enhanced the observed damage to the dopaminergic neurons [*Gao et al., 2002*].

1.2.5 Metals

It is known that, at least in some pathological conditions, traces of reactive metals are present in vivo either free or bound (chelated) to molecules/proteins, where this binding may not prevent the reactivity of these metals (actually it may enhance their reactivity/toxicity in some situations) [*Graf et al., 1984, Hallaway et al., 1989, Engelmann et al., 2003, Gutteridge, 1987, Sayre et al., 1999, Ong and Halliwell, 2004, Thompson et al., 2001, Darley-Usmar and Halliwell, 1996, Halliwell and Gutteridge, 1992, Liochev, 1996, Halliwell, 2006*]. In many cases, these metals exert their toxicity through some sort of a reaction with ROS. As mentioned earlier, some metals (usually iron or copper) can react with hydrogen peroxide to produce the very reactive and toxic hydroxyl radical. In a previous study, it was shown that hydrogen peroxide added to cultures of hepatocytes exerted an intracellular toxicity that was mediated by both intracellular iron and intracellularly generated superoxide [*Starke and Farber, 1985*]. Superoxide or peroxynitrite can deactivate some enzymes through interacting with their iron clusters. In this process, in addition to the deactivation of these enzymes, iron is released from the clusters in a reactive form capable of causing oxidative stress. Superoxide can also release iron in a reactive form from the storage protein ferritin. Also, peroxynitrite can release copper in a reactive form from the plasma protein caeruloplasmin. Hydrogen peroxide can degrade haem proteins, which results in the release of iron from them in a reactive form [*Liochev, 1996, Darley-Usmar and Halliwell, 1996*]. To mention an example of the diseases affected by ROS and metals interplay, it was discussed that a toxic interaction between some metals and some ROS might play a role in atherosclerosis [*Darley-Usmar and Halliwell, 1996, Ong and Halliwell, 2004*].

1.3 Fate of cells damaged by ROS

The fate of cells insulted by lethal amounts of ROS (or in disease models where ROS are secondarily produced) is said to be an eventual death induced through either apoptosis or necrosis. Apoptosis is a term used to describe the programmed, cascaded, controlled, active, and 'gentle' events that lead to cell death. In contrast, the acute, accidental, passive, and uncontrolled cell death with cell membrane rupture is termed necrosis [*McHugh and Turina, 2006, Chandra et al., 2000, Samali et al., 1999*]. The distinction between apoptosis and necrosis is vague, which is manifested in the attempts by some researchers to classify the mode of cell death into necrosis and programmed cell death (PCD), and then classifying PCD further into classical apoptosis, apoptosis-like PCD, and necrosis-like PCD [*Krantic et al., 2005*]. Although this classification might turn out to be correct, it is also possible that there might be a spectrum of cell death signals rather than just apoptosis and necrosis (and even rather than just PCD and necrosis). So these obscurities should be considered during reading the following discussion of apoptosis and necrosis. Apoptosis is usually achieved by the activation of several protease families, with caspases being the most prominent among them [*Chandra et al., 2000*].

It seems that mitochondria play an important role in triggering apoptosis and even necrosis [*Bras et al., 2005*]. Toxic ROS can release Cytochrome c from the mitochondria into the cytoplasm, where it can activate caspases there. ROS can also release a protein called apoptosis-inducing factor (AIF) from the mitochondria into the cytoplasm where it can induce apoptosis through caspase-independent mechanisms (see below). Treatment of lymphocyte cultures with hydrogen peroxide resulted in the appearance of Cytochrome c in the cytoplasm within 2 hours. One hour later, caspase activation was observed [*Stridh et al., 1998*]. Matsura and co-workers (1999) found that, by using human promyelocytic leukemia HL-60 cells, caspase-3 (but not caspase-1) was responsible for the hydrogen peroxide-mediated apoptosis observed in their study.

In acute neuronal toxicity (e.g. in stroke), the severely insulted neurons may die through necrosis, while the other neurons that are less severely insulted may die through apoptosis [*Xu et al., 2006, Hou et al., 2008*]. Although apoptosis in the case of glutamate receptor-mediated acute excitotoxicity in neurons can be induced by caspase activation, it seems that AIF is also a major trigger of apoptosis, where in this type of toxicity some

ROS are thought to be involved in the release of AIF from the mitochondria [*Cheung et al., 2005, Dawson and Dawson, 2004*]. Regarding chronic neurodegeneration, in an in vivo model of Parkinson's disease, it was proposed that cell death proceeds through apoptosis mediated by ROS-dependent AIF release from the mitochondria [*Wang et al., 2003*]. Regarding amyotrophic lateral sclerosis (ALS), an in vivo animal study showed that the cell death proceeds through an apoptotic pathway, where nitric oxide, superoxide, and possibly peroxynitrite play a crucial role, since NOS deficient mice or over-expression of SOD resulted in the protection against the observed apoptosis [*Martin et al., 2005*].

The interaction between oxidative stress and apoptosis is complex. Logically, since ROS exert their toxicity through attacking biological molecules, there is no reason for the 'active' enzymes that induce apoptosis not to be 'inactivated' by direct attack of ROS. In cultured hepatic HepG2 cells, it was observed that treatment with menadione, a toxic compound known to exert its toxicity through producing ROS (especially superoxide and hydrogen peroxide), treated at 250 μM for 6 hr killed the cells by necrosis, and did not activate caspases. In that study, adding menadione to cells undergoing apoptosis inhibited the apoptosis (and induced necrosis), where this anti-apoptotic effect of menadione was blocked by catalase (which deactivates hydrogen peroxide)! [*Samali et al., 1999*]. In another study that used lymphocytes it was shown that hydrogen peroxide at high concentrations was able to suppress the activation/activity of caspases possibly through oxidizing the cysteine residues on these enzymes, while at low concentrations hydrogen peroxide was able to activate caspases, suggesting that the level of ROS can determine the mode of cell death [*Hampton and Orrenius, 1997*]. In contrast to the above menadione study that used HepG2 cells, Sun and co-workers (1997) have shown that menadione, treated at 200 μM for 3 hr, induced apoptosis in cultured osteoblasts, suggesting that cell type might also be a determining factor. Also, using cell cultures, nitric oxide was shown to shift cell death from apoptosis to necrosis through an effect that might have involved S-nitrosylation (and hence inhibition) of the cysteine-containing apoptotic enzymes [*Melino et al., 1997*]. Nitric oxide was also shown to inhibit the apoptosis of Jurkat lymphoma cells by a mechanism different from S-nitrosylation of caspases, possibly involving an inhibition of mitochondrial synthesis of ATP, and hence inhibition of the energy-dependent release of apoptotic proteins from the mitochondria. Restoration of ATP levels by supplementation with glucose recovered the apoptotic ability of those cells. In that study, inhibiting the mitochondrial synthesis

of ATP by rotenone, an inhibitor of complex I of the respiratory chain, mimicked the effect of nitric oxide in inhibiting apoptosis and mediating necrosis [*Leist et al., 1999*].

From the above discussion, it seems that the exact conditions under which oxidative stress causes either apoptosis or necrosis need further investigation, though the concentration of ROS and the cell type seem to be determining factors. Also, it should be considered, as mentioned, that the distinction between apoptosis and necrosis is vague, and that there is possibly a spectrum of death signals rather than just apoptosis and necrosis.

1.4 Xanthine oxidoreductase (XOR)-generated ROS

In the last few decades, evidence has been accumulating for a role for xanthine oxidoreductase (XOR)-generated ROS in a variety of pathological conditions that affect different organ systems. Many examples will be mentioned later, but for a thorough reference, see [*Zweier et al., 1994, Brown et al., 1988, Harrison, 2002, Berry and Hare, 2004, Okuda et al., 1996, Wiezorek, 1994, Phan et al., 1989, Osarogiagbon et al., 2000, Thom, 1992, Terada et al., 1992a, Weinbroum et al., 1995, Nakazono et al.,1991, Jankov et al., 2008, Widmer et al., 2007, Ohta et al., 2007, Inkster et al., 2007, Castro-Gago et al., 2006, Flaishon et al., 2006, White et al., 1996, Schröder et al., 2006, Baldus et al., 2006, Nakai et al., 2006, Minhas et al., 2006, Zeki et al., 2002, Rieger et al., 2002, Desco et al., 2002, Saavedra et al., 2002, Kumagai et al., 2002, Matsumura et al., 1998, Beetsch et al., 1998, Suzuki et al., 1998, Lamarque and Whittle, 1995, Xia and Zweier, 1995, Terada et al., 1992b, Han et al., 2007, Pacher et al., 2006, Abramov et al., 2007*].

1.4.1 XOR structure and properties

The enzyme in mammals exists in two inter-convertible forms: xanthine dehydrogenase (XDH) (which is the predominant intracellular form under physiological conditions) and xanthine oxidase (XO). The enzyme is a homodimer (i.e. composed of two identical subunits), where each subunit works generally independently from the other, and thus it is strange that little investigation has been undertaken to reveal the reason(s) of the presence of two instead of one subunit. Each subunit contains three distinct parts/domains: a molybdenum (Mo) containing domain (contains one Mo atom), an iron-sulphur containing domain (contains four atoms of iron and four atoms of sulphur),

and a flavin adenine dinucleotide (FAD) containing domain (contains one FAD molecule) [*Harrison, 2002, Hille and Nishino, 1995, Berry and Hare, 2004*].

The conceived function of XOR is the conversion of hypoxanthine (HX) to xanthine (X), and X to uric acid, the final product of purine metabolism in humans [*Harrison, 2002*]. The enzyme couples the oxidation of X (or HX) to the reduction of either primarily NAD^+ or secondarily oxygen in the case of XDH, or the reduction of only oxygen in the case of XO (NAD^+ can not oxidize (i.e. can not be reduced by) XO). If oxygen is the oxidizing substrate, hydrogen peroxide and superoxide are directly produced (the enzyme here can be either XDH or XO), whereas NADH is produced if NAD^+ is the oxidizing substrate (the enzyme here will be only in the form of XDH) (Fig. 1-1) [*Harrison, 2002, Hille and Nishino, 1995, Berry and Hare, 2004, Fridovich, 1970, Olson et al., 1974*].

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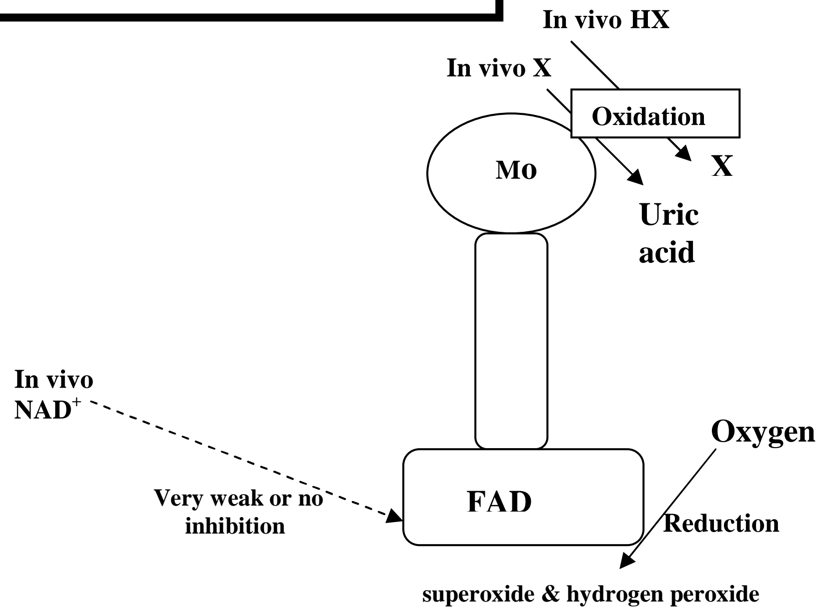
To add more complication to the picture, XOR can also oxidize NADH, and in this case, oxygen (not NAD^+) will always be the oxidizing substrate (this will generate hydrogen peroxide and superoxide directly) regardless of whether the enzyme is in the form of XDH or XO. Generation of ROS (i.e. superoxide and hydrogen peroxide) by the XDH form is strongly inhibited by NAD^+ (which is available in relatively high concentrations in cells) when oxidizing NADH or X. On the other hand, the generation of ROS by the form XO is less inhibited (compared to XDH) by NAD^+ when oxidizing NADH and even much less inhibited (compared to XDH) by NAD^+ when oxidizing X. The site of NADH binding, the FAD site, is different from that of X binding, the Mo site (Fig. 1-1) [*Harrison, 2002, Hille and Nishino, 1995, Berry and Hare, 2004, Gilbert, 1963, Landon and Myles, 1967, Rajagopalan and Handler, 1967, Nakamura et al., 1978, Harris and Massey, 1997, Maia et al., 2007, Sanders et al., 1997, Zhang et al., 1998*].

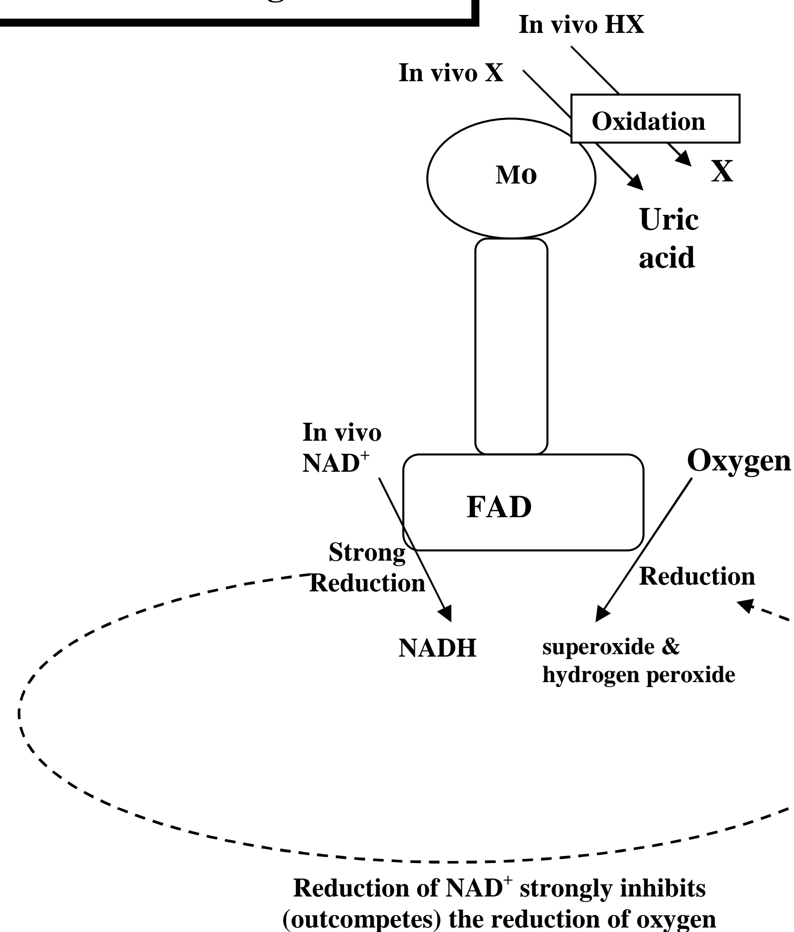
If the Mo site is removed/inhibited, the enzyme will of course not be able to oxidize X, but can still oxidize NADH and reduce oxygen leading to ROS generation. On the other hand, if the FAD site is removed/inhibited, the enzyme will of course not be able to oxidize NADH, but can still oxidize X (or HX) only in the presence of a suitable artificial oxidizing agent, but importantly not oxygen in this case, and hence no ROS will be generated [*Komai et al., 1969, Sanders et al., 1997, Olson et al., 1974, Nakamura, 1991, Berry and Hare, 2004, Harrison, 2002*]. In other words, in vivo, the FAD site on XOR is the site of oxygen reduction (and hence ROS generation) regardless of whether

the reducing substrate binds to the Mo site (i.e. X or HX) or to the FAD site (i.e. NADH) (Fig. 1.1).

The enzyme shows a striking variation in properties/functions among the different species, and also among the different organs in one species. For example, avian XOR is present only in the XDH form and does not undergo conversion to XO [*Landon and Myles, 1967*, *Harrison, 2002*]. Deficiency of XOR is fatal to mice, but not to humans [*Harrison, 2002*]. Surprisingly, some reports show that some human forms of XOR exhibits a much weaker X oxidase activity than the bovine XOR, although human XOR still keeps a potent NADH oxidase activity. Human milk XOR exhibits weaker X oxidase activity than human liver XOR [*Harrison, 2002, Sanders et al., 1997, Zhang et al., 1998*].

The enzyme exhibits an ability to oxidize, and to a lesser extent reduce, an unusually wide range of endogenous and artificial substrates [*Harrison, 2002, Berry and Hare, 2004*], which has left some researchers wondering if this enzyme has unknown important regularly functions, at least in some species. Relatively recently, the enzyme was shown to be able to reduce nitrate to nitrite, and nitrite to nitric oxide [*Harrison, 2002, Zhang et al., 1998, Millar et al., 1998, Li et al., 2004*]. The first thing that comes to the mind is that, as it was demonstrated, nitric oxide and superoxide that can be directly generated by XOR can join together to form the very reactive and oxidant molecule, peroxynitrite. Also, unlike NOS, XOR generation of nitric oxide can proceeds even under anaerobic conditions. This raises the possibility that, while NOS (which requires oxygen for its function) will fail to generate nitric oxide under pathological ischemic conditions, XOR will be able to do so, which might lead to nitric oxide-mediated beneficial or harmful effects in the vasculature and/or other tissues [*Harrison, 2002, Millar et al., 1998, Zhang et al., 1998, Li et al., 2004*]. The role of XOR in regulating nitric oxide system and other aspects of XOR structure and properties is now an active area of research. Although the research on XOR spans more than a century, the enzyme seems still to be hiding many unrevealed secrets.


XO action when X (or HX) is the available reducing substrate
A
In vivo HX
In vivo X
Oxidation
Mo
X
Uric acid
In vivo NAD+
Oxygen
FAD
Very weak or no inhibition
Reduction
superoxide & hydrogen peroxide


XDH action when X (or HX) is the available reducing substrate
B
In vivo HX
In vivo X
Oxidation
Mo
X
Uric acid
In vivo NAD+
FAD
Oxygen
Strong Reduction
Reduction
NADH
superoxide & hydrogen peroxide
Reduction of NAD+ strongly inhibits (outcompetes) the reduction of oxygen

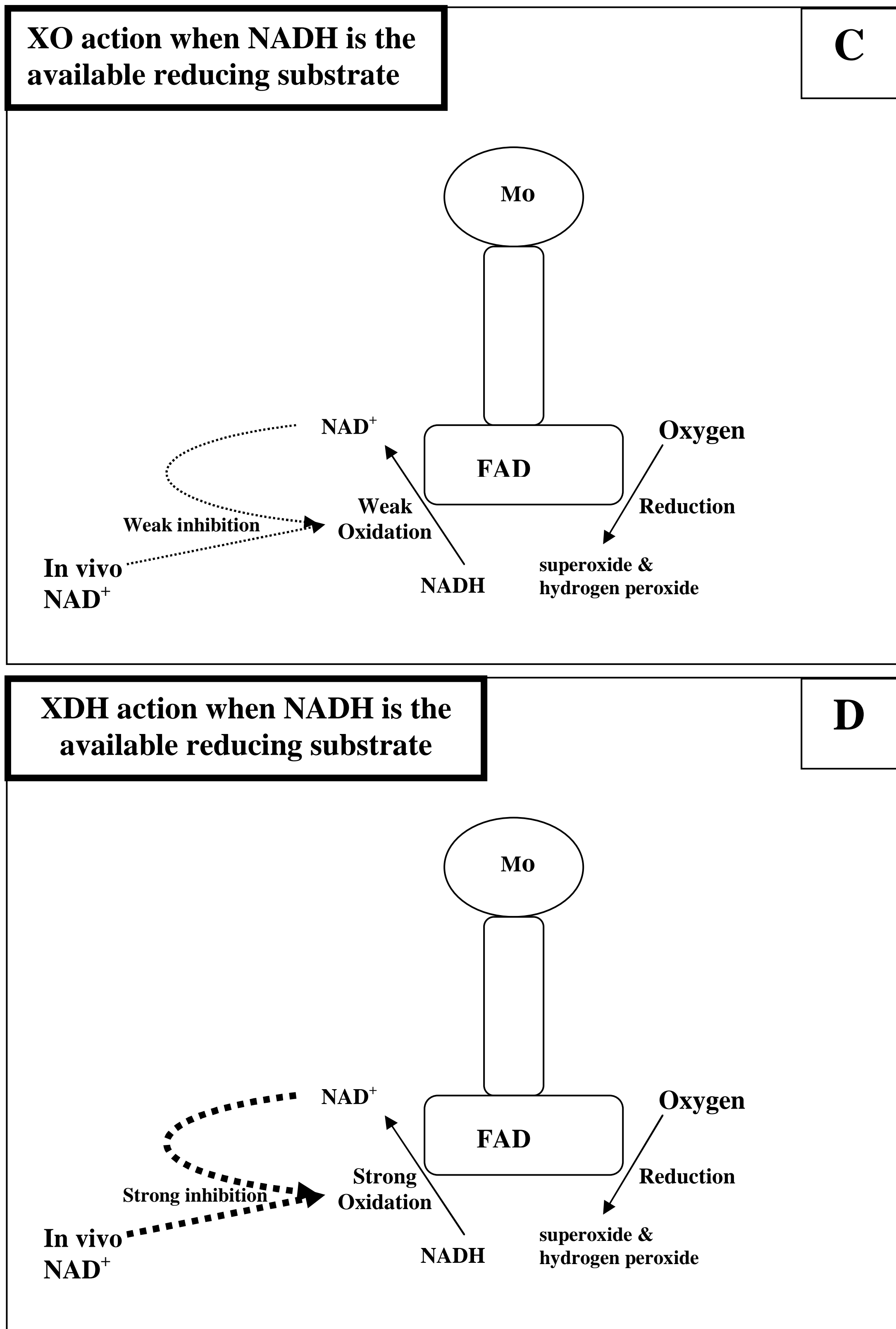


Figure 1-1: Expected action of in vivo XOR. **A:** action of XO when X (or HX) is the available reducing substrate. XO oxidizes X (or HX) at the Mo site and couples this oxidation to the reduction of oxygen at the FAD site. This process can be only very weakly inhibited by NAD^+. **B:** action of XDH when X (or HX) is the available reducing substrate. XDH oxidizes X (or HX) at the Mo site and couples this oxidation to the reduction of either primarily NAD^+ or secondarily oxygen at the FAD site. So the reduction of NAD^+ strongly inhibits (outcompetes) the reduction of oxygen, so ROS generation is inhibited. **C:** action of XO when NADH is the available reducing substrate. XO oxidizes NADH at the FAD site, and couples this oxidation to the reduction of oxygen at also the FAD site. Although XO weakly oxidizes NADH, this oxidation might be significant because NAD^+ only weakly inhibits this oxidation. **D:** action of XDH when NADH is the available reducing substrate. XDH oxidizes NADH at the FAD site, and couples this oxidation to the reduction of oxygen at also the FAD site. Although XDH strongly oxidizes NADH, this oxidation might be insignificant because NAD^+ (which is present in relatively high concentrations in cell) strongly inhibits this oxidation.

The enzyme distributes unevenly throughout the body organs. It is concentrated in the liver and intestine [*Harrison, 2002, Berry and Hare, 2004*]. Brain and heart, especially in humans, contain a minute amount of the enzyme based on a whole organ purification [*Harrison, 2002, Berry and Hare, 2004*]. However, further investigation showed that XOR is present at high levels in sub-localizations in the brain and heart. For example, endothelial cells of the cardiac and cerebral vasculature contain a significant amount of XOR [*Betz, 1985, Harrison, 2002, Berry and Hare, 2004, Zweier et al., 1994, Terada et al., 1991a*]. The enzyme is also present in the circulation in an active form (see later). Intracellularly, the enzyme is localized in the cytoplasm, and in possibly some sub-cellular organelles, but not in the mitochondria [*Berry and Hare, 2004*].

Since XO accepts electrons from X (or HX) and can then only transfer them to molecular oxygen producing ROS, whereas XDH can transfer the electrons to either primarily NAD^+ or secondarily molecular oxygen producing either primarily NADH or secondarily ROS, the in vivo intracellular conversion of XDH to XO was thought to be required for the toxicity of the enzyme [*Harrison, 2002*]. Xanthine dehydrogenase (XDH) was shown to be converted to XO in cells (or in tissues) under some pathological conditions [*Wiezorek, 1994, Phan et al., 1989, Osarogiagbon et al., 2000, Thom, 1992, Schröder et al., 2006, Ischiropoulos et al., 1996, Park et al., 1998*]. However, in many other cases, this conversion was shown to be either absent or too slow/too small to account for the observed tissue damage [*Harrison, 2002, Xia and Zweier, 1995, Terada et al., 1992b, Cighetti et al., 1990, Mink et al., 1990, Marubayashi 1991, Betz et al., 1991, Frederiks and Bosch, 1996, Kooij et al., 1994, Battelli et al., 1998*]. It can be argued, however, that this conversion may not be necessary for the toxicity of the enzyme for two reasons. First, if a pathological condition increases the activity/expression of the total enzyme (XDH + XO), as it was shown in some conditions, then the XO activity/expression will also increase in parallel even in the absence of a significant conversion from XDH to XO. Secondly, it was shown that XDH is also capable of producing significant amount of ROS. It should be remember that if NADH is the reducing substrate, the enzyme can only transfer the electrons to molecular oxygen (not to NAD^+), and hence ROS will be generated regardless of whether the enzyme is in the form of XO or XDH. However, although XDH is indeed more efficient than XO in oxidizing NADH, its generation of ROS is strongly inhibited by NAD^+ (which is available in relatively high concentrations in cells) when oxidizing NADH or X. On the other hand, the generation of ROS by XO is less inhibited (compared to XDH) by

NAD^+ when oxidizing NADH and even much less inhibited (compared to XDH) by NAD^+ when oxidizing X (see above in Fig. 1-1) [*Maia et al., 2007, Sanders et al., 1997, Zhang et al., 1998, Hille and Nishino, 1995, Harris et al., 1999, Maia et al., 2005*].

An important observation is that the enzyme is present in the circulation in an active form under normal conditions and gets even much increased (it can increase several hundred-fold) under some acute pathological states. In both cases the enzyme was found in the circulation to be largely in the form of XO or, under some cases of organ damage, was found to leak to the circulation largely in the form of XDH and then gets rapidly converted to XO [*Harrison, 2002, Terada et al., 1992a, White et al., 1996, Kooij et al., 1994, Friedl et al., 1990, Tan et al., 1995, McHale et al., 1979*]. From the discussion in this and the previous paragraphs, it does not seem clear which isoform of the enzyme will be more injurious under pathological conditions when oxidizing X (or HX) or especially NADH. Regardless of the isoform, and as we mentioned before (also see later), the evidence points out to the responsibility of the enzyme for many pathological conditions.

There are many fairly specific blockers of the molybdenum (Mo) site on XOR. On the other hand, to date, there are still no specific blockers of the FAD site on the enzyme. Although diphenyleneiodonium (DPI) can block the FAD site on XOR, it can also inhibit many other enzymes [*Harrison, 2002, Pacher et al., 2006*]. The two classical blockers of the Mo site on XOR are allopurinol and oxypurinol. They have been used clinically for decades to treat hyperuricemia-related disorders. Besides their blockade of XOR, these two compounds exhibit some other nonspecific activities e.g. scavenging hydroxyl radical and/or chelating copper ions [*Pacher et al., 2006, Ko and Godin, 1990, Lapenna et al., 1997, Moorhouse et al., 1987, Malkiel et al., 1993*]. From a clinical perspective, although these two compounds have fairly good tolerability, they have some unpleasant adverse effects e.g. allergy and, in patients with renal impairment, renal toxicity [*Pacher et al., 2006*]. Since the discovery of allopurinol and oxypurinol, there has been a quest for more selective and better XOR blockers, which resulted in the development of generations of XOR blockers. One of the newly developed and very selective blockers of the Mo site on the enzyme is febuxostat, which has already entered many clinical trials [*Pacher et al., 2006*].

Another elegant way to inhibit the Mo site on the enzyme that has been used frequently in in vivo animal studies is through feeding the animals with tungsten, which results

with time in the incorporation of tungsten instead of molybdenum at the Mo site on the enzyme, rendering it inactive (see later). However, in cases when tungsten was the only used way to inhibit XOR, it can not be ruled out that a protective effect of tungsten treatment was due to inhibiting enzymes other than XOR that have a molybdenum atom at their active sites e.g. aldehyde oxidase and sulfite oxidase (see later).

Like many other enzymes, XOR can be deactivated/inhibited by some (if not all) of its products: uric acid, NAD^+, NADH, or especially ROS [*Tan et al., 1993, Terada et al., 1991b, Sanders et al., 1997, Landon and Myles, 1967*]. This can complicate the interpretation of results of treatments that interfere with XOR. A theoretical example is that if scavenging ROS showed protection against a disease model where XOR involvement was suspected, then although these scavengers would appear to have been protective through preventing toxic effects of XOR-generated ROS, they might have actually been protective through blocking the deactivation of XOR by its produced ROS. This would result in the continuation of the concomitant production of uric acid and its mediation of an unanticipated protective effect against that disease model (uric acid is known to have some beneficial as well as harmful effects, see later).

1.4.2 Role of XOR-generated ROS in disease

In studies relating to cardiovascular system, Zweier and co-workers (1994) showed that subjecting cultured human aortic endothelial cells to anoxia resulted in severe damage (after reoxygenation) and intense production of free radicals that was prevented in the presence oxypurinol. Although in their study XOR level did not change during the insult, the concentrations of its substrate (HX) and its product (uric acid) increased sharply after the anoxia. This sharp rise in HX was paralleled with a sharp decrease in ATP concentration, which suggests that the source of the accumulated HX was the breakdown of ATP pathway. This can be considered as an example of toxic augmentation between energy depletion and oxidative stress. Brown and co-workers (1988), using isolated rat heart, found that inhibiting XOR by either feeding rats (before isolating the heart) with tungsten or infusing the isolated heart with allopurinol led to the attenuation of ventricular dysfunction induced by ischemia-reperfusion injury. Phan and co-workers (1989) have shown that, in cultures of rat pulmonary artery endothelial cells, the use of any of three different inhibitors of XOR (allopurinol, oxypurinol, or

lodoxamide) attenuated the damage induced by adding activated neutrophils, an example of a toxic augmentation between oxidative stress and inflammatory mediators.

In an in vivo study, Nakazono and co-workers (1991) showed that XOR has a role in increased blood pressure in spontaneously hypertensive rats (SHR), since oxypurinol (as well as a modified form of SOD) decreased the blood pressure in those rats. Although in that study the level of XOR was the same in SHR and normal rats, the levels of plasma uric acid was higher in SHR rats, suggesting that the enzyme substrate (HX and X) concentrations were higher in the SHR rats. In that study, oxypurinol did not decrease the blood pressure of normal rats. Another in vivo study showed that XOR played a role in a mouse model of atherosclerosis [*Schröder et al., 2006*]. In that model, inhibiting XOR through feeding mice with tungsten led to the normalization of endothelial function and the decrease in free radical generation as well as the attenuation of plaque formation. However, as the authors mentioned, since oxypurinol or alloppurinol could not be used in that study, it can not be ruled out that this protective effect of tungsten treatment was due to inhibiting enzymes other than XOR that have a molybdenum atom at their active sites e.g. aldehyde oxidase and sulfite oxidase.

In a recent clinical study, oxypurinol was seen to improve myocardial contractility in patients with ischemic cardiomyopathy [*Baldus et al., 2006*]. Another clinical study showed that allopurinol improved endothelial function in patients with chronic heart failure [*George et al., 2006*]. Doubling the allopurinol dose in that study resulted in more than twice the improvement in endothelial function, and based on this finding, the authors argued that allopurinol doses used in many previous clinical trials were sub-optimal. The other important finding of the study of George and co-workers was that merely decreasing uric acid (which can have either detrimental or beneficial effects, see later) concentration by a means other than inhibiting XOR did not result in any improvement in endothelial function. The implication is that allopurinol was likely protective through inhibiting XOR-mediated ROS production (coupled to the oxidation of HX and X to uric acid) and not through merely decreasing the in vivo concentration of uric acid per se. However, it should be kept in mind that, as mentioned earlier, allopurinol (and oxypurinol) can exert some other beneficial actions unrelated to inhibiting XOR. Another clinical study showed that oxypurinol improved coronary endothelial function in patients with coronary artery disease [*Baldus et al., 2005*], whilst others observed that treatment with XOR inhibitors has beneficial cardiovascular effects

in smokers and hypercholesterolemic patients [*Guthikonda et al., 2003, Cardillo et al., 1997*]. However, all these clinical studies are limited by the small sample size, demanding more validation with bigger clinical trials.

Regarding neuronal/cerebral disease, using primary cultures of rat striatum, it was found that intracellular XOR, through a non clear mechanism, exacerbated the toxicity of an endogenous neurotoxicant, 3-Hydroxykynurenine (3-HK), when this toxicant was applied externally at concentrations of 1-10 µM. The importance of this finding is that these concentrations are comparable to 3-HK concentrations found in some neurodegenerative diseases, implying that 3-HK as well as XOR might be important players in the development of some neurodegenerative diseases [*Okuda et al., 1996*]. Using cortical neuronal cultures, Tagami and co-workers (1998) showed that allopurinol attenuated the damage induced by hypoxia-reoxygenation.

Widmer and co-workers (2007) showed that inhibiting XOR with oxypurinol attenuated lipid peroxidation as well as cellular damage in cultures of microglia cell line (microglia are considered resident macrophages in the brain) induced by anoxia-reoxygenation. It is worth mentioning that, in that study, oxypurinol was protective at a low concentration (10 µM) which rules out that its protection was due to directly scavenging hydroxyl radical (not inhibiting XOR), a side activity that might occur only at a high concentration of oxypurinol (or allopurinol) (≥ 500 µM). Actually, since oxypurinol (and allopurinol) was shown to scavenge hydroxyl radical at such a high concentration only in cell-free experiments in the presence of only one competing detector (i.e. scavenger), even if oxypurinol (or allopurinol) was used at such a high concentration in the above toxicity study (or other toxicity studies), its protective effect in a cellular milieu is unlikely to be due to its ability to directly scavenge hydroxyl radical. The reason is that, in a cellular milieu, oxypurinol needs to compete with many biological targets (i.e. scavengers) of hydroxyl radical, and thus a much higher concentration than 500 µM of oxypurinol might be needed for it to significantly scavenge hydroxyl radical. Abramov and co-workers (2007) showed that 20 µM oxypurinol attenuated cell damage induced by oxygen-glucose deprivation in cultures of either cortical or hippocampal neurons. In other studies, XOR inhibitors attenuated the damage induced by kainate (which activates a subclass of glutamate receptors called AMPA/kainate receptors) in cortical, retinal, or cerebellar neurons [*Dykens et al., 1987, Cheng and Sun, 1994, Dutrait et al., 1995*].

Takuma and co-workers (1999) showed that, in primary cultures of astrocytes (which are glial cells that exert usually protective/supportive roles in the brain), allopurinol (100 μM) attenuated the damage induced by increasing intracellular Ca^{2+} concentration. Allopurinol attenuated the damage induced by mechanical trauma in cultured brain endothelial cells [*Gidday et al., 1999*]; whilst oxypurinol was shown to attenuate the damage induced by anoxia-reoxygenation in similar brain endothelial cell cultures [*Beetsch et al., 1998, Wu et al., 1997*]. This damage to brain vascular endothelial cells is thought to be an important traumatic event in acute neurodegenerative diseases e.g. stroke, and as mentioned before, cerebral (as it is the case with the cardiac) vascular endothelial cells contain significant amount of XOR [*Betz, 1985, Harrison, 2002, Berry and Hare, 2004, Zweier et al., 1994, Terada et al., 1991a*].

MacGregor and co-workers (1996) showed that oxypurinol, and to a lesser extent allopurinol, attenuated the neuronal membrane damage induced by a systemic in vivo administration of kainate in rats. Palmer and co-workers (1993), in an in vivo study, showed that a high dose of allopurinol, although administered after the period of ischemia, attenuated acute and chronic brain injuries in rats subjected to cerebral ischemia-reperfusion. Phillis (1989) showed that in vivo administration of oxypurinol attenuated hippocampal damage and the associated neurological deficits in gerbils subjected to ischemia. Thom (1992) showed that inhibiting XOR through either feeding rats with tungsten for a month or pre-treatment with allopurinol resulted in the attenuation of brain lipid peroxidation induced by carbon monoxide poisoning (followed by reoxygenation). Phillis and co-workers (1995) showed that oxypurinol restored cerebral cortical ATP content (during the early period of insult) and also improved physiological indices in rats subjected to ischemia-reperfusion injury. These authors suggested that oxypurinol may have been protective by inhibiting XOR, which would result in inhibition of ROS generation and would result also in the accumulation of HX which can in turn be converted (salvaged) to adenine nucleotides including ATP.

Peeters-Scholte and co-workers (2003) observed a protective effect of allopurinol, although given after the period of ischemia, against brain damage induced by ischemia-reperfusion in newborn piglets. In a small sample size clinical trial, allopurinol was observed to decrease free radical generation and improve cerebral hemodynamics and

electrical activity in human newborns suffering, during birth, from severe asphyxia followed by cerebral perfusion [*Van Bel et al., 1998*].

A toxic role has been also observed for XOR in many other disease models of different organs of the body (in addition to the cardiovascular and cerebral systems). In cultures of the Kupffer cells of rat liver, it was shown that allopurinol significantly attenuated the damage induced by hypoxia-reoxygenation [*Wiezorek et al., 1994*]. In cultures of mouse retinal endothelial cells, it was shown that either DPI (which can inhibit XOR through blocking the FAD site, but can also inhibit some other enzymes) or oxypurinol attenuated the damage induced by glucose/oxygen deprivation followed by restoration to normal glucose and oxygen levels [*Rieger et al., 2002*].

Terada and co-workers (1992b) showed that inducing intestinal ischemia-reperfusion resulted in injury to the lung tissue, suggesting that the damaged intestine released toxic circulating mediators that caused distal injury to the lung tissue, where inhibiting XOR by either feeding the rats with tungsten or pre-treatment with allopurinol attenuated the lung injury. These authors suggested that XOR released from the damaged intestine (in addition to XOR present in the lung tissue) played a role in the lung injury. Nielsen and co-workers (1996) showed that inducing ischemia-reperfusion of rabbit liver resulted in damage to both of the liver and the lungs, where feeding the rabbits with tungsten attenuated the damage observed in both of the organs. Ohta and co-workers (2007), in an in vivo study, showed that rat liver damage induced by D-galactosamine (a toxicity model resembles the liver damage observed in acute viral hepatitis in humans) was attenuated by allopurinol even though it was administered 6 hours after administering D-galactosamine. Zeki and co-workers (2002), using an in vivo model of chronic pancreatitis, showed that feeding the animals with tungsten resulted in the attenuation of the observed injury.

Kumagai and co-workers (2002) showed that in vivo inhibition of XOR with either BOF-4272 (a highly specific inhibitor of the Mo site on the enzyme) or allopurinol attenuated rat testicular damage induced by surgical cryptorchidism. Also, Lamarque and Whittle (1995) showed that in vivo pre-administration of allopurinol attenuated rat gastric mucosal damage induced by local intra-arterial infusion of nitric oxide donors. These authors attributed the allopurinol-inhibitable toxicity of the nitric oxide donors to

the reaction between superoxide produced by XOR and nitric oxide to produce peroxynitrite, which can be very toxic.

In all disease models where inhibiting XOR-generated ROS appears to be either beneficial or non beneficial, it is difficult to know where to put uric acid (whose level will be reduced by inhibiting XOR) in the equation. This is because that uric acid seems to have both beneficial and harmful effects [*Feig et al., 2008, Dimitroula et al., 2008*]. Thus, if inhibiting XOR shows protection, a question arises: is this protective effect of XOR inhibition is due to decreasing ROS levels or due to decreasing uric acid level (here we assume that uric acid is detrimental)? On the other hand, when inhibiting XOR does not show protection, another question arises, is this lack of protection of XOR inhibition is due to the lack of a role of XOR-generated ROS, or is it due to the beneficial effects of decreasing ROS were antagonized by preventing beneficial effects of uric acid? A possible way to address these questions is through decreasing ROS levels by a means other than inhibiting XOR (e.g. direct scavenging of ROS) and/or decreasing uric acid levels by a means other than inhibiting XOR (e.g. direct scavenging of uric acid).

Because of the above mentioned evidence for a toxic role of XOR in many pathologies, and also because XOR is considered one of very few convenient tools to produce superoxide experimentally, a combination of XO and its substrate X (or other substrates that bind to the Mo site) is a widely used model to generate ROS and to study their effects in many cell culture studies [*Rieger et al., 2002, Fatokun et al., 2007a, Matesanz et al., 2007, Van Grevenstein et al., 2007, Knorpp et al., 2006, Casalino-Matsuda et al., 2006, Mander et al., 2006, Wu et al., 2002, Lee et al., 2001, Atlante et al., 2000, Mitobe et al., 2000, Bellmann et al., 1995, Satoh et al., 1998, Link and Riley, 1988, Mohsen et al., 1995, Duell et al., 1995, Simon et al., 1981, Hiraishi et al., 1987, Ito et al., 1992, Valencia and Morán, 2004, Michikawa et al., 1994*]. However, there are not many cell culture studies which have studied the effect/toxicity of the combination of XO (or XDH) and the substrate NADH (which binds to the FAD site). A possible reason for this lack of interest is that, unlike X, NADH is oxidized by many enzymes other than XO and is involved in many cellular processes, which can obscure the mechanism of action of ROS generated by applying NADH / XO combination. Another reason for this lack of interest may be that NADH is known to be a much weaker substrate than X for the most studied form of the enzyme, the bovine milk XO [*Gilbert, 1963, Liochev et al., 1989, Nakamura, 1991*]. However, some

other forms of the enzyme are much more potent than the bovine milk XO in oxidizing NADH. Actually, recent studies have shown that XO (and especially XDH) isolated from certain human tissues have a potent NADH oxidase and ROS generating activity, while some of these human forms have, surprisingly, a low xanthine oxidase activity [*Sanders et al., 1997, Zhang et al., 1998, Maia et al., 2007*]. Also, as mentioned earlier, in cell-free experiments, it was shown that blocking the site of X binding on the enzyme (the Mo site) does not significantly prevent NADH oxidation and the concomitant ROS generation [*Nakamura, 1991, Berry and Hare, 2004, Harrison, 2002, Sanders et al., 1997, Olson et al., 1974*]. This led some authors to warn against overlooking the NADH oxidase activity of XOR when interpreting results of studies that looked for a role for this enzyme in certain diseases. In particular, the failure of allopurinol (a blocker of the site of X binding, but not NADH binding) in preventing tissue damage in previous studies where XOR-mediated damage was proposed [*Allen et al., 1990, Benders et al., 2006, Mosler et al., 2005, Coetzee et al., 1996*] could be theoretically explained by the inability of allopurinol to prevent NADH oxidation by XOR, and hence its inability to prevent the tissue damage [*Berry and Hare, 2004, Harrison, 2002, Sanders et al., 1997, Zhang et al., 1998*].

1.5 Cell culture technique

Cell cultures derived from different organ systems are now widely used for different physiological, pathological, and pharmacological studies. They have several advantages over the in vivo studies: they allow for investigations on specific cell types; test compounds can be applied in defined concentrations, and a precise control of the environment around cells can be achieved [*Smith and Jiang, 1994, Freshny, 2004*]. The ability of a test compound to penetrate the membrane of the cell or the subcellular organelles can be assessed; specific extracellular, membrane, or intracellular targets/effects of the test compounds can be identified; interactions between two or more types of cells can be studied (e.g. interaction between neurons and astrocytes); availability of multi-well plates allows for testing a large number of treatments at the same time and under the same conditions, and ethical concerns about animal experimentation are avoided [*Freshny, 2004*]. A challenging aspect in studying oxidative stress is how to directly detect and measure ROS which are unstable, short lived, and present at very low levels? It is often done in vivo through indirect measurements e.g.

(1) measuring the end products of oxidative stress attack on lipids, proteins, or DNA (2) measuring the alteration in the activity of the antioxidant enzymes [*Halliwell and Whiteman, 2004*]. Cell cultures, however, make it much easier to directly and indirectly measure ROS.

Cell cultures, however, have their clear limitations e.g. lack of the complex biological environment around the cells, and hence the lack of the resemblance to the actual physiological and pathological states. Regarding oxidative stress in particular, it is possible that the isolation of cells and the subsequent culturing 'stress' would force the cells, in order to survive in the new strange environment, to induce survival/protective pathways that would not be induced under normal in vivo conditions. This means that cells that survive in vitro might be more resistant to oxidative stress insults than cells in vivo. However, it can be argued for an opposite possibility, where in vivo tissues might be better equipped with protective mechanisms (e.g. they have richer antioxidant environment) to cope with insults than cells in vitro [*Halliwell, 2003*], and hence higher concentrations of toxic insults might be required to kill cells in vivo than if applied in vitro.

Another important fact that should be considered when conducting cell culture studies is that cells in cultures are usually exposed to higher oxygen levels than its levels in most in vivo tissues [*Halliwell, 2003*]. However, there are some details that require discussion. Physiological oxygen levels in most in vivo tissues (with some exceptions e.g. some pulmonary cells) are estimated to be around 1-60 mmHg [*Halliwell, 2003, de Groot and Littauer, 1989, Taylor and Camalier, 1982*], where in vivo brain interstitial oxygen levels were reported to be around 30-40 mmHg [*Liu et al., 2004*]. In cell cultures plated under standard conditions (5% CO_2, 95% air, and 37˚C), the levels of dissolved oxygen around cells (in the culture medium) were reported to be more than 100 mmHg after the first minutes/hours of plating (or renewing the medium) [*Metzen et al., 1995, Wolff et al., 1993, Hanson et al., Hanson et al., 2007, Taylor and Camalier, 1982*]. What happens to the dissolved oxygen level around cells after that (i.e. after the first minutes/hours of plating (or renewing the medium)) is not clear, but seems to depend on (among other factors) the oxygen consumption efficiency of the cultures when maintained in a static environment i.e. without shaking the plates [*Metzen et al., 1995, Jensen, 1976, Bader et al., 1999*]. For example, Metzen and co-workers (1995) showed that although in rat renal mesangial primary cultures the dissolved oxygen level around cells was more than 100

mmHg after 24 hours of renewing the medium, in some cell line cultures (renal LLC-PK1 and LLC-MK2 or hepatic HepG2 and Hep3B epithelial cells) the dissolved oxygen level around cells dropped to less than 0.2 mmHg (the detection limit) after 24 hr of renewing the medium (i.e. the cells become very hypoxic despite the fact that the air in the incubator was maintained at 95%). These authors attributed the observed hypoxia in these cell lines to a high oxygen consumption efficiency of the cells that exceeded the ability of oxygen in the incubator air to dissolve in the culture medium and diffuse to the cells attached to the bottom of the plate wells (i.e. oxygen consumption far exceeded oxygen supply). Similar findings were observed by others [*Holzer and Maier, 1987*]. Therefore, in cultures (under static environment and standard conditions) with high oxygen consumption efficiency the cells might be exposed to hyper-oxic conditions (initially) and then be exposed to either transient or sustained hypoxic conditions, where both cases are non-physiological. To overcome these problems, some researchers have tried culture plates with gas-permeable bottoms instead of the standard (polystyrene) culture plates (which are poorly permeable to gases) [*Holzer and Maier, 1987, Wolff et al., 1993, Bader et al., 1999, Jensen, 1976, Metzen et al., 1995*]. On the other hand, in cultures with low oxygen consumption efficiency (many primary cultures might be under this category) the cells might be under hyper-oxic (i.e. oxidative stress) conditions both initially and throughout their maintenance in culture, which is also non-physiological.

With these limitations in mind, however, cell culture is an indispensible technique to investigate many biological/pathological conditions including oxidative stress. Many important discoveries would have been difficult or at least delayed in the absence of the cell culture technique e.g. the demonstration that activating some glutamate receptors in cultured CNS neurons generates intracellular superoxide [*Patel et al., 1996, Lafon-Cazal et al., 1993b*].

Oxidative stress in cell cultures can be induced in different ways. ROS can be applied/generated directly e.g. applying hydrogen peroxide solution, applying XOR with its substrate, or applying nitric oxide donors. Also, oxidative stress can be induced indirectly through applying specific disease model inducers e.g. hypoxia, glucose deprivation, serum/growth factors deprivation, or (in cultures of CNS neurons) glutamate receptor activation. Also oxidative stress can be induced by inhibiting cellular antioxidant mechanisms e.g. inhibiting the activity or knocking out the gene of SOD or catalase.

To test the possible involvement of ROS in some pathological conditions in cell cultures, many detection/measurement methods have been developed. For example, 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) is used as a probe for general intra-cellular oxidative stress. The principle of this assay is that DCFDA readily crosses the cell membrane and accumulates in the cytosol. Intracellularly, DCFDA is converted by esterases to a non-fluorescent species, 2′,7′-dichlorodihydrofluorescein (DCFH), where many reactive species can oxidize DCFH into a fluorescent species, 2′,7′-dichlorofluorescein (DCF), which can be measured [*Halliwell and Whiteman, 2004*].

A recently developed method is considered one of the most available specific intracellular detection methods of superoxide, which involves the oxidation of the probe hydroethidine by superoxide to yield a fluorescent species (2-hydroxyethidium) [*Zhao et al., 2005*]. This specific species (2-hydroxyethidium) was shown to be only produced by reaction between hydroethidine and superoxide, but not by reaction between hydroethidine and any of the following reactive species: hydrogen peroxide, hydroxyl radical, peroxynitrite, or hypochlorous acid [*Zhao et al., 2005*]. The involvement of oxidative stress in some pathological conditions in cell cultures can also be measured indirectly e.g. measuring the stable end products of the oxidative stress attack on lipids, proteins, or DNA.

In studying oxidative stress or other damaging events in cell cultures, the overall damage to the cells is usually assessed using viability tests. One type of viability tests is measuring membrane integrity through examining the ability of the cells to uptake a dye that is normally excluded by cells e.g. trypan blue or naphthalan black, so dead cells will uptake the dye while viable cells will exclude it. This can also be done the other way around through applying a dye that is known to be excluded by dead cells while being taken up by viable cells e.g. neutral red [*Freshny, 2004*]. The membrane integrity can also be assessed through observing the leak of some intracellular components into the extracellular medium e.g. lactate dehydrogenase [*Lin and Maiese, 2001*]. Another type of viability tests is measuring the enzymatic activity of cells. For example, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and Alamar blue are dyes that can be reduced by some cellular enzymes, where the extent of their reduction reflects the viability status of the cultures [*Hamid et al., 2004, Fatokun et al., 2007b*]. Since cells in cultures have characteristic morphology, a very important way of assessing the culture viability is also through examining the morphological appearance

of the cells under the microscope. For example, cultured neurons have a characteristic morphology, and when damaged, clear changes in their morphology are observed e.g. degenerated axons and also shrinking or even lysed cell bodies. To know whether the morphological changes are reversible or irreversible, the morphology can be periodically checked for an extended period of time.

In this project cultured neurons were chosen to investigate the toxicity of XOR. Although this thesis attempted to answer some relatively general questions regarding XOR toxicity (and hence any other type of cells might have been applicable for this study), neurons were selected for several reasons. As mentioned earlier, brain is thought to be particularly vulnerable to oxidative stress, and hence delicate oxidative stress-related levels of toxicity/effects might be observed in neurons by applying relatively low concentrations of ROS. Also, since a toxic interplay in the CNS neurons between oxidative stress and glutamate-receptor activation was previously postulated, we were keeping in mind that we might want to do some experiments to have more analysis of this toxic interplay. Also, it was mentioned earlier that cellular XOR potentiated the toxicity of an endogenous neurotoxicant, 3-hydroxykynurenine (3-HK), in cultured neurons through a non clear mechanism. So, we were also keeping in mind that we might want to do some experiment to investigate the mechanism of this interesting toxic augmentation.

Using neuronal cultures derived from the brain of adult animals is preferable to younger ones, since the oxidative stress-related neurondegenrative diseases (e.g. stroke and Parkinson's disease) are age related. At each stage of body development, neurons and cells in general have characteristic genetic, structural, metabolic, and redox status e.g. the expression pattern of neuropeptide Y in the guinea-pig sympathetic neurons differs significantly between embryos and adults [*Matsumoto, 1993*]. Also neurons may switch their dependency from one growth factor to another at different stages of development.

However, neuronal cultures derived from adult animal brain are difficult to produce and sustain. The reasons for this difficulty might include that neurons in the adult brain tissue are embedded in a network of adhesive macromolecules that physically retard the neurons from dissociating from the tissue during the isolation procedure [*Brewer, 1997*]. Also, some molecules in the adult tissue were shown to specifically inhibit the attachment of neurons to the surface of culture plates e.g. phosphacan and neurocan are

large proteoglycans present in the extracellular matrix in the nervous system which have been shown to impair the neurite outgrowth and the attachment to the culture plate of adult rat dorsal root ganglion neurons in a dose dependent manner [*Sango et al., 2003*]. Also, with increased age, there is an impairment of the fundamental properties of the cell membrane such as fluidity and elasticity [*Horie et al., 1990*].

Some researchers have tried different approaches/techniques to improve culturing conditions in order to produce and sustain a good yield of adult neuronal cultures e.g. using specialised media, adding specific growth factors, and/or using techniques such as density gradient fractionation technique [*Brewer, 1997*]. However, these approaches are relatively expensive and complex for a routine use. So, neuronal cultures derived from younger animals, embryos or neonates, are widely used instead in research. Among the different types of brain-derived cultures, the primary cultures of neonatal cerebellar granule neurons (CGNs) have a particular attraction [*Contestabile, 2002, Smith et al., 2008, Fatokun et al., 2007b*]. They contain a very homogeneous population of neurons. The cerebellum is anatomically distinct and easy to dissect. Also, these cultures seem to be very vulnerable to at least some types of oxidative stress e.g. it was observed that treatment with hydrogen peroxide (100 μM) for just 15 minutes killed 75% of CGNs, while it was observed in another study that the same percent of death in cultured brain cortical neurons required 24 hours application of the same concentration of hydrogen peroxide [*Klein and Ackerman, 2003*].

1.6 Aim/Objectives

The aim of this project was to investigate some aspects of the in vitro toxicity of XOR, which might provide more insights into its in vivo toxicity. So we were interested to know the answer of the following questions:

A:

Since there are not many cell culture studies which have investigated the toxicity of the NADH / XO combination (see before), what are the differences and similarities between X / XO and NADH / XO toxicities? In particular:

1- What are the concentrations of NADH and X that produce the same toxicity level?

2- What is the effect of blocking the different sites on XO on the two toxicity systems?

3- What is the effect of deactivating superoxide or hydrogen peroxide on the two toxicity systems?

4- What is the effect of adding metal chelators on the two toxicity systems?

5- What is the effect of adding hydroxyl radical scavengers on the two toxicity systems?

B:

We were also interested to clarify some secondary issues regarding X / XO toxicity. In particular:

1- Since many previous studies (one of them was conducted previously in this laboratory using CGNs [*Fatokun et al, 2007a*]) showed that catalase was protective against X / XO toxicity, while SOD had no effect, what is the reason for the lack of effect of SOD? Is it because superoxide generated from this combination was not involved in the toxicity?

2- Since some previous studies, that used some tissues other than CGNs, showed that intracellularly generated superoxide mediated the X / XO toxicity (or similar models, where extracellular hydrogen peroxide was a main product) [*Ito et al., 1992, Kyle et al., 1988, Hiraishi et al., 1994*], is this also the case with CGNs i.e. does intracellularly generated superoxide mediate X / XO toxicity in CGNs? And if so, is the lack of protection of SOD, observed in CGNs and other tissues, due to its inability to cross the cell membrane? And also if intracellular superoxide is involved, does the lack of protection by SOD mean that superoxide generated extracellularly from X / XO combination was unable to cross the cell membrane?

3- Since some previous studies showed that some commercial preparations of XO are contaminated with iron [*Britigan et al., 1990*], is this also the case with our preparation of XO? And if so, is this iron active and does it contribute to the effects that we observe in X / XO toxicity?

4- Since many previous studies suggested that intracellular hydroxyl radical is involved in X / XO toxicity [*Satoh et al., 1998, Link and Riley, 1988, Mohsen et al., 1995, Duell et al., 1995, Hiraishi et al., 1987, Zigler et al., 1985*], can this also be demonstrated here? If so, can directly scavenging hydroxyl radical intracellularly provide protection?

C:

Since in the start of this project we found some difficulties with CGNs cultures, one aim was to establish the optimal culturing conditions required in order to successfully

perform our experiments with XO. In particular, we wanted to know why the neurons (including those in the control group) die at the experiments day by manipulating the cultures (i.e. aspirating the culture medium, adding treatment and control vehicles, and adding the restoration medium).

2 Materials and methods

2.1 Chemicals (ordered alphabetically)

- AlamarBlue®; Invitrogen (DAL1100)
- Albumin; Sigma (A2153)
- Allopurinol; Sigma (A8003)
- Catalase; Sigma (C1345)
- Cytochrome c; Sigma (C7752)
- Cytosine β-D-arabinofuranoside (Cytosine arabinoside); Sigma (C1768)
- Deferoxamine mesylate salt; Sigma (D9533)
- Diethyldithiocarbamate (DDC); Sigma (228680)
- Diphenyleneiodonium (DPI); Sigma (D2926)
- DNAse I ; Sigma (AMPD1)
- Dulbecco's Phosphate-Buffered Saline (DPBS); Invitrogen (14190)
- Ethylenediaminetetraacetate (EDTA); Sigma (D2SS)
- Fetal bovine serum (FBS); Sigma-Aldrich (F9665)
- Glutamic acid (glutamate); Acros Organics (156212500)
- Glutamic-Pyruvic Transaminase; Sigma (G8255)
- Hydrogen peroxide (H_2O_2) solution; Sigma (H1009)
- Manganese-superoxide dismutase (Mn-SOD); Sigma (S5639)
- Minimum Essential Medium (MEM); Invitrogen (32360-034)
- (+)-MK-801; Sigma (M107)
- NG-nitro-L-arginine methyl ester (L-NAME); Sigma (N5751)
- Poly-D-lysine hydrobromide ; Sigma (P0899)
- α-(4-Pyridyl N-oxide)-N-tert-butylnitrone (POBN); Sigma (215430)
- Reduced β-Nicotinamide adenine dinucleotide (NADH); Sigma-Aldrich (N4505)
- S-Nitroso-N-acetyl-DL-penicillamine (SNAP); Sigma (N3398)
- Superoxide dismutase-1 (SOD-1) (Copper,Zinc-superoxide dismutase); Sigma-Aldrich (S5395).
- Tiron (4,5-Dihydroxy-1,3-benzenedisulfonic acid) disodium salt; Sigma (33724)
- Trypsin; Sigma (T-4799)
- Trypsin inhibitor; sigma (T6414)

- Xanthine; Sigma (X4002)
- Xanthine oxidase (from bovine milk); Sigma-Aldrich (X4376)
- XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt); Sigma (X4626)

2.2 Equipments

- Laminar flow hood.
- Different sizes of automatic pipettes.
- 96-well plates.
- Inverted contrast-field microscope (Olympus IX50) with Olympus DP50 software for image processing.
- Centrifuge (MSe; Harrier 18/80).
- CO_2 incubator.
- Water bath.
- Plate reader (DYNEX TECHNOLOGIES; *Opsys* MR).

2.3 Treatment solutions and media

HEPES-sol: Contains (in distilled water): Sodium chloride (140 mM), potassium chloride (5 mM), calcium chloride (2mM), N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) (for buffering) (20 mM), magnesium chloride (0.8 mM), and glucose (3 mM). pH= 7.2-7.6. When this solution was used, the cultures were moved to an incubator that contains zero% CO_2.

This solution was used as a vehicle for the test compounds in many viability studies that were performed after solving the problem of fresh culture medium toxicity. This solution was also used in all cell-free experiments (except one cell-free experiment that will be indicated later on).

MEM-HEPES-sol: MEM medium (Invitrogen (32360)), which already contains: HEPES (for buffering) (25 mM) and sodium bicarbonate ($NaHCO_3$) (also for buffering) (2.2 mg/ml). This solution was modified to contain: glutamine (2 mM), gentamicin (50

μg/ml), and potassium chloride (25 mM) as final concentrations. When this solution was used, the cultures were maintained under 5% CO_2.

This solution was used as a vehicle for the test compounds in many viability studies that were performed after solving the problem of fresh culture medium toxicity.

Fresh culture medium: the same as MEM-HEPES-sol but also contains 10% fetal bovine serum (FBS).

This was the solution that was used in the isolation and plating process of neurons at day zero. So, the neurons were maintained in this solution for 8-9 days until the time of experiments. A fresh culture medium was also used as a vehicle for the test compounds in all of the experiments that were performed before solving the problem of fresh culture medium toxicity. This solution was also the solution to which the neurons were restored after the treatment period in all of the experiments that were performed before solving the problem of fresh culture medium toxicity.

Conditioned medium: This was the culture medium collected from plates that contained neurons grown for 6-7 days. This medium will not contain significant (toxic) amount of glutamate (that is already present in the fresh culture medium), because glutamate gets taken up/degraded by neurons during the 6-7 days of plating [*Aronica et al., 1993*]. So, this solution was the solution to which the neurons were restored at the end of treatment period in all of the experiments that were performed after solving the problem of fresh culture medium toxicity.

2.4 Test compounds stock solutions

-Bovine milk XO powder was dissolved in 0.001 M sodium hydroxide (NaOH) (prepared in normal saline) to a concentration of 0.5 Units/ml. Aliquots of this stock solution were stored at -20°C until use (notice that the NaOH presence and freezing the enzyme might cause damage to the enzyme, and thus these should be avoided in future experiments. A better way is to dissolve the enzyme in a neutral buffer and then use it immediately).

-SOD-1 powder was dissolved in normal saline to a concentration of 10000 Units/ml. Aliquots of this stock solution were stored at -20˚C until use (notice that freezing the enzyme might cause damage to the enzyme, and thus this should be avoided in future experiments. A better way is to dissolve the enzyme in a neutral buffer and then use it immediately).

-Catalase (Cat.) powder was dissolved in phosphate buffer (pH= 7.4) to a concentration of 10000 Units/ml. This stock solution was stored at 4-8 C and used in the same day.

- NADH powder (100 mg) was dissolved in 1 ml of 0.01 M NaOH to generate a solution of 134.8 mM NADH, and this solution was then diluted by adding 5.74 ml of normal saline to make 6.74 ml of 20 mM NADH stock solution. Aliquots of this solution (pH = 10–11) were protected from light and stored at – 40 °C until use (notice that the high pH might cause damage to NADH, and thus should be avoided in future. A better way is to dissolve NADH in a neutral buffer and then use it immediately without freezing).

-POBN powder was dissolved in normal saline to a concentration of 100 mM. Aliquots of this solution were protected from light and stored at -20˚C until use.

-Deferoxamine powder was dissolved in distilled water to a concentration of 76 mM. Aliquots of this solution were stored at -20˚C until use.

-Cytochrome c powder was dissolved in distilled water to a concentration of 2 mM. Aliquots of this solution were protected from light and stored at -20˚C until use (notice that freezing the protein might cause damage to it, and thus this should be avoided in future experiments. A better way is to dissolve the protein in a neutral buffer and then use it immediately).

-XTT powder was dissolved in distilled water to a concentration of 2 mM. Aliquots of this solution were protected from light and stored at -20˚C until use.

-Allopurinol powder was dissolved in 1 M NaOH to a concentration of 100 mM. Aliquots of this solution were stored at -20˚C until use.

-Xanthine powder was dissolved in 0.1 M NaOH to a concentration of 10 mM. Aliquots of this solution were stored at -20˚C until use.

-EDTA powder was dissolved in distilled water to a concentration of 50 mM. Aliquots of this solution were stored at -20˚C until use.

- Tiron powder was dissolved in distilled water to a concentration of 200 mM. Aliquots of this solution were stored at -20˚C until use.

-To prepare a stock solution of diphenyleneiodonium (DPI), an initial concentrated solution of 15.9 mM was prepared, and some of this solution was diluted in distilled water to 0.1 mM. Aliquots of this 0.1 mM solution were stored at -20˚C until use.

Notice: It was missed to record the identity of the solvent that was used to prepare the initial concentrated solution (i.e. 15.9 mM) of DPI. Tracing our memory back could not reveal the identity of this solvent. However, it is very likely that this solvent was DMSO, for two reasons. First, we usually follow the supplier instructions to dissolve our compounds, and in this case, Sigma mentioned that although DPI can be dissolved in water or ethanol to generate low concentrations of stock solutions, the only solvent they mentioned that can dissolve DPI to generate concentrations of stock solutions as high as the one prepared here (i.e. 15.9 mM) was DMSO. Second, when we thawed this concentrated solution of DPI (15.9 mM) (which we were still keeping it) it gave the distinctive odour of DMSO. Assuming this was DMSO, this means that in the viability experiments where DPI was tried (it was always tried at 100 nM) the DMSO concentration present in the treatment solution applied to cells was in the micromolar range. This is unlikely to have an effect, since DMSO used at 20 mM against the same toxicity insults that DPI was protective against them had no effect (Results section; Fig. 3-66 and Fig. 3-67).

Also, in a pilot experiment, new DPI powder was obtained from Sigma, and distilled water was used to dissolve it. In agreement to the supplier instructions, it was not possible to generate 15.9 mM, and a DPI stock solution of only 0.636 mM in distilled water was prepared. Using DPI diluted from this 0.636 mM stock solution in a pilot viability experiment at 100 nM produced substantial protection (in the morphological examination) against NADH / XO toxicity i.e. produced the same effect against NADH / XO toxicity as DPI (100 nM) derived from the 15.9 mM concentrated solution. This substantial protection (observed in the morphological examination) in this pilot study was also observed when the experiment was repeated in the subsequent day (this is not n = 2, since n = 2 in the viability experiments in this project represents experiments repeated in two separate weeks) (no Alamar blue viability assay was performed for this pilot study).

Notice: In viability experiments where NaOH was used in preparing the stock solutions of the test compounds, the levels of NaOH added to cultures (by adding the treatment solutions) do not exceed few hundreds micromolar concentrations in the majority of experiments. In few experiments, the level of NaOH added can be around 1 mM. In rare

cases (only in two experiments: Fig. 3-23 (the fourth treatment group) and Fig. 3-36 (column C)) there will be around 2 mM NaOH added in the treatment solutions. However, adding MEM-HEPES-sol containing 2 mM NaOH to the cultures did not have any effect on the viability (data not shown).

2.5 Using 96-well plates

96-well plates allow for testing many treatment groups at the same time and under the same conditions (Fig. 2-1 shows a photo and a diagram of a 96-well plate). These plates were used for both of viability (cell-containing) and cell-free experiments.

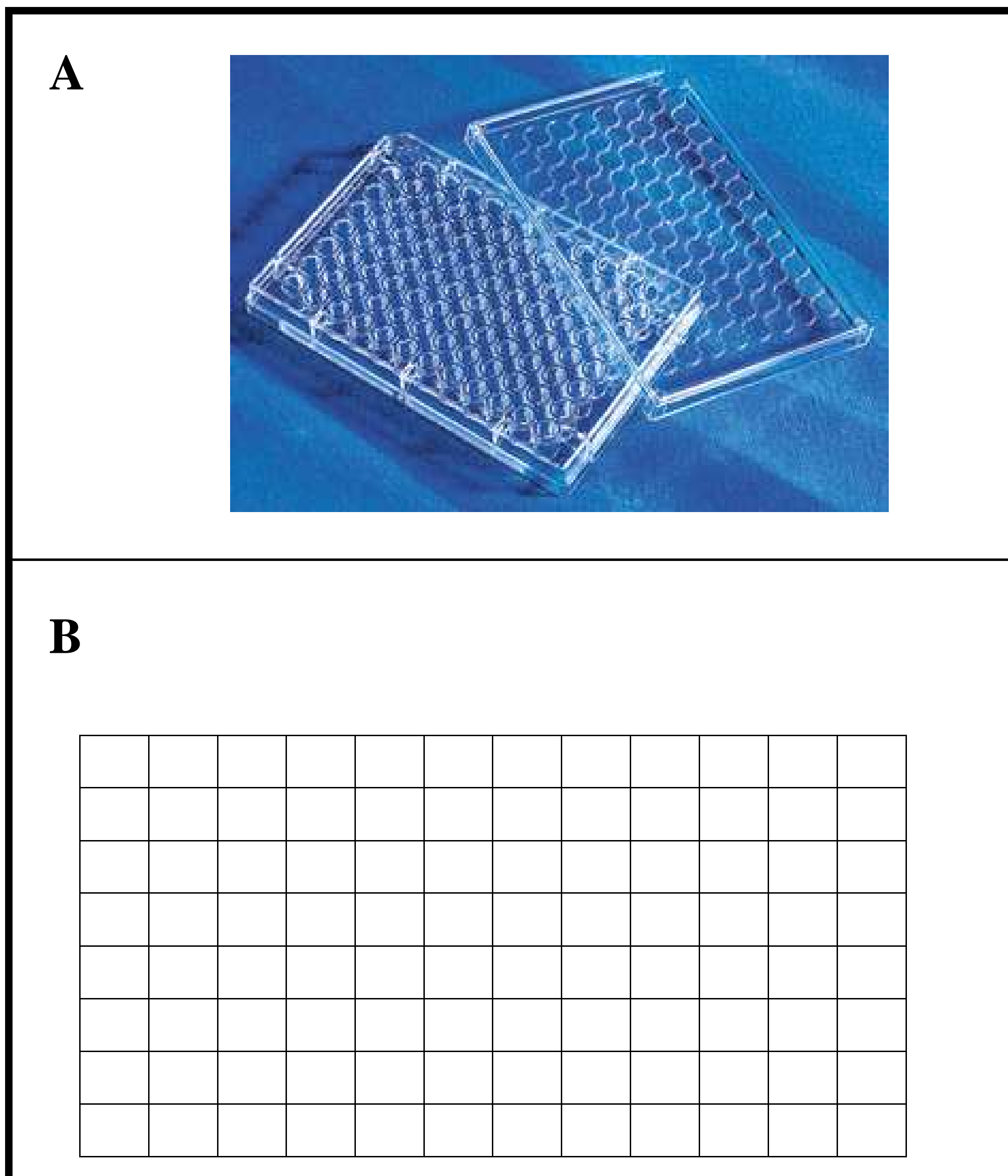


Figure 2-1: A photo and a diagram of a 96-well plate. A: A photo of a 96-well plate with its cover. **B:** A diagram of a 96-well plate. This diagram will be shown later in this thesis for many purposes. Notice that the surface of the wells is not square as it appears in the diagram, but it is actually round as it appears in the shown photo, but will be shown as square in the subsequent diagrams only for convenience.

2.6 Primary cultures of cerebellar granule neurons (CGNs)

Every set of cerebellar granule neurons (CGNs) cultures was prepared as follows:

1- Cerebella were isolated from 7- to 8-day old Sprague-Dawley rats (6-9 cerebella were used), cleared from meninges and blood vessels using forceps, chopped thoroughly by a blade to small pieces.

2- The chopped pieces were transferred to trypsin solution: 0.25 mg/ml in 20 ml of DPBS buffer solution (this buffer is a DPBS with the following added: albumin (3 mg/ml), glucose (2.5 mg/ml), and magnesium sulphate (0.382 mg/ml)), and incubated for 20 minutes at 37°C.

3- To the same tube, an equal volume (20 ml) of a weak trypsin inhibitor solution (contains in the DPBS buffer solution: trypsin inhibitor (8 µg/ml) and DNAse I (8 Units/ml)) was added and the tube was centrifuged at 1200 RPM for two minutes.

4- The supernatant was discarded and 2 ml of a concentrated trypsin inhibitor solution (DPBS buffer solution containing trypsin inhibitor (50 µg/ml) and DNAse I (50 Units/ml)) was added to the cell pellet. The cell suspension was triturated with three Pasteur glass pipettes with a decreasing pore size, 10 times each.

5- The DPBS buffer solution was then added up to 20 ml, and the tube was centrifuged again at 1200 RPM for two minutes.

6- The supernatant was discarded and 2 ml of fresh culture medium was added, and the same steps of trituration with Pasteur glass pipettes were repeated.

7- Fresh culture medium was then added up to 20 ml. This dilution of cell suspension eases cell counting.

8- The cells in this suspension were counted under the microscope as follows: one drop of the suspension was added to a chamber of a haematocytometer slide and the slide was placed under the microscope. A 10x objective was selected, and the slide was moved so that the field seen is the central area of the grid. A large square will appear filling the field. This square contains 25 smaller squares, and each one of these 25 squares is bounded by three parallel lines. The number of cells was counted in 5 of these 25 squares. The number obtained was multiplied

by 5 (to get the approximate number in the 25 squares). The number obtained is number of cells in a volume of 0.1 mm^3. This number was multiplied by 10000 to give the number of cells in one cm^3 i.e. the number of cells in one ml.

9- The cell density in the cell suspension was adjusted (diluted) with fresh culture medium to give a cell density of 1 million cells / ml.

10- This adjusted cell suspension (which contains a density of 1 million cells / ml) was plated into 96-well plates (pre-coated with poly-D-lysine hydrobromide (50 µg/ml)), where 100 µl of this cell suspension was added to each well (so each well contained 0.1 million cells).

11- The cultures were incubated at 37˚C and maintained in a humidified atmosphere under 5% CO_2 / 95% air.

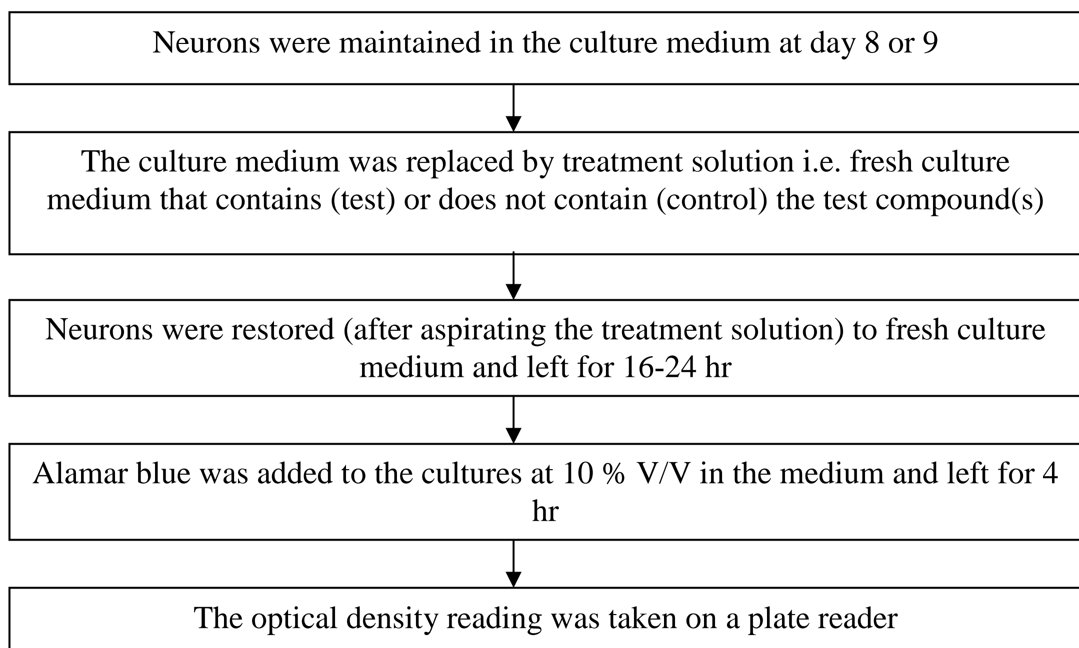
12- After 24 hours of plating, 10 µM of cytosine arabinoside was added to inhibit the growth of non-neuronal cells.

13- Viability experiments were performed at day 8 or 9 after plating.

2.7 Experimental design of viability studies

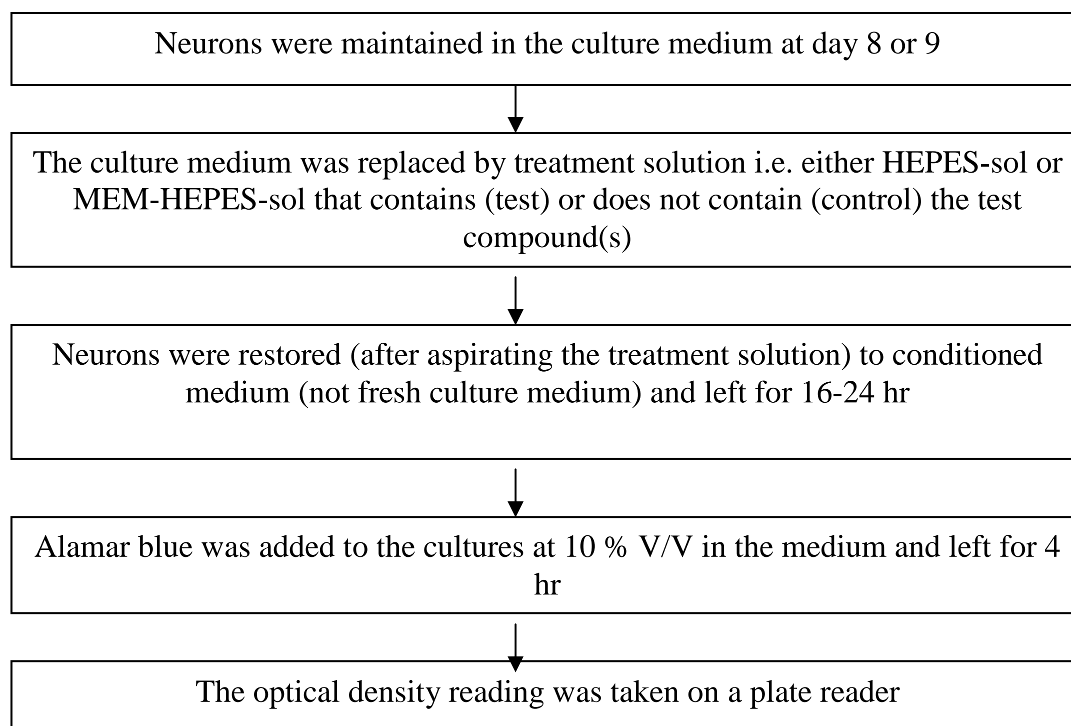
1- Experimental design of viability studies performed before solving the problem of fresh culture medium toxicity

The exact experimental protocol will be stated for each experiment in the figures of the Results section. In general the design is as follows:



2- Experimental design of viability studies performed after solving the problem of fresh culture medium toxicity

The exact experimental protocol will be stated for each experiment in the figures of the Results section. In general the design is as follows:



2.8 Viability assay

In each experiment, at the end of the treatment period, the cultures were restored to a culture medium and left for 16-24 hours. The viability status of the cultures was then assessed by measuring the reduction of the Alamar blue dye, added as 10 % V/V in the medium and left for 4 hr before taking the optical density (OD) reading on a plate reader. The principle is that the more reduction of the dye is, the more viable the neurons are. The assay was performed according the instructions of the manufacturer. We will show here the steps of calculating the viability of the control and treatment groups. The steps will be shown using data obtained in a real experiment that will be shown later in the Results section (the experiment shown in Fig. 3-68 in the Results section):

1- The optical density readings for the culture plate wells were taken at two wavelengths, 540 and 595 nm. All included wells contained cells with Alamar

blue added to them as 10% V/V in the culture medium, with the exception of two wells. One of these two wells contained only culture medium but did not contain either Alamar blue or cells (a blank well), and the other well contained culture medium that contained Alamar blue as 10% V/V but did not contain cells (see Fig. 2-2).

2- The reading taken at 540 nm of the well that contained only 10% Alamar blue (did not contain cells) was divided by the reading taken at 595 nm of the same well. The obtained number was called the factor. From Fig. 2-2, this factor was: 0.191 / 0.479 = 0.399.

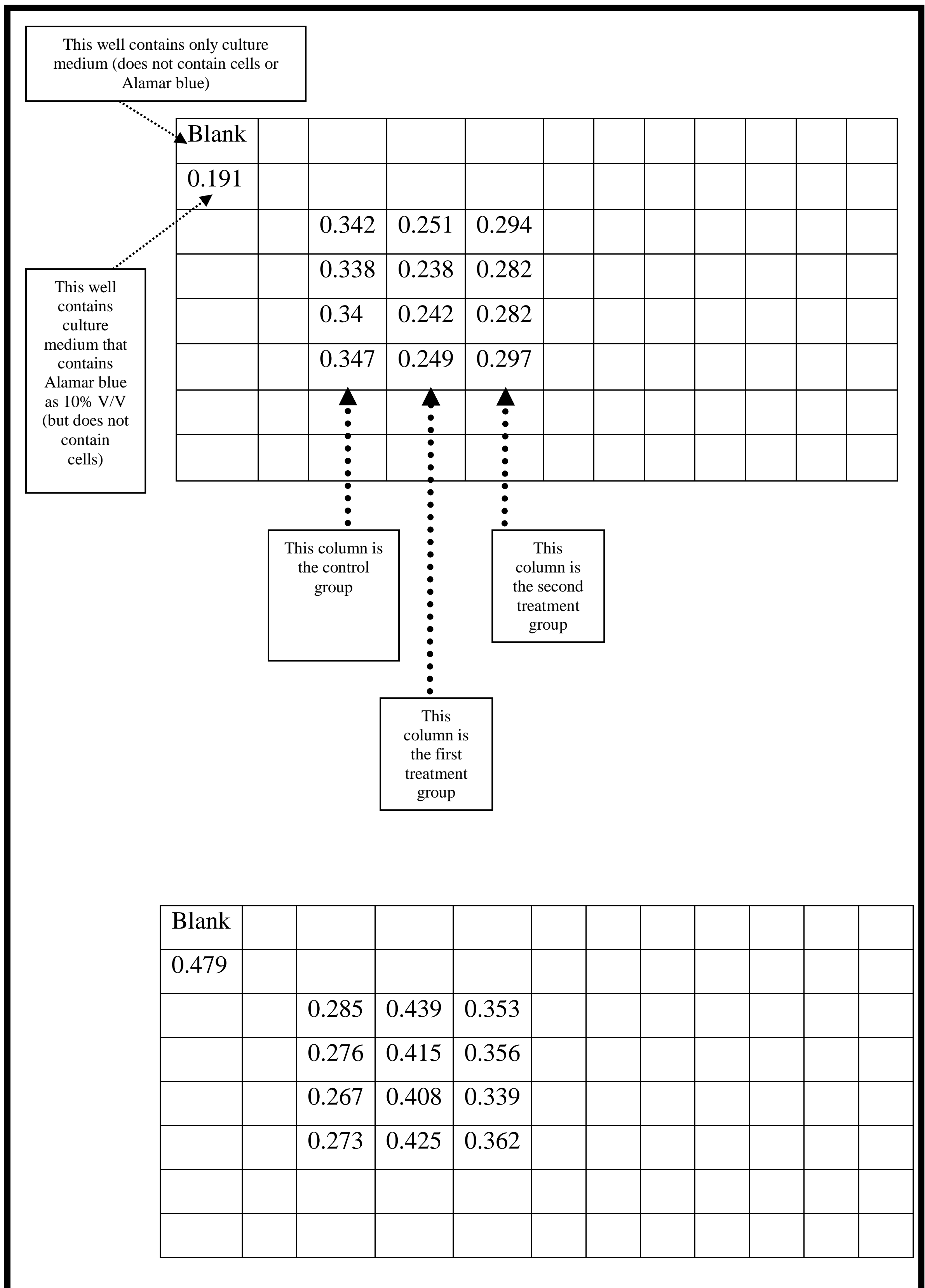
3- The following calculations were performed: the OD readings taken at 595 nm of the wells in the groups were averaged. From figure 2-2, the averaged OD readings at 595 were: Control group = (0.285 + 0.276 + 0.267 + 0.273) ÷ 4 = 0.275; First treatment group = (0.439 + 0.414 + 0.408 + 0.425) ÷ 4 = 0.422; Second treatment group = (0.353 + 0.356 + 0.338 + 0.362) ÷ 4 = 0.352. The average was also taken for OD readings taken at 540 nm for the wells in the groups. So, from Fig. 2-2, the averaged OD readings at 540 were: Control group = (0.342 + 0.338 + 0.34 + 0.347) ÷ 4 = 0.342; First treatment group = (0.251 + 0.238 + 0.242 + 0.249) ÷ 4 = 0.245; Second treatment group = (0.294 + 0.282 + 0.282 + 0.297) ÷ 4 = 0.289.

4- To obtain what is called the adjusted OD value for each group, the averaged OD reading taken at 595 nm for a group was multiplied by the factor obtained in step 2. The obtained number was subtracted from the averaged OD reading taken at 540 nm for the same group. The obtained number is the adjusted OD value for that group. So, the adjusted OD values for the groups were: Control group = 0.342 - (0.275 × 0.399) = 0.232; First treatment group = 0.245 – (0.422 × 0.399) = 0.0766; Second treatment group = 0. 289 – (0.352 × 0.399) = 0.149.

5- By obtaining the adjusted OD values, the groups can now be compared with each other. Because we wanted to express the viability of each group as % of the control group, we considered the adjusted OD value of the control group to be 100%, and we divided the adjusted OD of each treatment group by the adjusted OD value of the control group and then multiplied by 100 to get the viability value expressed as % of the control. Therefore, the viabilities of the groups were: Control = 100%, First treatment group = (0.0766 / 0.232) × 100 = 33%, Second treatment group = (0.149 / 0.232) × 100 = 64%.

6- The viability percents obtained in step 5 were those viabilities obtained in only one set of cultures (one week). Since we repeat each experiment using many sets of cultures (prepared in different weeks), we calculate the mean ± the standard error of mean (S.E.M) for the viabilities obtained in the different sets of cultures (i.e. obtained in the different weeks). In the case of the experiment shown in the Figure 3-68 in the Results section, the legend of that figure indicated that n=5, which means that that experiment was repeated in 5 different weeks. The means ± S.E.M of the viabilities obtained in the 5 different weeks for that experiment were: Control group = (100% + 100% + 100% + 100% +100%) ÷ 5 = 100% ± zero; First treatment group = (33% + 21% + 15% + 54% + 27%) ÷ 5 = 30% ± 6.7; Second treatment group = (64% + 40% + 40% + 89% + 44%) ÷ 5 = 55.4% ± 9.5. Notice that the means ± S.E.M. obtained in this step are those shown in Figure 3-68 in the Results section.

In addition to the Alamar blue assay, the viability status of the cultures was checked by observing the morphology of the neurons under the microscope (see the Results and Discussion sections for comments on the Alamar blue assay and references therein).



Blank											
0.191											
		0.342	0.251	0.294							
		0.338	0.238	0.282							
		0.34	0.242	0.282							
		0.347	0.249	0.297							

Blank											
0.479											
		0.285	0.439	0.353							
		0.276	0.415	0.356							
		0.267	0.408	0.339							
		0.273	0.425	0.362							

Figure 2-2: Diagram shows the Alamar blue OD readings at 540 and 595 nm for one set of an actual viability experiment. This is the reading in one of the 5 weeks in which we repeated the experiment shown in Figure 3-68 (see the Results section). The upper diagram of the plate shows the reading at 540 nm. The lower diagram is for the same plate but with the reading taken at 595 nm.

2.9 Cell-free assays

2.9.1 Cell-free detection of superoxide production

Since superoxide is known to be directly produced by the X / XO and NADH / XO combinations (see Introduction), it was of interest to confirm this in cell-free experiments. These cell-free experiments of detecting superoxide production also helped us answer many questions we faced during the progress of this project (please see the Results and Discussion sections). Two different cell-free detection methods of superoxide production were performed:

1- Cytochrome c reduction

The principle of this assay is that superoxide reduces the oxidized Cytochrome c in a SOD-inhibitable manner, where this reduction can be detected calorimetrically through observing the increase of Cytochrome c absorbance at 550 nm [*McCord and Fridovich, 1969*]. Since hydrogen peroxide (which will be directly generated from XO and will also be produced by the spontaneous dismutation of superoxide) interferes with this method, catalase has to be present in the reaction mixture. Also, EDTA is usually added in the reaction mixture to prevent the interference of metals that might be present as contaminants. In this assay, the blank was the assay solution (HEPES-sol) free of reagents.

Experiments in Fig. 3-46 and Fig. 3-47 were performed as follows: test cell-free wells contained the indicated treatment groups in HEPES-sol and left for 1 hr. After that, the optical density at 550 nm was recorded on a plate reader. Experiment in Fig. 3-51 was was performed as follows: test cell-free wells contained the indicated treatment groups and the optical density at 550 nm was recorded on a plate reader at the indicated time points.

2- XTT reduction

This is similar to Cytochrome c reduction method. Superoxide reduces XTT in a SOD-inhibitable manner, where this reduction results in the release of an orange product

(water soluble formazan) that can be detected calorimetrically through observing the increase of its absorbance at 450 nm (maximum absorption at 570 nm) [*Ukeda et al., 1997, Benov and Fridovich, 2002*]. The advantage of this method is that, since hydrogen peroxide is unlikely to interfere with this method, catalase does not need to be added to the reaction mixture. In this assay, the blank was the reaction solution (HEPES-sol) free of reagents.

Experiment in Fig. 3-48 was performed as follows: test cell-free wells contained the indicated treatment groups in HEPES-sol and left for 1 hr. After that, the optical density at 450 nm was recorded on a plate reader. Experiment in Fig. 3-50 was was performed as follows: test cell-free wells contained the indicated treatment groups and the optical density at 450 nm was recorded on a plate reader at the indicated time points.

2.9.2 Cell-free detection of hydrogen peroxide production

Since hydrogen peroxide is known to be directly produced by the X / XO and NADH / XO combinations, and will also be produced by the spontaneous dismutation of superoxide (see Introduction), we were interested to confirm that in cell-free experiments. These cell-free experiments of detecting hydrogen peroxide production also helped us answer important questions we faced during the progress of this project (please see the Results and Discussion sections).

It is observed that assay for hydrogen peroxide is difficult in systems that contain NADH, because NADH interferes with some components of some commonly used assays for hydrogen peroxide [*Rapoport et al., 1994, Votyakova and Reynolds, 2004*]. Therefore, we used one of the few suitable detection methods [*Rapoport et al., 1994*], which is principled as follows:

Catalase converts hydrogen peroxide to water, where catalase is converted in this process to the so-called compound-1. Compound-1 can convert another molecule of hydrogen peroxide intro water plus oxygen, and compound-1 in this process is converted back to catalase. Alternatively, in the presence of a suitable substrate e.g. methanol, compound-1 can convert (oxidize) methanol to formaldehyde, and compound-1 in this process is also converted back to catalase. So, catalase can work as

a peroxidase i.e. uses hydrogen peroxide to oxidize some molecules e.g. methanol. Hence, the production of formaldehyde from methanol is dependent on the presence of hydrogen peroxide. Formaldehyde can by detected calorimetrically by reacting it with Nash's reagent [*Nash, 1953*], producing a yellow product that can be detected by observing the increase of its absorbance over a wide spectrum range (400-450 nm) (we used 405 nm) (Fig. 2-3 shows a diagram for the principle of this assay). In this assay, the blank was the reaction solution (HEPES-sol) free of reagents plus an equal volume of Nash's reagent.

The experiments were performed as follows: test cell-free tubes contained the indicated treatment compounds in HEPES-sol and left for 1 hr. After that methanol (to get 5% V/V) and catalase (to get 100 Units/ml) were added to each of these tubes and left for 10 minutes. After that an equal volume of Nash's reagent (which contains the following (in distilled water): ammonium acetate (150 mg/ml), acetic acid (0.3% V/V), acetyl acetone (0.2% V/V)) was added to each of these tubes and left for 40 minutes. After that, the mixture solutions in the test tubes were aliquoted into a 96-well plate with each mixture solution (i.e. treatment group) aliquoted into 4 wells, and then the optical density at 405 nm were taken on a plate reader. The readings of the 4 well for each group were averaged, and this average was considered n=1. Each experiment is repeated in 3 different days, and hence the figures legends of these experiments state that n=3.

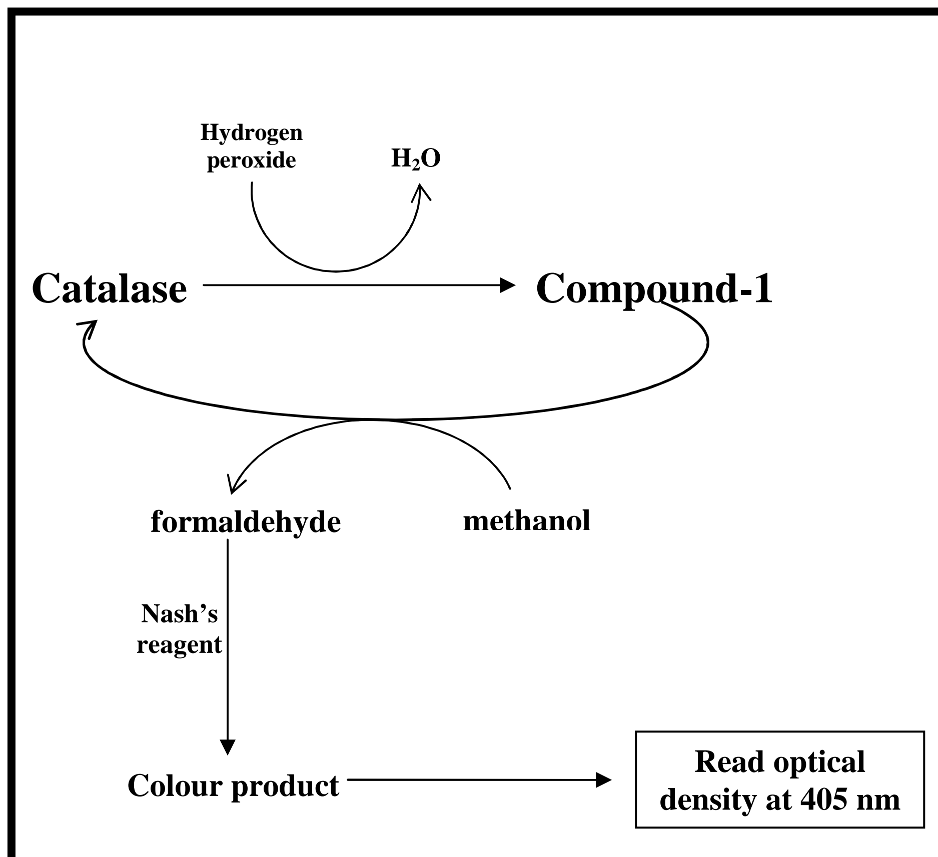


Figure 2-3: A diagram shows the principle of detecting hydrogen peroxide using catalase.

2.10 Statistical analysis

- Figures in the Results section show the mean values, where the error bars represent standard error of mean (S.E.M).
- If comparing one test group to the control group, a one sample t test was performed, where the mean of the test group was compared to a hypothetical mean of either 100 or zero depending on the expression of the treatment group. So when we wanted to express the value of the test group as viability relative to group A (i.e. control) (%), then the hypothetical mean was 100. On the other hand, when expressing the value of the test group as improvement in viability relative to group A (%), then the hypothetical mean was zero.
- If comparing more than one test group to the control group, a one-way ANOVA followed by Dunnette's multiple comparisons was performed.

- To determine the differences among more than two groups including the control group, a one-way ANOVA followed by Tukey's comparisons was performed.
- In the one experiment where only selected pairs of treatment groups were compared (Results section, Fig. 3-29), a one-way ANOVA followed by Bonferroni's comparisons was performed.
- In all tests, the difference between two groups was considered significant at $p \leq 0.05$.
- The n number that will be shown later in the figures of the Results section differs depending on whether the experiment is a viability (cell-containing) or a cell-free experiment. In viability experiments, the n number is the number of sets of cultures in which the experiment was repeated. Since every set of cultures is prepared in a separate week, this means that n=5 for example means that the experiment was repeated 5 times using 5 sets of cultures prepared in 5 different weeks. In cell-free experiments, the n number is the number of times in which the experiment was repeated using reagents diluted from the thawed aliquots of the stock solutions at each time. Since each experiment is performed in a different day, this means that n=3 for example is the number of the different days in which the experiment was repeated using reagents diluted from the thawed aliquots of the stock solutions at each day.

3 Results

3.1 Culturing and experimental conditions

3.1.1 Morphology of the cultures

The culturing process produced viable CGNs. Immediately after plating, the cells were round with no processes (Fig. 3-1). After 24 hours of plating, the cells showed extended processes, and tended to migrate and group with each other (Fig. 3-2). Also at this time, many cells appeared to be dead, even before addition of the cytosine arabinoside. After 8 days in cultures, the cells showed the known characteristic morphology of cultured CGNs (Fig. 3-3).

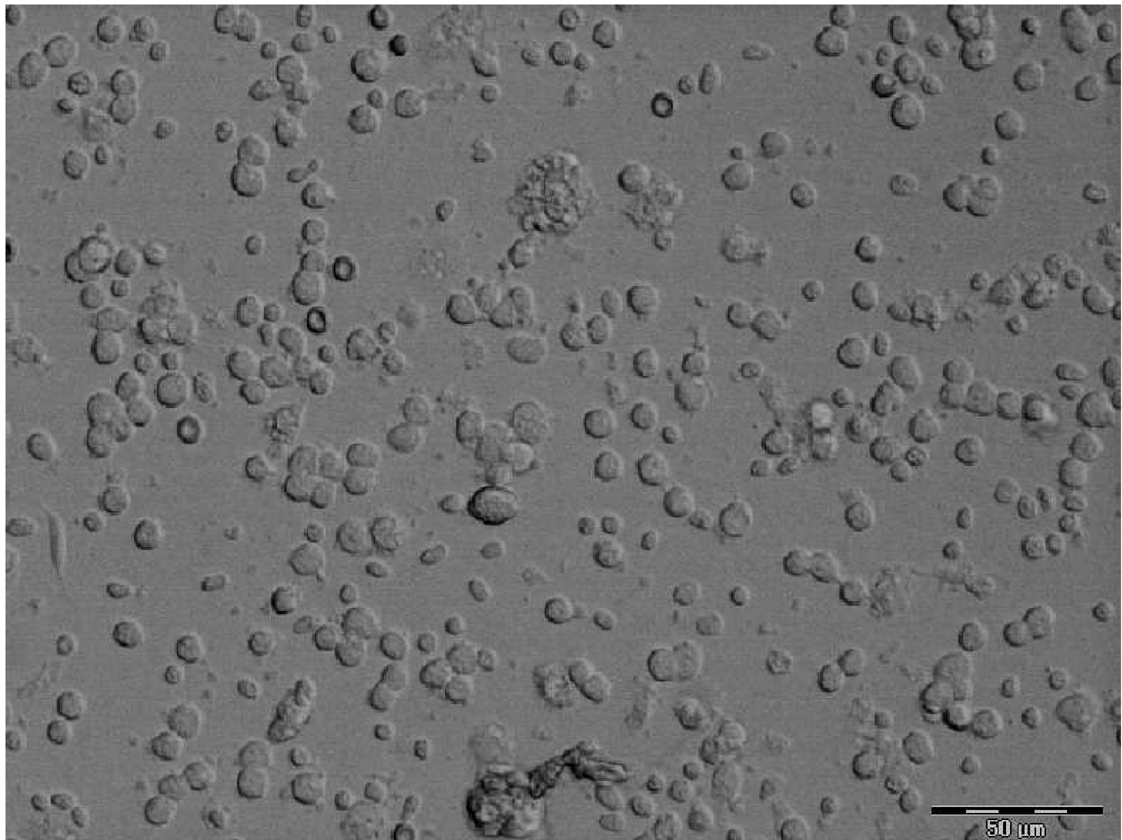


Figure 3-1: Morphology of the cultures immediately after plating. The cells look round with no processes. Scale bar = 50 µm.

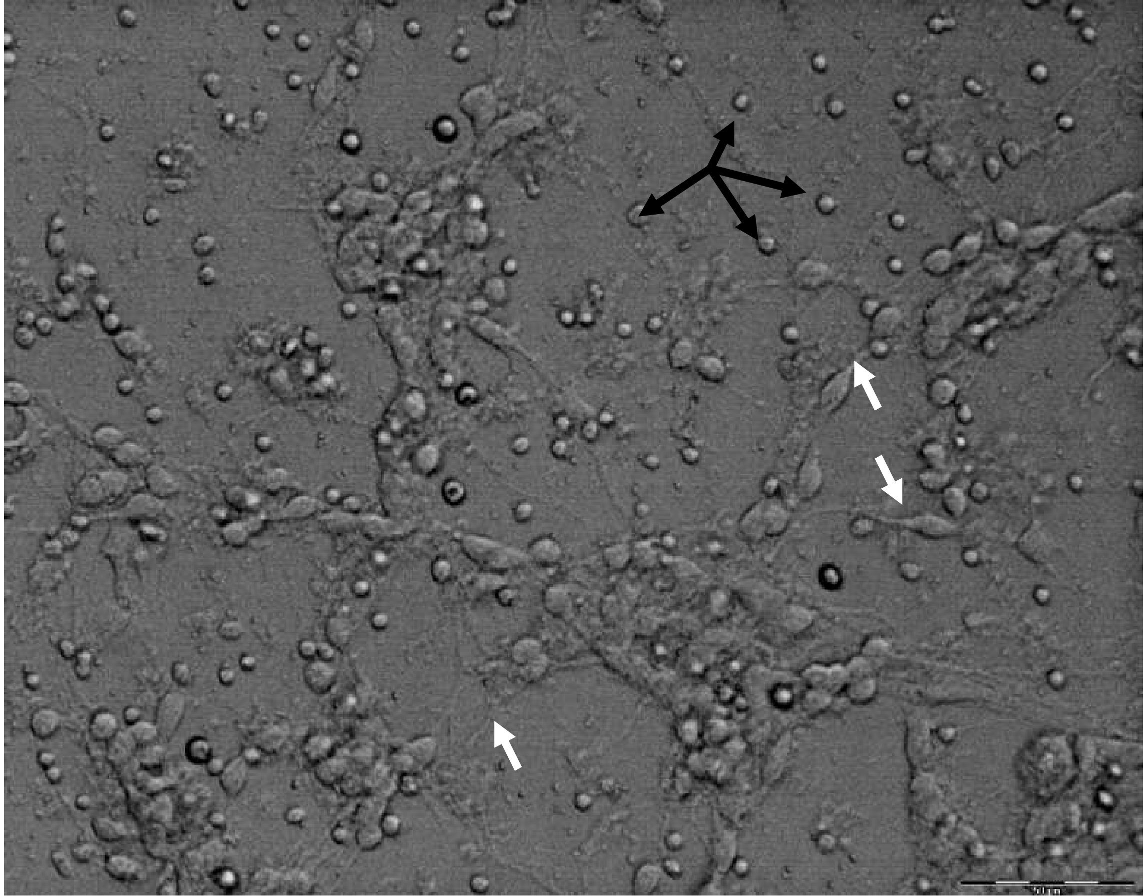


Figure 3-2: Morphology of the cultures 24 hr after plating. The cells began to show extend processes (white arrows), and tended to group with each other. Also, many dead cells were evident (black arrows). This photo was taken before adding cytosine arabinoside. Scale bar = 50 μm.

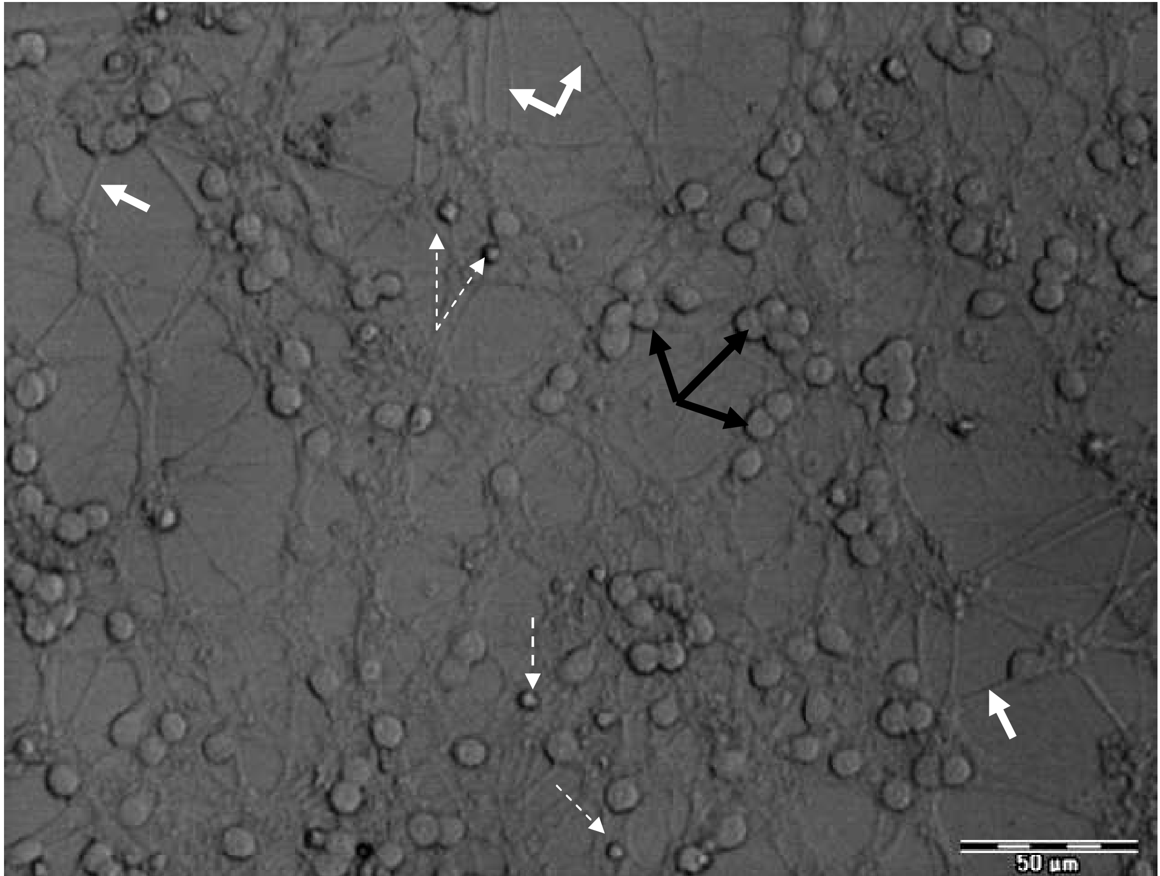


Figure 3-3: Morphology of the cultures after 8 days of plating. Black large arrows = cell bodies. White large arrows = processes. Also notice that most of those cells that died in the first 24 hours of plating (shown in Fig. 3-2) seemed to have disappeared, though some are still remaining (white dashed small arrows). Scale bar = 50 µm.

3.1.2 Effect of the position in the plate on the viability of cultures

In the initial stages of this project the neurons were plated into all wells in a 96-well plate (apart from the top left well and the well below it). After 8 days of plating, the neurons in the wells located at the edge of the plate (edge wells) (see Fig. 3-4) looked much stressed. The cultures in those wells did not look healthy and they contained a lot of debris. So, from the start of this project, the cultures in the edge wells were not included in the viability studies.

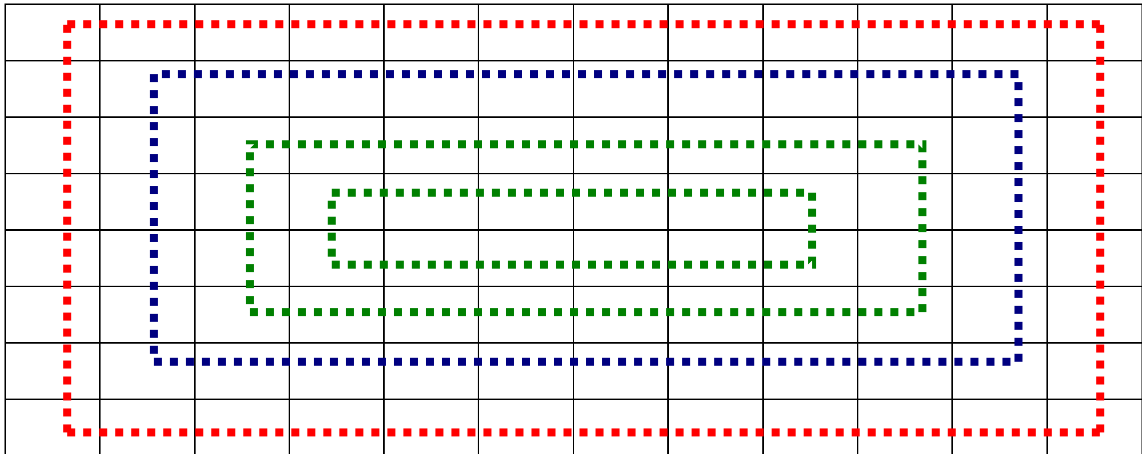


Figure 3-4: Diagram of a 96-well plate showing edge, next-edge, and inside wells. 1) edge wells dotted with red = 36 wells; 2) next-edge wells dotted with blue = 28 wells; 3) inside wells dotted with green = 32 wells.

In addition, since the cultures in the edge wells appeared much stressed, there was a possibility that the cultures in the wells located next to the edge wells (next-edge wells) (see Figures 3-4 and 3-5) were being affected by this effect, even though they did not appear to be stressed. So, an experiment was performed to compare the viability of the neurons in the next-edge wells with the viability of the neurons in the wells located inside the plate (inside wells) (see Figures 3-4 and 3-5). The result of this experiment is shown in Fig. 3-5.

From the upper diagram in Fig. 3-5 it is clear that the wells in both of group A and group F are next-edge wells, whereas the wells in groups B, C, D, and E are inside wells. This experiment was designed in a way that all treatment groups (A-F) were manipulated exactly the same way, leaving the only difference between them is their position in the plate (see Fig. 3-5 for the exact treatment procedure).

The result shows that, interestingly, although there was no statistically significant difference in the viability between group A and group F (the wells in these two groups are next-edge wells), there was statistically significant difference in the viability between any of the groups B, C, D, or E (the wells in these groups are inside wells) and group A (Fig. 3-5).

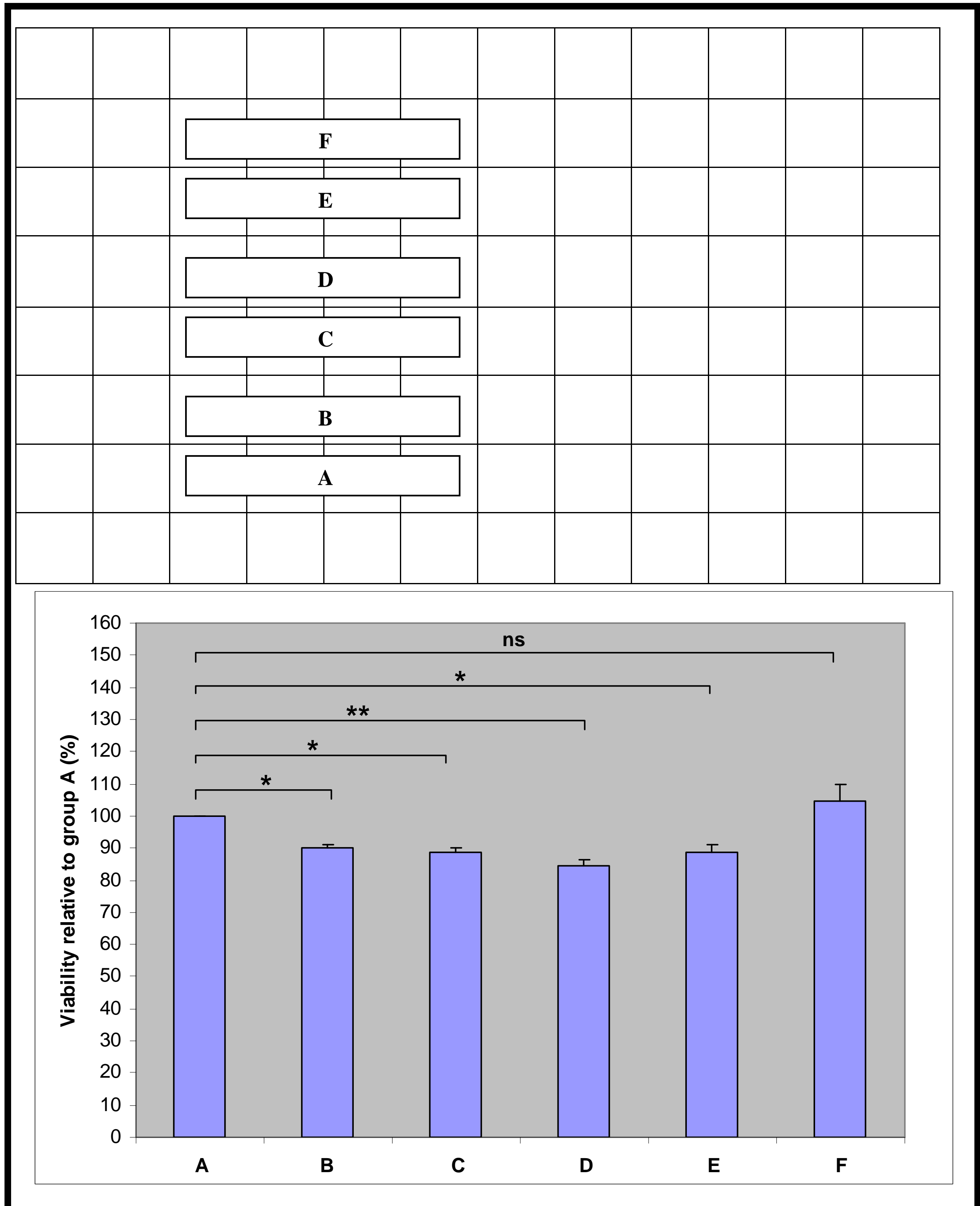


Figure 3-5: The effect of the position in the plate on the viability of neurons. The upper diagram shows the position of each group (where each group contained 4 wells) in the 96-well plate. The experiment was performed as follows: at day 8 or 9 after plating, culture medium was replaced by fresh culture medium and left for 1 hour (this was done to all groups) → the neurons were restored (after aspirating the fresh culture medium) to also fresh culture medium and left for 16-24 hours until the viability assay (this was done to all groups). So, the only difference between the groups was their position in the plate. This shows that neurons in next-edge wells (A & F) give slightly higher viability values than neurons in inside wells (B,C,D, and E). *p<0.05, **p<0.01, ns: not significant. (n=5).

Also when the groups in the plate were aligned vertically, there was a statistically significant difference in the viability between any of the inside groups and the next-edge group (Fig. 3-6). Importantly, there was no statistically significant difference when the viability of the inside groups were compared with each other (Fig. 3-6, see the note in the figure legend).

Therefore, the results in Figures 3-5 and 3-6 show that the cultures in the next-edge wells give consistently slightly (around 10-15 %) higher viability values (measured by Alamar blue assay) compared to the cultures in the inside wells. Also, it was noticed that this was reflected in the morphology of neurons seen under the microscope. However, it was not possible to rely on the morphological examination in this case i.e. it was not possible to judge that, based on the morphological examination, the neurons in the next-edge group looked undoubtedly more viable than the neurons in the inside groups. The reason is that, as noticed throughout this project, the morphological examination can be very helpful, and also very objective, only if the difference in the Alamar blue viability readings between two groups is high (≥ 20 %), in which case there will be observed in correlation a clear difference in the morphology of the neurons. On the other hand, if the difference in the Alamar blue readings is small (as in this experiment) there will be no completely clear (although it can be noticed) difference in the morphology, and the examination under the microscope may result in a subjective judgment. So, in this case, it was not possible to decisively confirm the Alamar blue viability readings by the morphological examination.

It should be noticed that this experiment was conducted before solving the problem of fresh culture medium toxicity, and since fresh culture medium was used in this experiment, the neurons were likely affected by glutamate (already present in the fresh culture medium) excitotoxicity (see Discussion for the implication of this fact).

Since the difference in the viability reading between the next-edge group and any of the inside groups was statistically significant, and also since the difference in the viability of the inside groups (aligned vertically) when compared with each other was not statistically significant (Fig. 3-6), it was decided not to include cultures in the next-edge wells in the experiments conducted in the rest of this project, and only cultures in inside wells were included. Therefore, in the experiments conducted afterwards, the neurons were plated into only the inside wells, and a cell-free medium was added to both of the

edge wells and next-edge wells. Also in the experiments conducted afterwards, it was decided to align the treatment groups vertically, where each group contained 4 wells, which means that it was possible to use maximum of 8 treatment groups with each group containing 4 wells (Fig. 3-7).

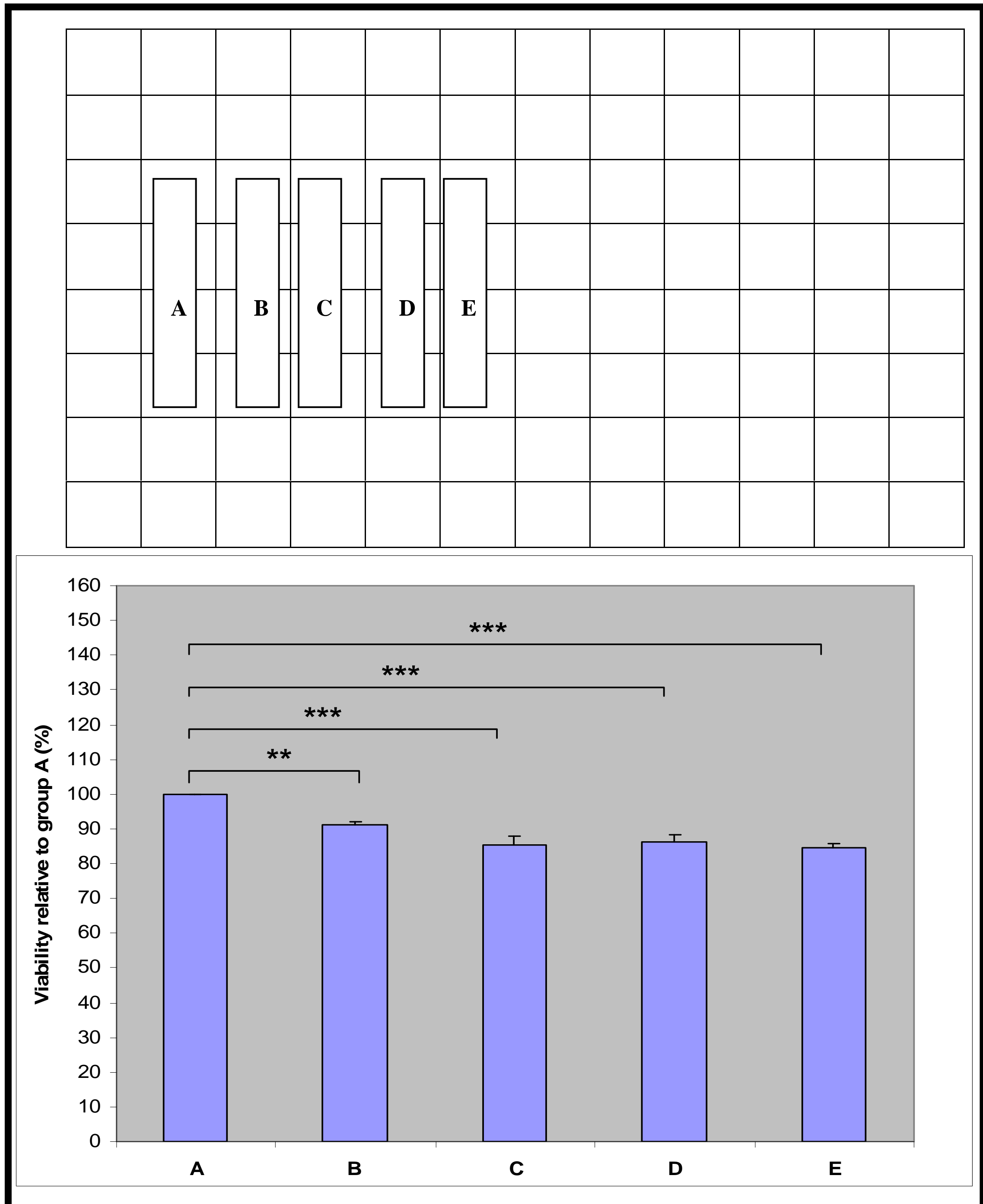


Figure 3-6: The effect of the position in the plate on the viability of neurons with the groups aligned vertically in the 96-well plate. The upper diagram shows the position of each group (where each group contained 4 wells) in the 96-well plate. The experiment was performed as follows: at day 8 or 9 after plating, the culture medium was replaced by fresh culture medium and left for 1 hour (this was done to all groups) → the neurons were restored (after aspirating the fresh culture medium) to also fresh culture medium and left for 16-24 hours until the viability assay (this was done to all groups). So, the only difference between the groups was their position in the plate**. Note:** there was no statistically significant difference when the viabilities of the inside groups (i.e. B, C, D, and E) were compared with each other. **$p<0.01$, ***$p<0.001$. (n=5).

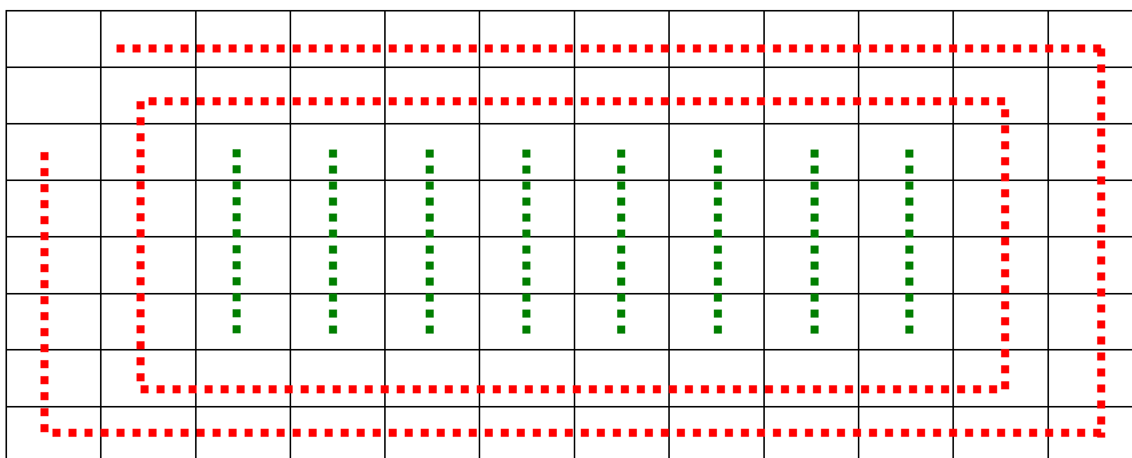


Figure 3-7: alignment of treatment groups in the viability studies that were performed after finding that next-edge group gives higher viability values than inside groups. So, in the experiments conducted afterwards, the neurons were being plated into only the inside wells (dotted in green), and cell-free medium was being added to both of the edge wells and next-edge wells (dotted in red). Also the treatment groups were being aligned vertically, as shown, where each group contained 4 wells. Notice that both of the well at the left upper corner of the plate and the well below it were always left empty at the plating day (i.e. day zero) because they were needed for the viability assay at day 9 or 10 (see Materials and Methods).

3.1.3 Toxicity of fresh culture medium

The biggest problem encountered with CGNs cultures in the early stage of this project was the severe and immediate damage to the neurons (including those in the control groups) induced, during the experimental day i.e. day 8 or 9, by manipulating the cultures (i.e. aspirating the culture medium, adding treatment and control vehicles, and adding restoration medium). Several months were spent before solving this problem, and it turned out that it was due to glutamate excitotoxicity (through activating its N-methyl-D-aspartate (NMDA) receptors). The source of glutamate was the fresh serum (which we were unaware of its content of glutamate) which is present at 10% V/V in the fresh culture medium that is added to the cultures at that day. Fresh culture medium addition occurred at the experimental day for two purposes; (i) as a vehicle that contains (treatment) or does not contain (control) the test compounds, and (ii) after that it is used as the restoration medium following treatment. Immediately after performing the experiment in which we discovered the role of NMDA receptors, we found an early report in the literature which showed that an NMDA receptor-mediated action, likely through the activation of these receptors by glutamate already present in the fresh serum, is responsible for fresh serum toxicity in CGNs cultures [*Schramm et al., 1990*]. We had, therefore, reached the same conclusion independently. In this section, there

will be shown some of the observations/experiments that either led eventually to discovering the reason of fresh culture medium toxicity or were explained only after discovering that reason. After that, there will be shown another set of experiments that were conducted in order to add more proof to the conclusion that we and others reached. Notice that the experiments whose results are shown in this section (i.e. Section **3.1.3**) were performed after finding that the neurons in next-edge wells give consistently higher viability reading than neurons in inside wells, so in these experiments only inside wells were used, which gives more validity to their results.

3.1.3.1 Was fresh culture medium responsible for the death?

The culture medium did not seem to kill the neurons when they were maintained in it before the intervention day. Although there were many cells that died in the first 24 hr of plating, fresh culture medium used to plate the cultures at day zero was not suspected to cause this death for two reasons. Firstly, this culture medium is universally used to culture many types of cells including neurons, and there was no reason to suspect that it might be toxic. Secondly, it indeed did not kill the viable neurons when they were maintained in it from day 1 until the intervention day.

This was the reason that in the beginning a toxic effect of the added fresh culture medium was not suspected to cause the damage observed at the intervention day, where alternative explanations were explored at that time. One of the explanations explored was that, from day zero to the experiments day, the neurons release growth factors that in turn maintain their viability, where the neurons become dependent on those factors, and when the medium that the neurons are maintained in is aspirated at the experiments day, the neurons die due to growth factor withdrawal. However, it was not feasible to test this explanation in the context of this project.

3.1.3.2 Previous projects

A strange observation was that the damage induced to the cultures at the experiments/intervention day was severe in this project but not severe in two previous projects undertaken in this laboratory [*Fatokun, 2006, Smith, 2008*]. This turned out to be related to the cell density obtained after plating at day zero (higher in this project than in these previous two projects, see Discussion).

3.1.3.3 Effect of pH

A reason for suspecting the fresh culture medium added at the experimental day as the reason for the seen cell death was the consistent observation that the damage tends to be more severe if this fresh culture medium was looking more pinkish i.e. more alkaline. The reason for the fresh culture medium getting alkaline is that this medium bottle was being opened many times (to take out the needed amount at each time), which was likely causing release of CO_2 from the medium, and since bicarbonate (HCO_3-) does not get released like CO_2, this will result in the presence of more bicarbonate that is not balanced by CO_2, which will result in the increase of the medium pH.

To overcome this problem, a modification was performed by keeping the fresh culture medium in a vented cap flask i.e. permeable to gases (not a closed cap bottle as before) and keeping it in the incubator (not in the water bath as before). This meant that any CO_2 that is released from medium by taking the flask out of the incubator (and opening the flask cap to take out the needed amount of medium) gets quickly replenished when the flask is returned to the incubator. This was evident by the observation that the medium colour (i.e. Phenol red colour) was kept constant all the time under this modification. This resulted in considerable improvement in the viability of cultures and made it possible to conduct reproducible experiments.

Despite the considerable improvement in the viability by the close adjustment of pH, the problem of the fresh culture medium toxicity added at the experimental day remained, and the damage was still severe. Also, there was an observation that was not possible to explain, which was that when a plate containing the cultures was placed outside the incubator for more than an hour, although this made the culture medium that the neurons were maintained in very pinkish (i.e. very alkaline) and although this also likely reduced the temperature of the cultures to the room temperature, this did not damage the neurons (at least there was no immediate damage observed). So, it seemed that although the increase in the pH per se (at day 8 or 9) of the culture medium that the neurons are maintained in does not kill the neurons, the increase in pH of the fresh culture medium added at that day potentiates its toxicity.

3.1.3.4 Effect of Ethanol and Dimethyl Sulfoxide (DMSO)

In parallel to the efforts to know the reason of cell death induced at the experimental day, there were also some experiments set out to study oxidative stress models that involved addition of different test compounds. Ethanol and dimethyl sulfoxide (DMSO) were used at some stage as hydroxyl radical scavengers, but it was necessary to check the effect of applying them alone to CGNs before testing them against any hydroxyl radical-producing insult. So the following experiments with ethanol and DMSO were not intentionally designed to investigate fresh culture medium toxicity. Surprisingly, instead of decreasing the viability of the neurons or having no effects, increasing concentrations of ethanol or DMSO tried alone (both added as V/V in fresh culture medium) resulted in increased improvement in cell viability i.e. these compounds attenuated the toxicity of the fresh culture medium.

Ethanol at 1.5 % V/V attenuated the toxicity of fresh culture medium (Fig. 3-8). Notice that in this figure (and some other subsequent figures), the y axis is not the viability relative to group A (%), but is rather the improvement in viability relative to group A (%). Since there will be no improvement in viability of group A relative to itself, column A gives zero value, as shown (so, zero does not mean that the cultures in group A are completely dead). Although DMSO at 0.1 or 1% V/V had no effect on the toxicity of fresh culture medium (data not shown), DMSO at 5 or 10% V/V greatly attenuated the toxicity of fresh culture medium (Fig. 3-9).

It is very likely that the attenuation of fresh culture medium toxicity by these two compounds was real and was not due to an artefact in the Alamar blue viability assay, for many reasons. The attenuation of the toxicity by these two compounds was undoubtedly clear in the morphological examination e.g. when Alamar blue assay indicated that ethanol protected at 1.5% V/V more than at 0.3% V/V, it was observed in correlation under the microscope that there was undoubtedly very little damage in the 1.5% V/V group compared to the 0.3% V/V group, and that there was less damage in the 0.3% V/V group compared to the group where no ethanol was added (i.e. group A). Also, it is unlikely that Alamar blue was reacting with these compounds, since at the end of treatment period, the treatment medium that contained these compounds was aspirated and the neurons were restored to fresh culture medium for at least 16 hr of

restoration period before adding Alamar blue. It is even unlikely that Alamar blue was interfering indirectly with these compounds through interfering with their delayed effects since the neuronal damage/morphology seen under the microscope stabilized within 8 hr of the restoration period (and cells did not deteriorate or recover after that) i.e. the damage/morphology stabilized at least 8 hr before adding the Alamar blue.

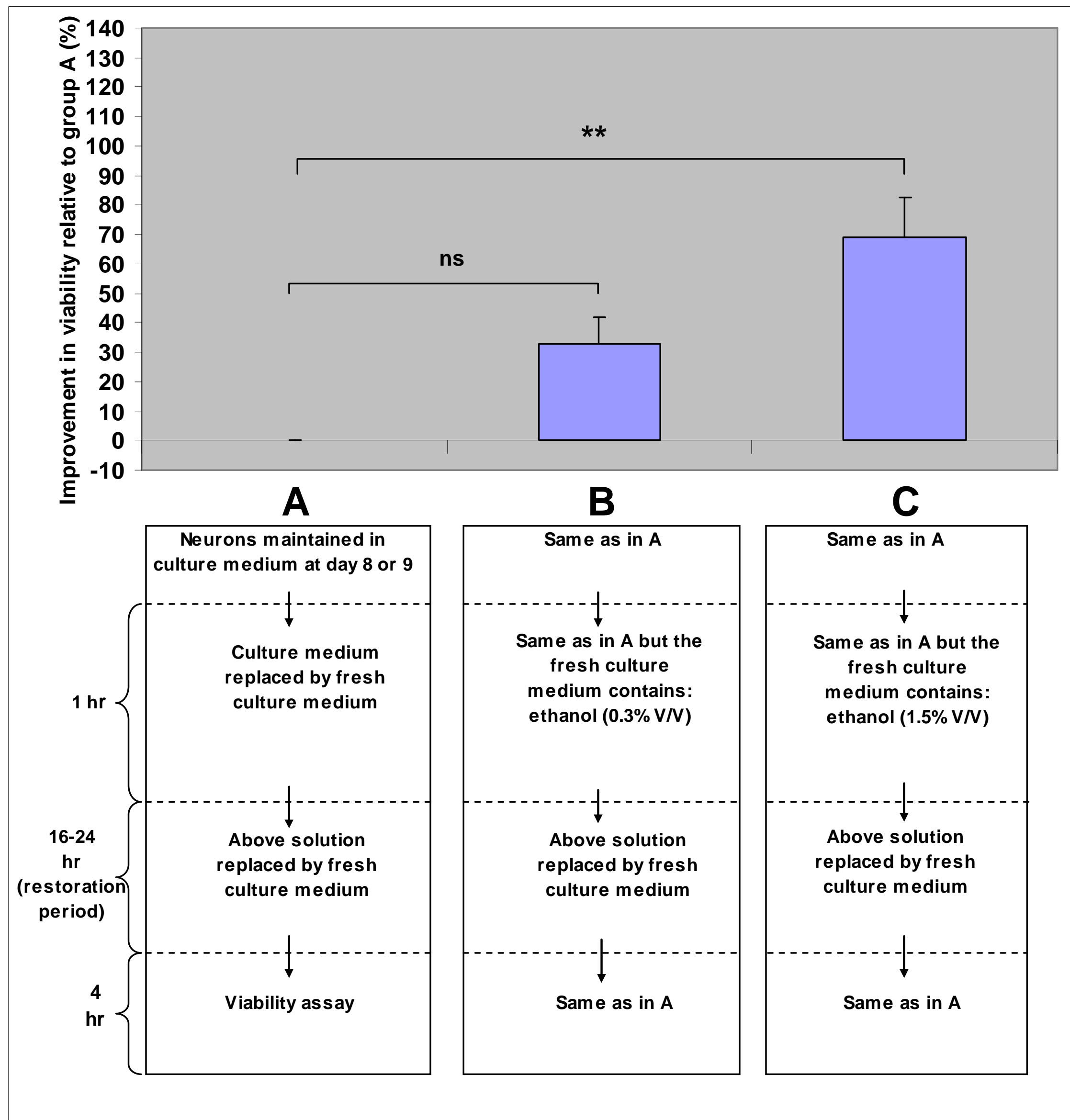


Figure 3-8: Effect of ethanol on fresh culture medium toxicity. It shows that ethanol, probably through blocking NMDA receptors, protects against fresh culture medium toxicity in a dose dependent manner. $^{**}p<0.01$, ns: not significant. (n=5).

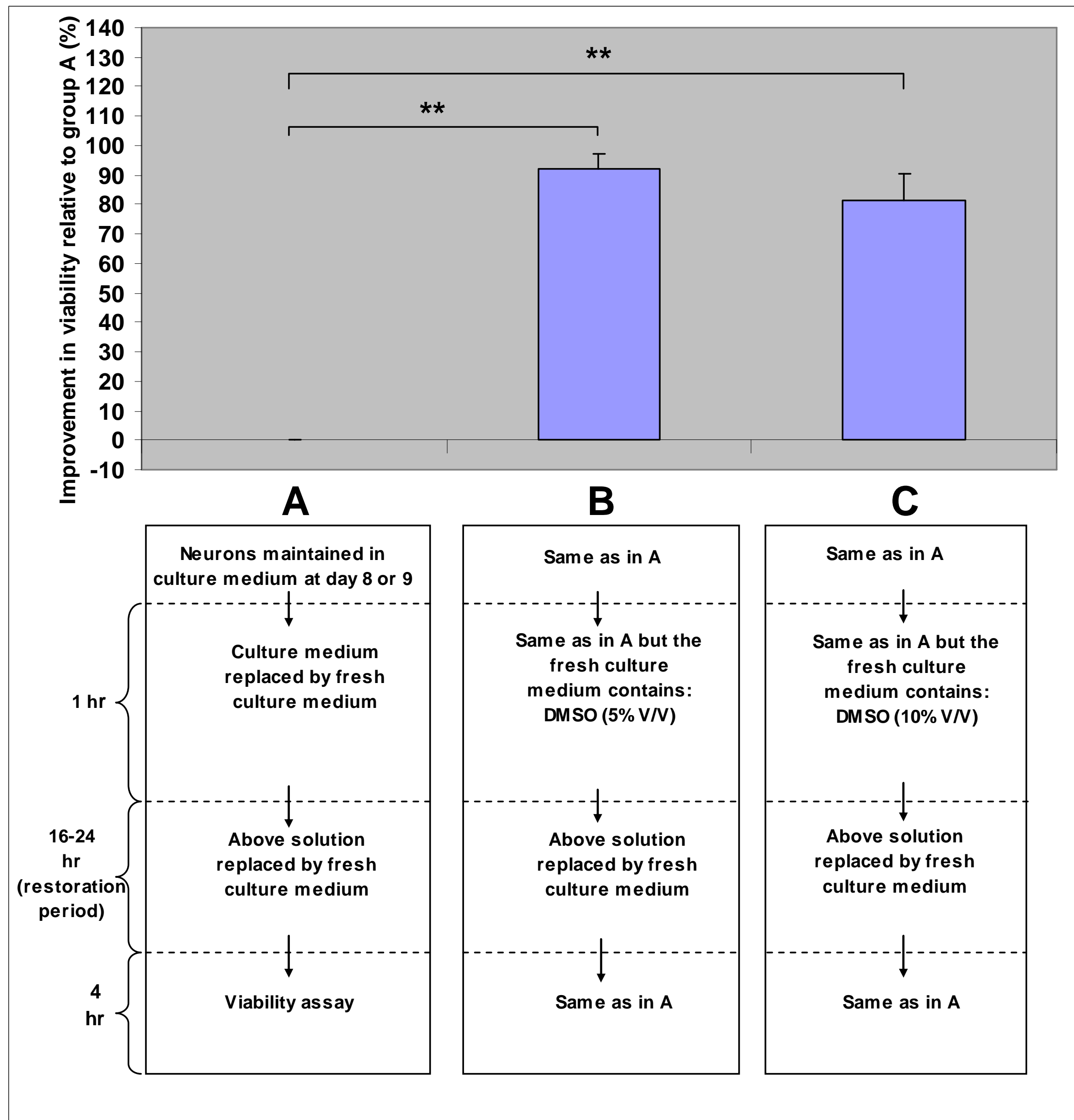


Figure 3-9: Effect of DMSO on fresh culture medium toxicity. It shows that DMSO, probably through blocking NMDA receptors, protects substantially against fresh culture medium toxicity. **$p<0.01$. (n=3).

3.1.3.5 NMDA receptors

Because it was very likely that the serendipitously discovered protection of ethanol and DMSO against fresh culture medium toxicity was real and not due to an artefact in the Alamar blue assay, it was expected that if the reason(s) of their protective effect is revealed, this may lead to identifying and solving the problem of fresh culture medium toxicity. After searching out the literature, it was found that ethanol can block NMDA receptors in CGNs and some other types of neurons [*Lin et al., 2003, Dildy and Leslie, 1989, Lovinger et al., 1989, Cebere and Liljequist, 2003*]. This effect of ethanol was shown

to inhibit the toxicity of NMDA [*Danysz et al., 1992, Cebere and Liljequist, 2003*]. It was also found in the literature that DMSO prevents both the action and excitotoxicity of glutamate in hippocampal neuronal cultures in a dose dependent manner [*Lu and Mattson, 2001*]. Therefore, a specific NMDA receptor blocker, MK-801 (20 μM), was tried and found to provide substantial protection against fresh culture medium toxicity (Fig. 3-10), which was also reflected in the morphological examination (Fig. 3-11). Also MK-801 at a very low concentration (20 nM) provided large and statistically significant protection (improvement in viability relative to the viability in the absence of MK-801 was 59.4% ±10.1, $p<0.05$, n = 4).

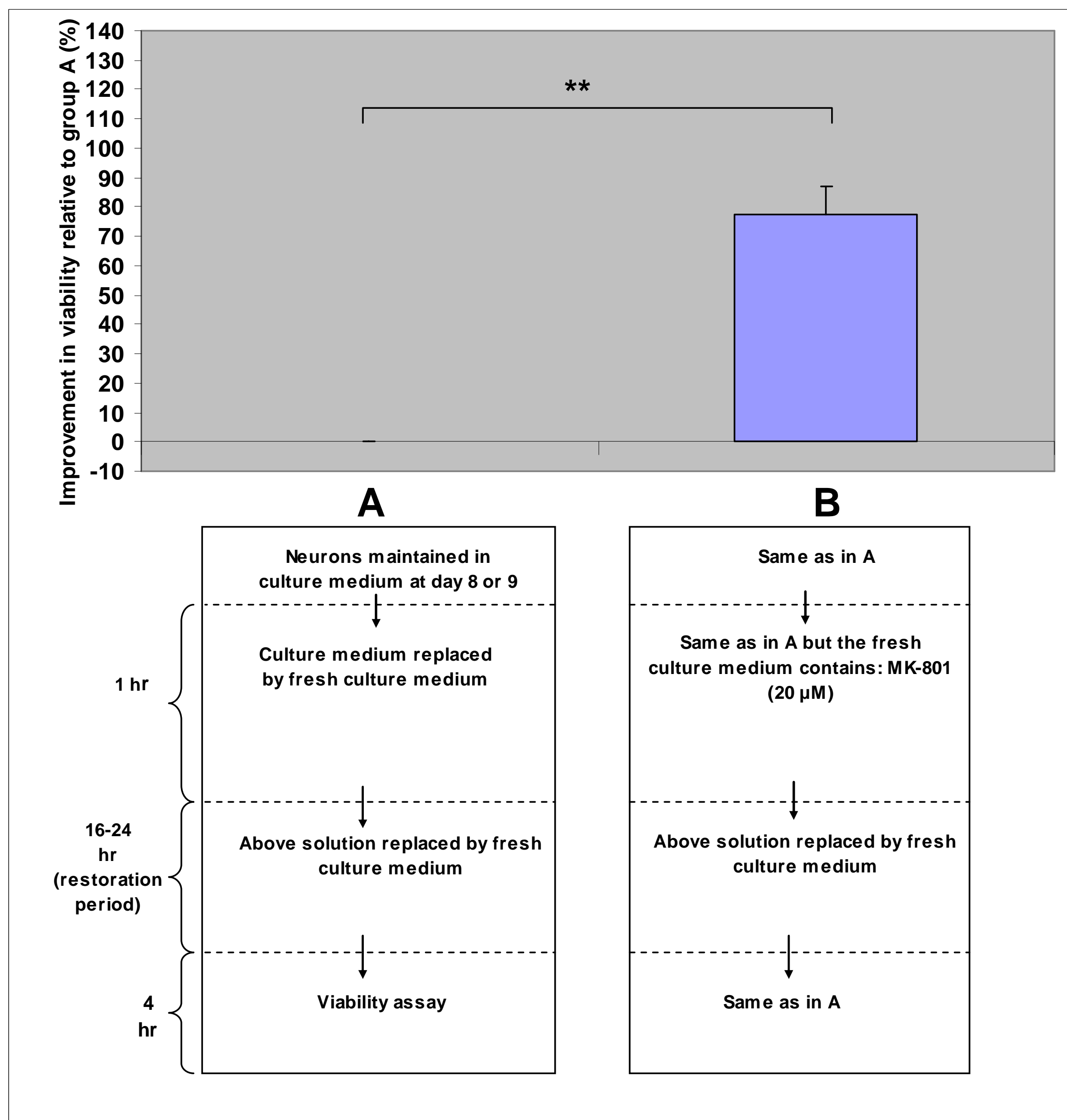


Figure 3-10: Effect of MK-801 on fresh culture medium toxicity. It shows that MK-801, a specific NMDA receptor blocker, blocks fresh culture medium toxicity. **$p<0.01$. (n=5).

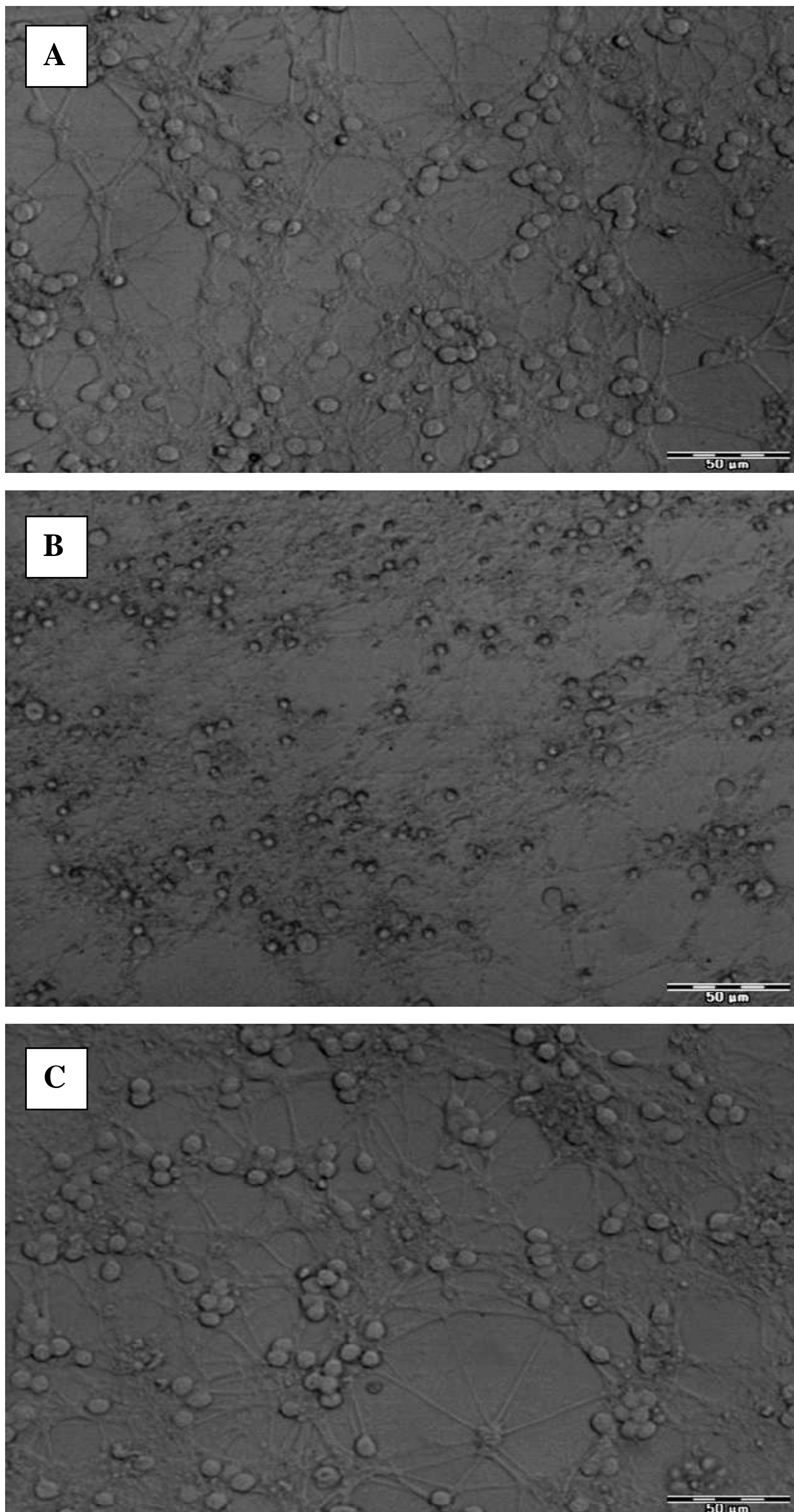


Figure 3-11: Photos of CGNs showing protection by MK-801 against fresh culture medium toxicity. A: CGNs culture not subjected to any intervention (i.e. were not touched). All areas in wells in this group were as healthy as the shown area. **B:** CGNs cultures exposed to fresh culture medium at day 8 after plating: severe damage to the cell bodies and processes is evident. Notice that there were still some viable cell bodies. There were some areas in the wells (not shown) (around 20-30 % of the areas) in this group that were healthy (not damaged like the shown area). **C:** CGNs cultures exposed to fresh culture medium at day 8 after plating but with MK-801 (20 µM) added. All areas in wells in this group were as healthy as the shown area. Scale bar = 50 µm. For the experimental design, see Fig. 3-10.

As mentioned at the beginning of this section, this role of NMDA receptors was discovered before finding that Schramm and co-workers (1990) reached a similar conclusion. In the following series of experiments, more proof is added.

3.1.3.6 Kynurenic acid

In addition to MK-801, another blocker of NMDA receptors, kynurenic acid, was tried. Kynurenic acid is an endogenous metabolite known to block NMDA receptors [*Perkins and Stone, 1982, Fatokun et al., 2008b*], and although this effect is relatively weak in cell cultures [*Hilmas et al., 2001*], the mechanism of blocking NMDA receptors by kynurenic acid is somewhat different from that of MK-801 [*Fatokun et al., 2008b*]. Thus it was useful to try it to see if protection against fresh culture medium toxicity can be provided by two different ways of blocking NMDA receptors. When tried, it provided protection in a dose dependent manner (Fig. 3-12).

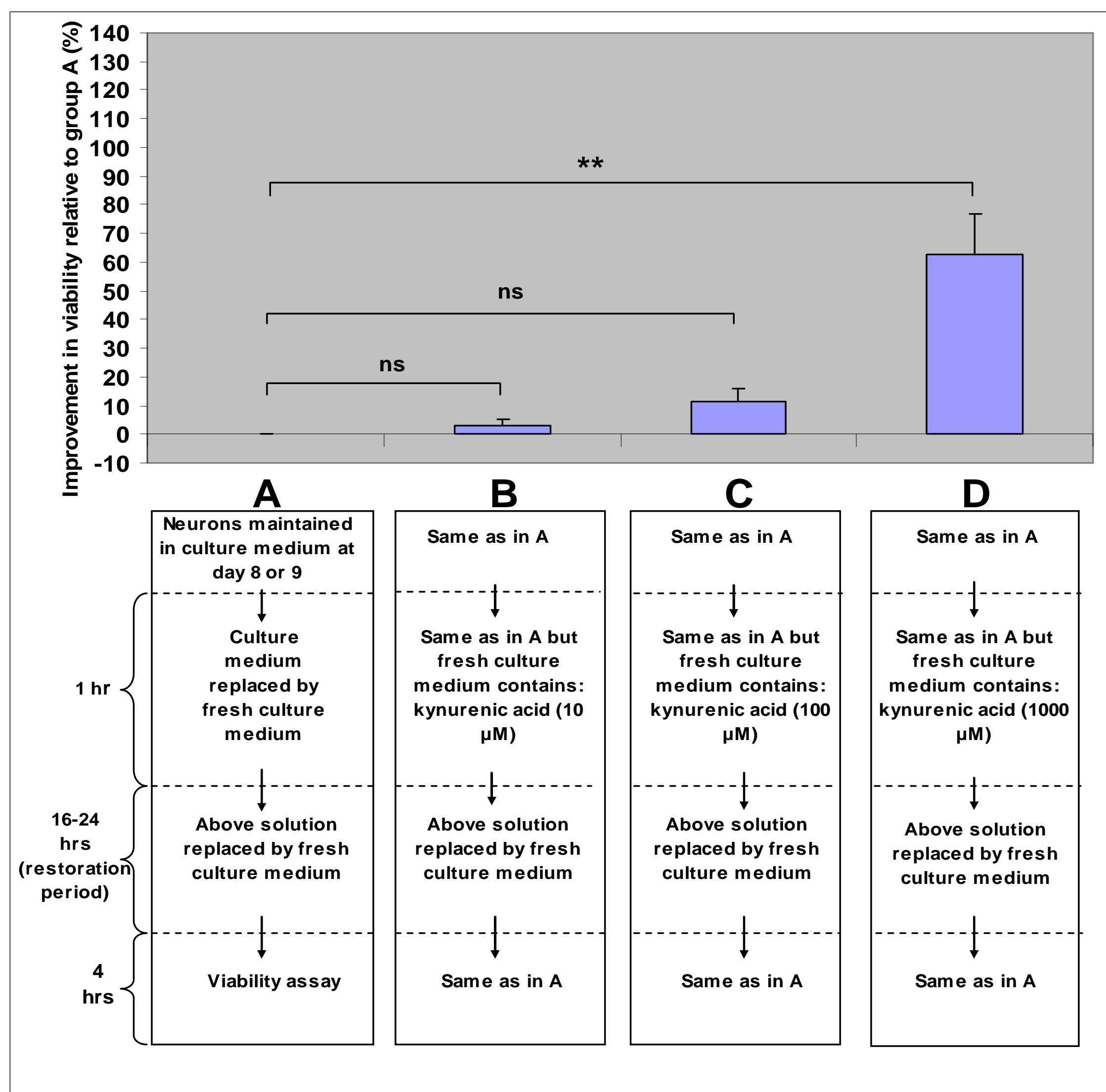


Figure 3-12: Effect of kynurenic acid (KA) on fresh culture medium toxicity. It shows that KA, a blocker of the NMDA receptors, protects against fresh culture medium toxicity in a dose dependent manner. $^{**}p<0.01$, ns: not significant. (n=4).

3.1.3.7 Glutamic-pyruvic transaminase

It was thought that if glutamate already present in the fresh culture medium was responsible for its toxicity, then degrading glutamate should prevent the toxicity. When tried, a glutamate degrading enzyme, glutamic-pyruvic transaminase [*Matthews et al., 2000*], provided substantial protection (Fig. 3-13). Likely because this enzyme requires its other substrate, pyruvate, the enzyme alone (or pyruvate alone) was not protective, and the protection was provided only in the presence of both of the enzyme and pyruvate (Fig. 3-13). In this experiment, the treatment period (24 hr) was immediately followed by the viability assay i.e. there was no restoration period. This was done to avoid, after degrading glutamate, exposing the neurons before the viability assay to fresh culture medium (which contains glutamate). Since Alamar blue is always added to the medium around neurons, this experimental design (it does not apply to the other experiments) means that Alamar blue was present together with the test compounds (the enzyme and pyruvate). However, it is unlikely that the protection by this enzyme was due to an artefact due to interaction between Alamar blue and the enzyme (or pyruvate), for two reasons. First, the substantial protection by the enzyme was undoubtedly reflected in the morphological examination of the neurons (long before adding Alamar blue). Secondly, the enzyme alone or pyruvate alone did not show significant difference in the Alamar blue reading compared to its reading in their absence (Fig. 3-13).

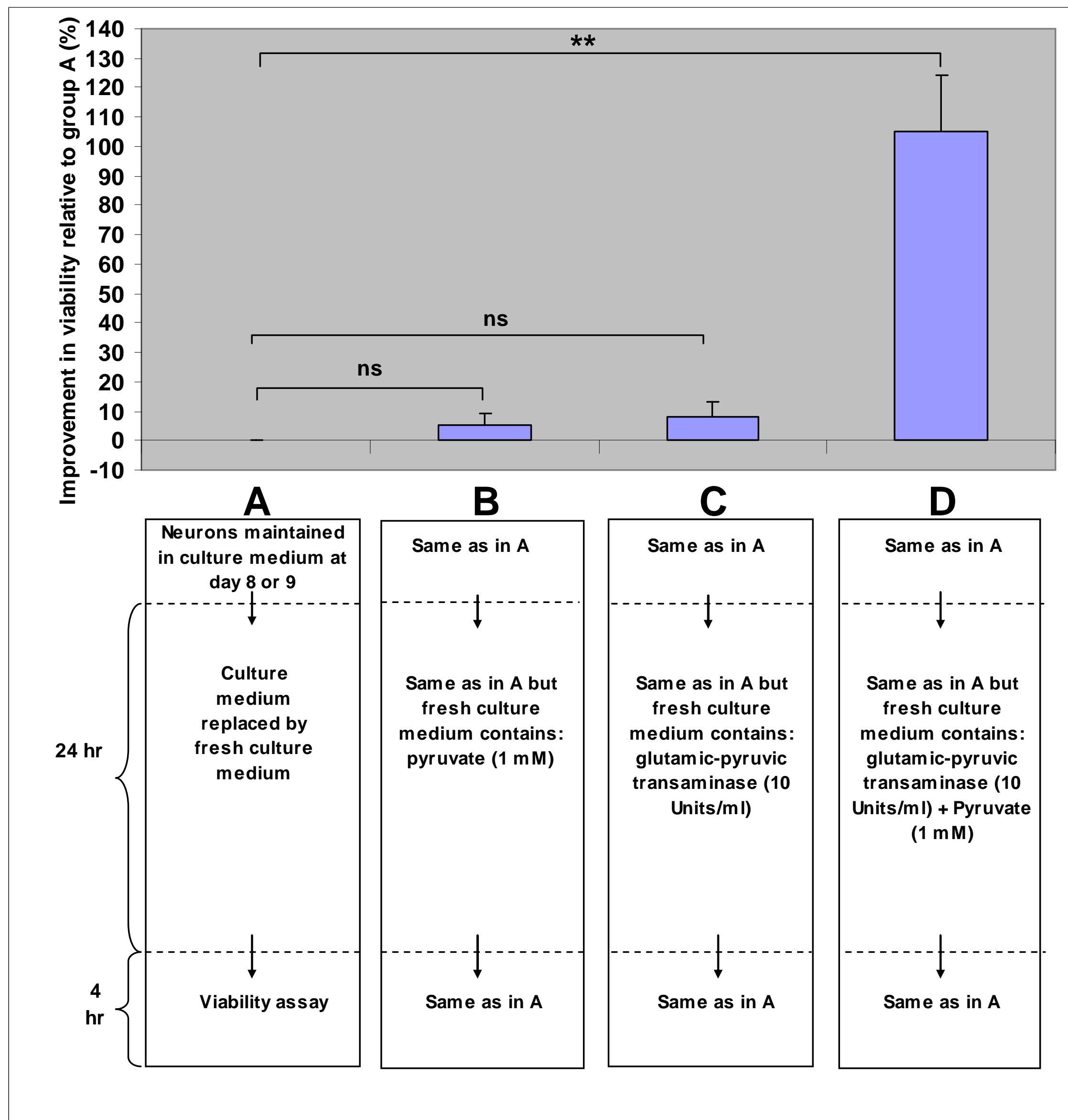


Figure 3-13: Effect of glutamic-pyruvic transaminase on fresh culture medium toxicity. It shows that degrading glutamate by this enzyme substantially protects against fresh culture medium toxicity. It also shows that pyruvate is a required co-substrate for the enzyme to degrade glutamate. **$p<0.01$, ns: not significant. (n=5).

3.1.3.8 Protection by pre-treatment with reduced amount of fresh culture medium

Since acute pre-treatment (minutes/hours) with a subtoxic concentration of glutamate in CGNs is known to protect against a subsequent lethal exposure to glutamate itself [*Marini and Paul, 1992*] (acute preconditioning effect), and since the fresh culture medium used here contains glutamate, it was sought to see if pre-treatment with reduced amount of fresh culture medium protects against a subsequent exposure to a lethal amount (full amount) of fresh culture medium itself, which can add more proof to the

conclusion that glutamate already present in fresh culture medium is responsible for its toxicity. Interestingly, this was found to be the case (Fig. 3-14).

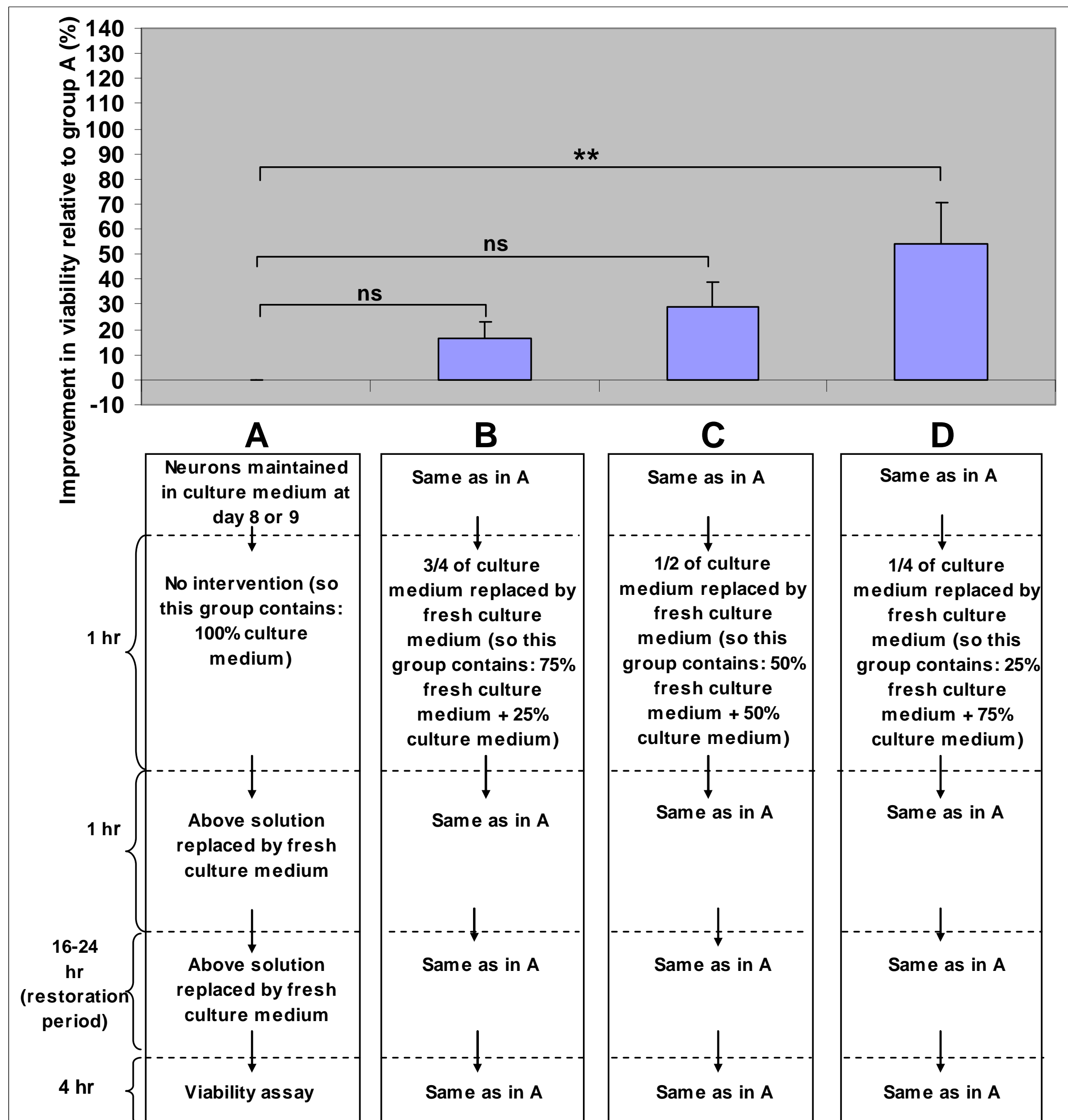


Figure 3-14: Effect of pre-treatment with reduced amount of fresh culture medium on the toxicity of a full amount of fresh culture medium itself. It shows that as the amount of fresh culture medium added as pre-treatment is reduced more and more, the protection against a subsequent lethal (full) mount of fresh culture medium increases. **$p<0.01$, ns: not significant. ($n=5$).

3.1.3.9 Further investigation

The experiment shown below in Fig. 3-15 shows many things at once (notice that the y axis in this figure shows the viability relative to group A (%) (and not the improvement in viability relative to group A (%)). It shows that physical intervention (through aspirating and replacing solutions) per se does not kill the neurons, evident by that groups B and C which were subjected to physical interventions (but did not contain fresh culture medium) gave the same viability values as group A which was not subjected to any physical intervention (i.e. was not touched). Therefore, fresh culture medium (in particular glutamate already present in it) seems to be required for the neurons to die (group D). This figure also shows that 1 hr as an exposure time is enough for the fresh culture medium to cause significant toxicity (group D, notice that after 1 hr exposure, the fresh culture medium was replaced by conditioned medium). Also, it is clear from groups B and C that restoring the neurons to conditioned medium (see Materials and Methods) does not cause toxicity. This was an important finding, and in all the subsequent viability experiments in this project, the neurons were restored to conditioned medium at the end of the treatment period.

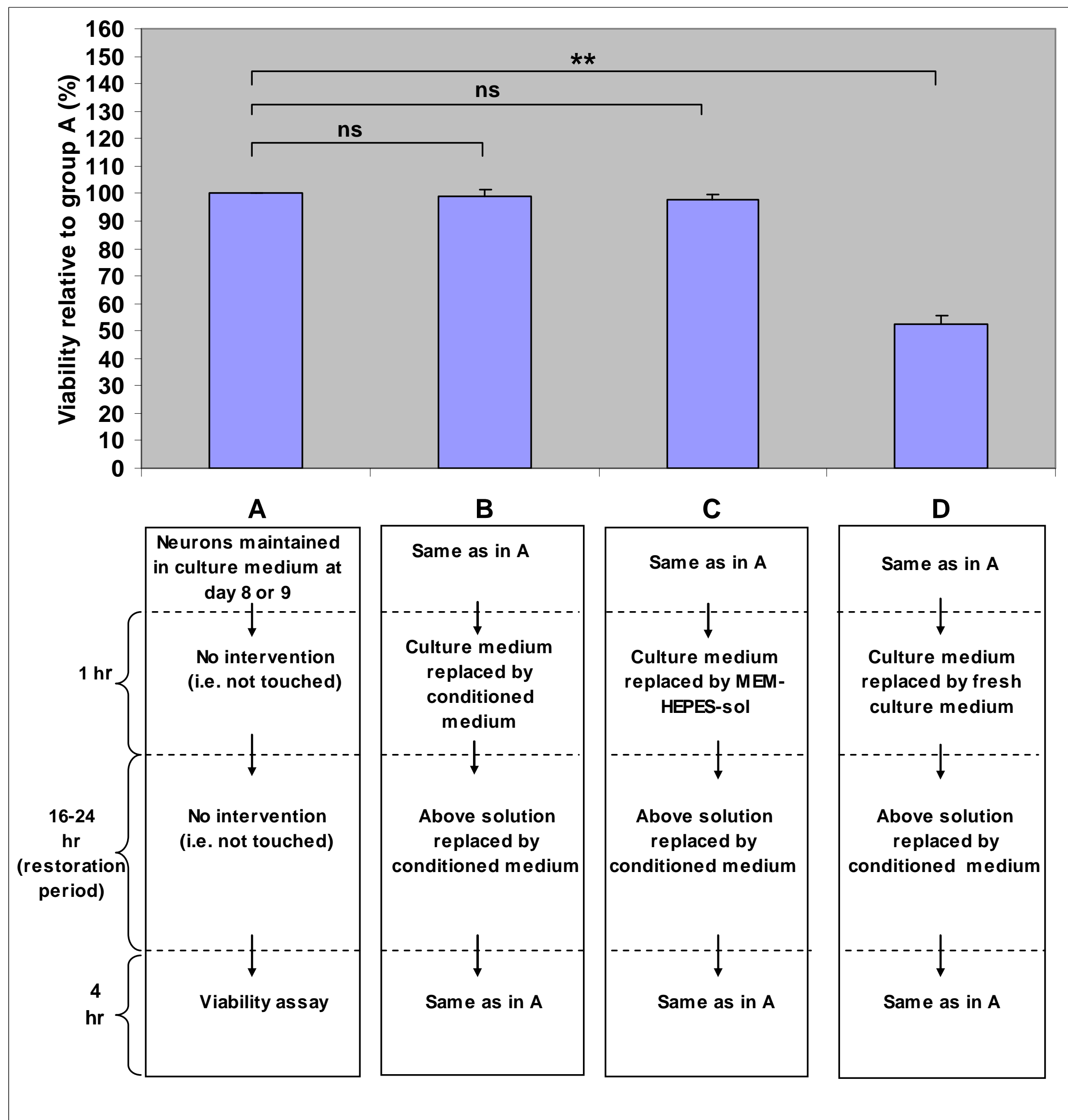


Figure 3-15: Further investigation of fresh culture medium toxicity. See explanation in the text. **p<0.01, ns: not significant. (n=5).

3.1.3.10 Externally added glutamate

Taking together, all the above experiments (in section **3.1.3**) build strong evidence that glutamate already present in fresh culture medium was responsible, through activating NMDA receptors, for this medium toxicity at the intervention day. The last experiment performed in order to add more proof to this conclusion was externally applying glutamate to the neurons, which was also a necessary experiment to show that these CGNs are indeed susceptible to glutamate excitotoxicity. When glutamate (300 µM) was applied in a glutamate-free solution (MEM-HEPES-sol) for only 1 hr, it caused significant toxicity to the neurons (Fig. 3-16). Notice that MEM-HEPES-sol does not contain serum, which means that glutamate does not require co-application of serum for

its toxicity (although serum can potentiate its toxicity as was shown in a previous report [*Eimerl and Schramm, 1991*]). There was also toxicity when the experiment was repeated but with glutamate applied at 10 times lower concentration i.e. 30 µM (Mean viability was 70.8% ± 5 of the viability in the absence of glutamate. $p<0.05$, n = 4).

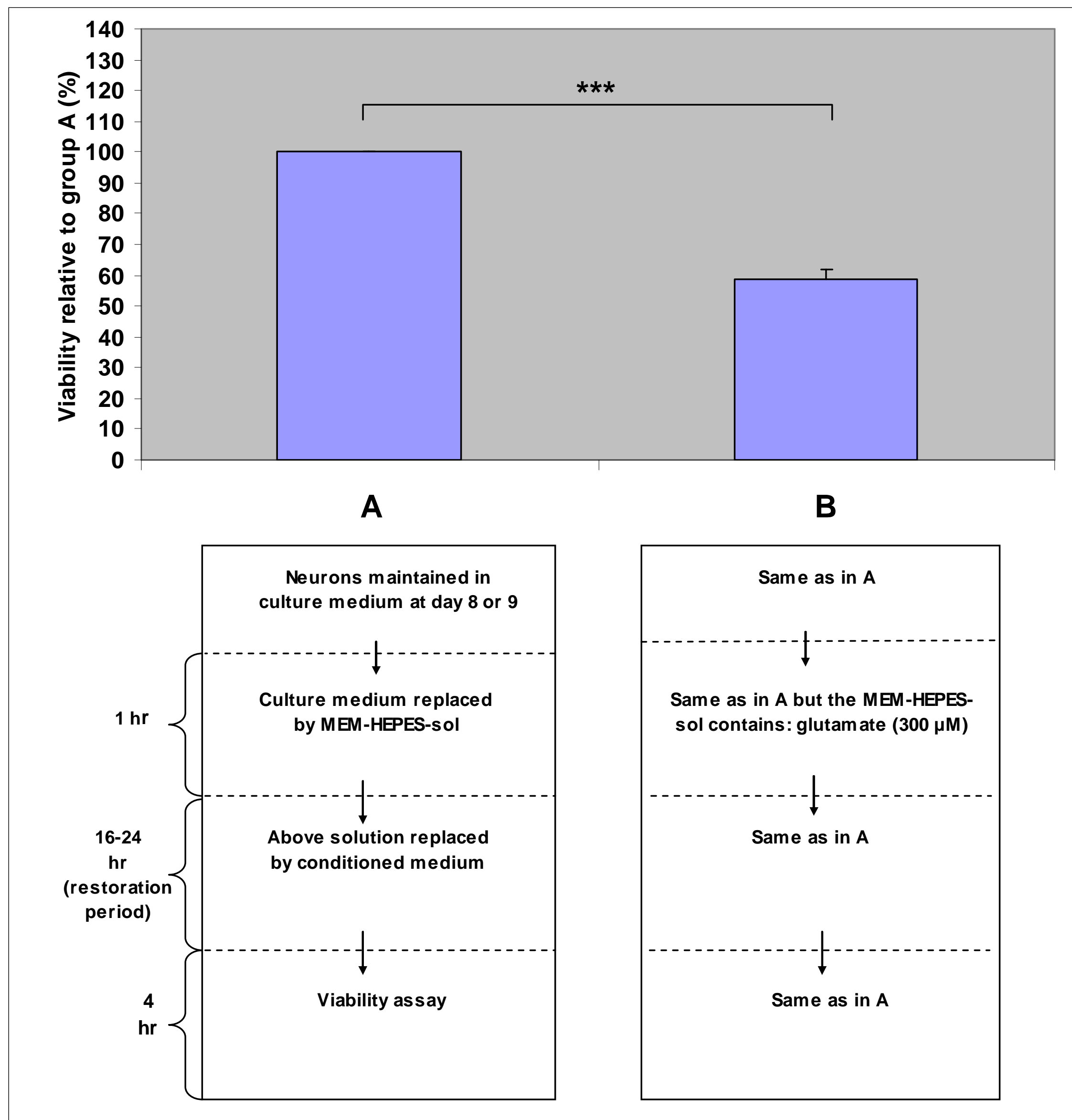


Figure 3-16: Toxicity of externally added glutamate. It shows that glutamate when applied in glutamate-free and serum-free medium for only 1 hr can cause significant toxicity to CGNs. ***$p<0.001$. (n=5).

3.2 Oxidative stress experiments performed before solving the problem of fresh culture medium toxicity

The experiments that will be shown in this section were performed before solving the problem of fresh culture medium toxicity, which means that the cultures (including those in the control groups) were under the influence of glutamate excitotoxicity. Also, some of these experiments were performed before finding that the neurons in next-edge wells give consistently slightly higher viability readings than the neurons in inside wells, so some of the treatment groups in these experiments contained next-edge wells. For these two reasons, it will be hard to interpret the results of these experiments. However, it was appropriate to show some of those experiments (performed in that period) that were consistent and provided some valuable information.

3.2.1 Examining the susceptibility of CGNs to oxidative stress insults

In the beginning it was appropriate to examine the susceptibility of CGNs to different types of oxidative stress insults.

3.2.1.1 Dose response curve of hydrogen peroxide toxicity

When externally applied to CGNs, hydrogen peroxide showed a dose dependent toxicity (Figure 3-17).

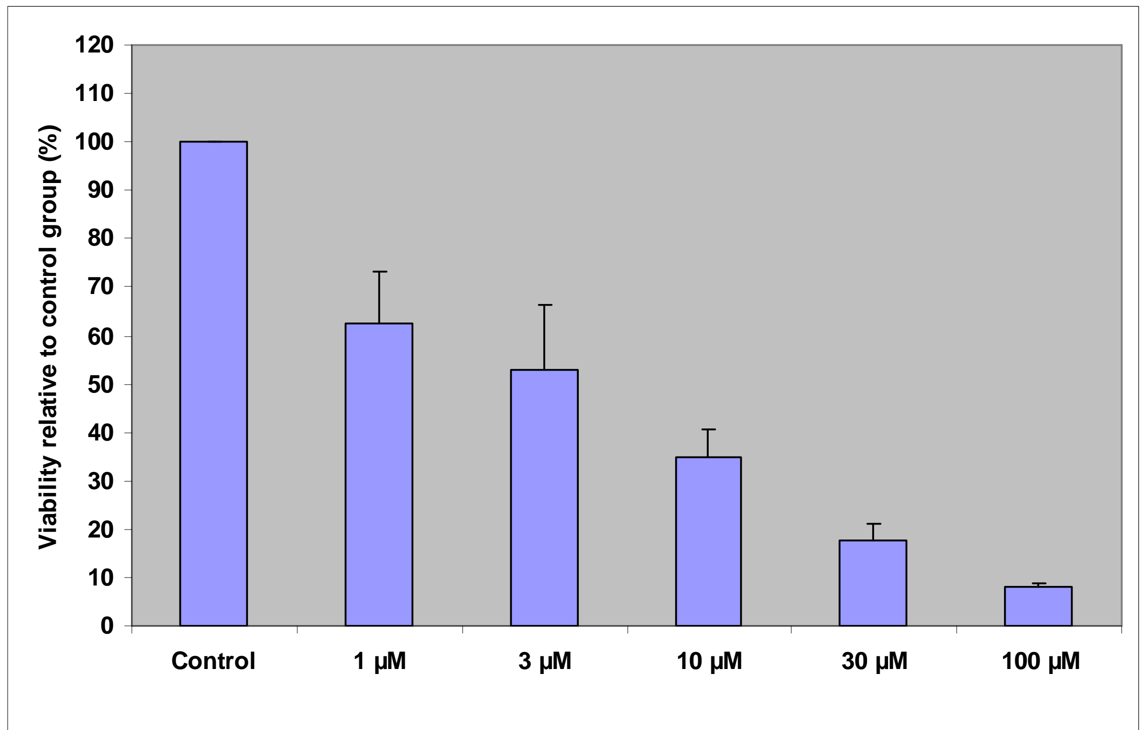


Figure 3-17: Dose response curve of hydrogen peroxide toxicity. The experiment was performed as follows: at day 8 or 9 after plating, the culture medium was replaced by fresh culture medium that does not contain (control) or contains hydrogen peroxide at the indicated concentrations, and left for 1 hour → the neurons in all groups (including control) were restored to fresh culture medium and left for 16-24 hours until the viability assay. **Notice:** all treatment groups showed statistically significant difference when compared to the control group. (n=5).

3.2.1.2 Toxicity of the combination of xanthine and xanthine oxidase (X / XO)

The X / XO combination is known to directly generate superoxide and hydrogen peroxide, and this type of insult is known to cause damage to many types of cells (see Introduction). An experiment was performed to determine the best combination of X and XO that gives consistent and significant toxicity levels, which can be used in subsequent experiments that use X / XO as a toxicity model. It was found that a combination of X (100 µM) and XO (0.02 Units/ml) was the best (Fig. 3-18).

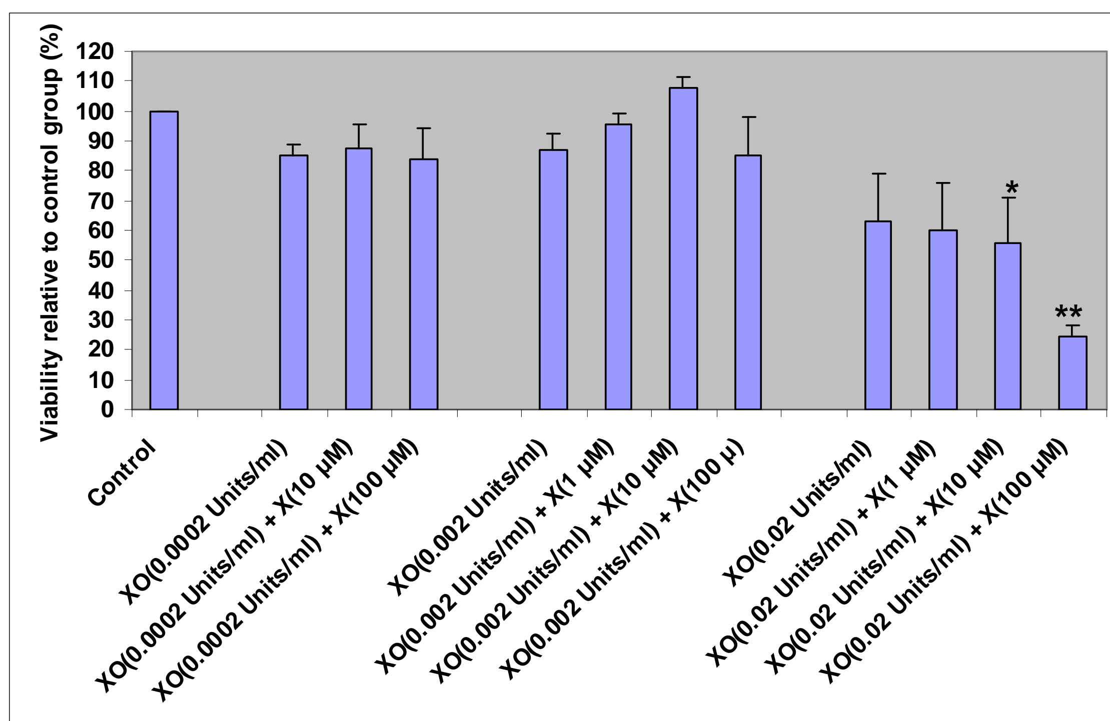


Figure 3-18: Toxicity of X / XO combinations. The experiment was performed as follows: at day 8 or 9 after plating, the culture medium was replaced by fresh culture medium that does not contain (control) or contains X / XO combinations at the indicated concentrations, and left for 1 hour → the neurons in all groups (including control) were restored to fresh culture medium and left for 16-24 hours until the viability assay. *p<0.05 compared to control, **p<0.01 compared to control. (n=4).

3.2.1.3 Toxicity of S-Nitroso-N-acetyl-DL-penicillamine (SNAP)

Nitric oxide is a free radical that can exert damaging effects under some conditions. Therefore, in this experiment a nitric oxide donor, SNAP, was tried to see if CGNs are susceptible to this type of oxidative stress insult. SNAP treated for 24 hr showed a dose dependent toxicity (Fig. 3-19). However, it may be that this effect of SNAP was not due to providing nitric oxide. The reason is that the SNAP powder was dissolved in distilled water and the aliquots of the solution kept in a freezer until the experiment day. If SNAP in solution instantly generates nitric oxide (as expected), this raises the possibility that the nitric oxide, which is a short lived free radical, generated would have been long degraded before the experiment day. A better way would have been to dissolve SNAP powder and then add it to the cultures instantly (see Discussion for possible explanations for the toxicity observed with SNAP).

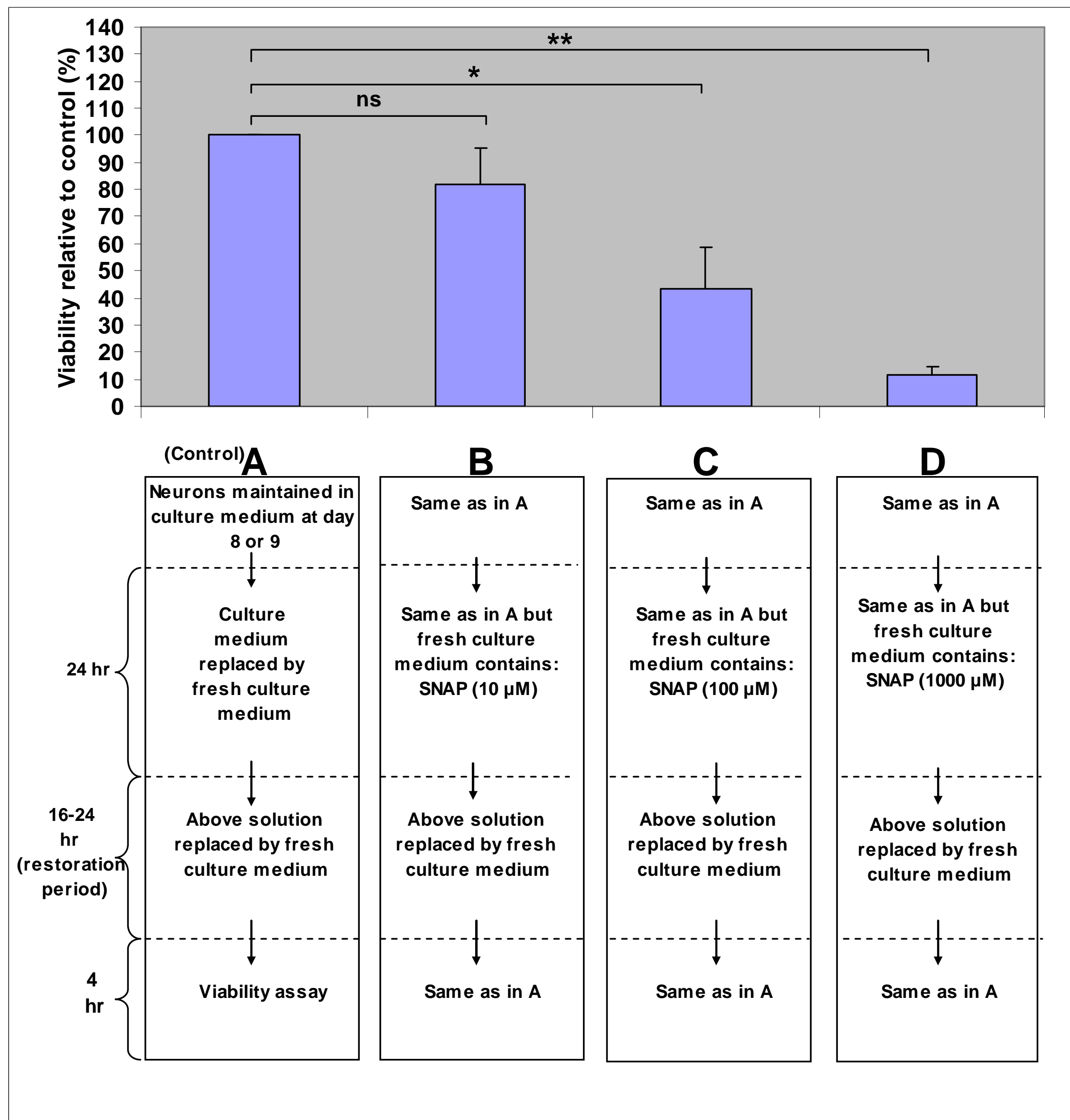


Figure 3-19: Dose response curve of SNAP toxicity. *p<0.05, **p<0.01, ns: not significant. (n=3).

3.2.2 Effect of different antioxidants on the toxicity of oxidative stress insults

After establishing the susceptibility of the CGNs culture to different types of oxidative stress insults, it was appropriate to examine the effect of different antioxidants on these insults.

3.2.2.1 Antioxidants against hydrogen peroxide

Since hydrogen peroxide can exert its toxicity through its conversion to the very reactive and toxic hydroxyl radical, and since this conversion can be mediated by a metal (usually iron or copper) (see Introduction), the effect of an iron chelator on hydrogen peroxide toxicity was examined. Deferoxamine (deferox.) (also called desferrioxamine) is an iron chelator with high affinity for the oxidized form of iron i.e. Fe^{3+} [*Keberle, 1964*]. Deferoxamine alone had no statistically significant effect on cell viability (Fig. 3-20), but showed substantial protection against hydrogen peroxide toxicity (Fig. 3-21). In this experiment, deferoxamine was present one hour before and also during the one hour treatment with hydrogen peroxide.

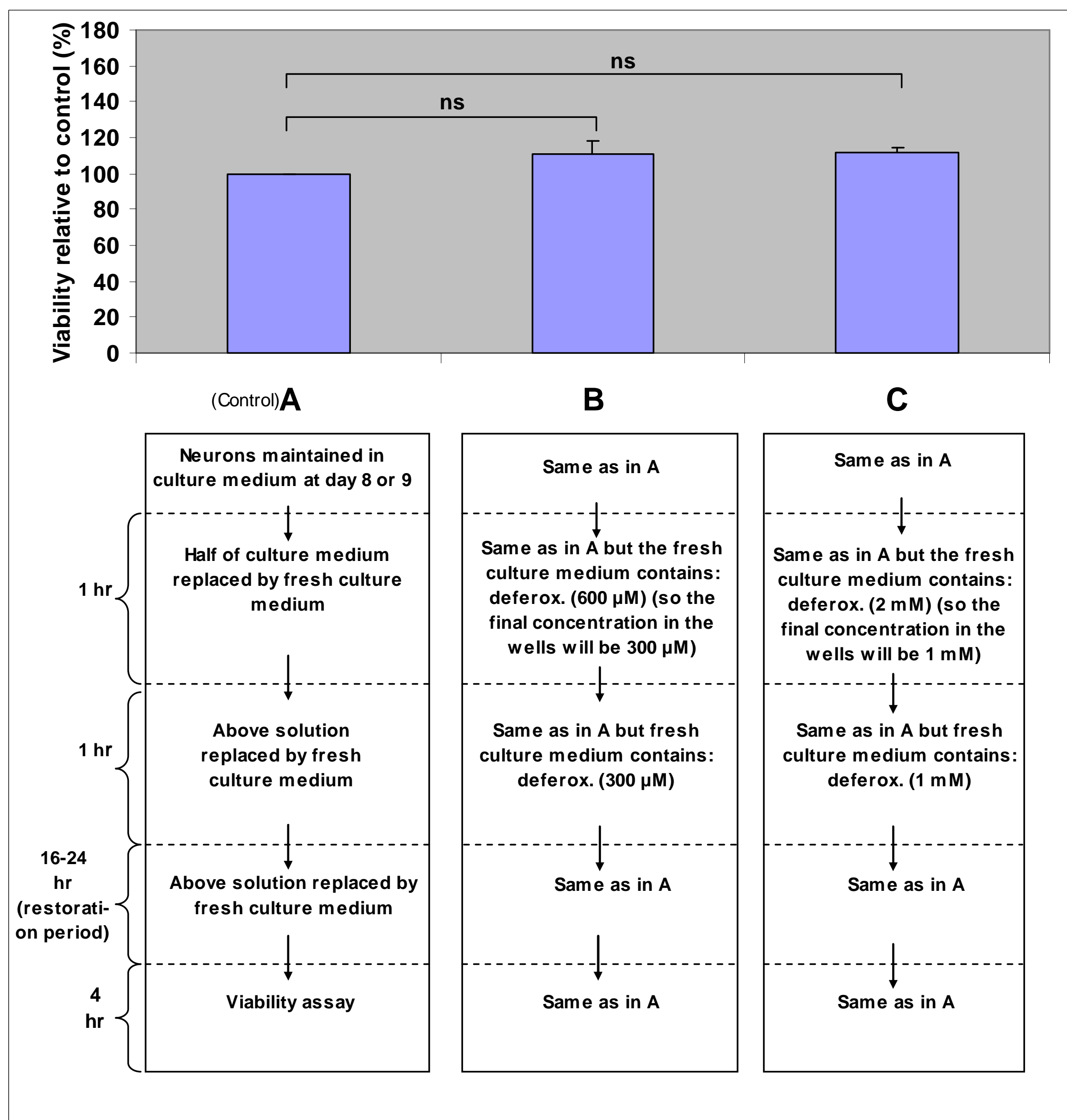


Figure 3-20: Effect of deferoxamine (deferox.) alone on cell viability. It shows that this iron chelator added alone has no significant effect on the neuronal viability. ns: not significant. (n=3).

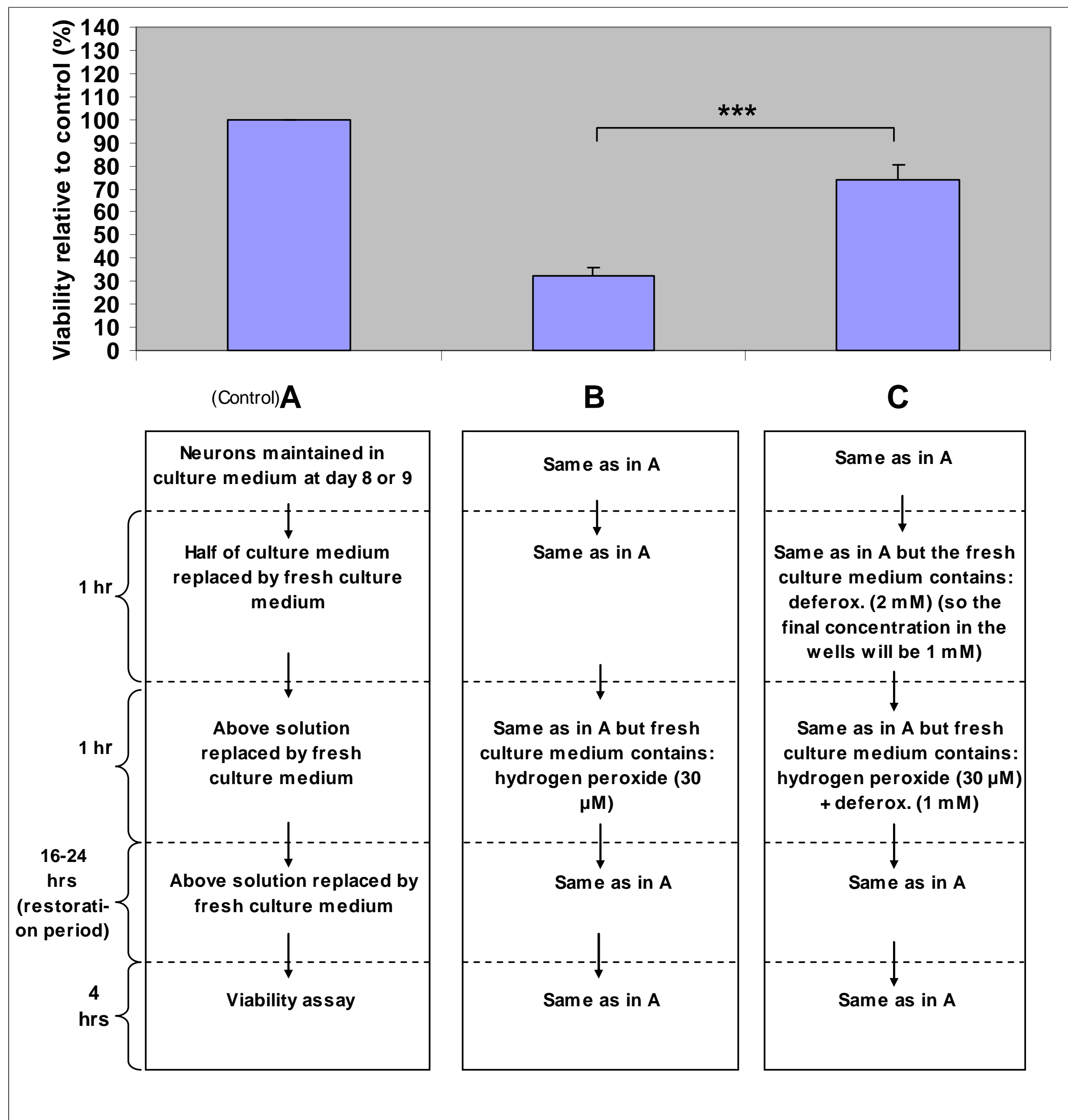


Figure 3-21: Effect of deferoxamine (deferox.) on the toxicity of hydrogen peroxide. It shows that this iron chelator substantially protects against hydrogen peroxide toxicity. Notice that deferoxamine was present 1 hr before and also during the 1 hr application of hydrogen peroxide. ***$p<0.001$. (n=5).

This result of deferoxamine suggests that, without excluding other possibilities, the toxicity of hydrogen peroxide was due to its interaction with an iron ion to produce the toxic hydroxyl radical. To test the possibility of hydroxyl radical involvement, one of its known scavengers, mannitol [*Babbs and Griffin, 1989*], was tried. Although mannitol alone at 1 and 10 mM has no effect on cell viability, mannitol alone at 100 mM showed slight but statistically significant toxicity to CGNs cultures (data not shown). When mannitol was tried at 1 or 10 mM, it did not protect against hydrogen peroxide toxicity (Fig. 3-22). Although the failure of mannitol to protect can be explained by the lack of a role of hydroxyl radical in hydrogen peroxide toxicity, there are many alternative explanations (see Discussion).

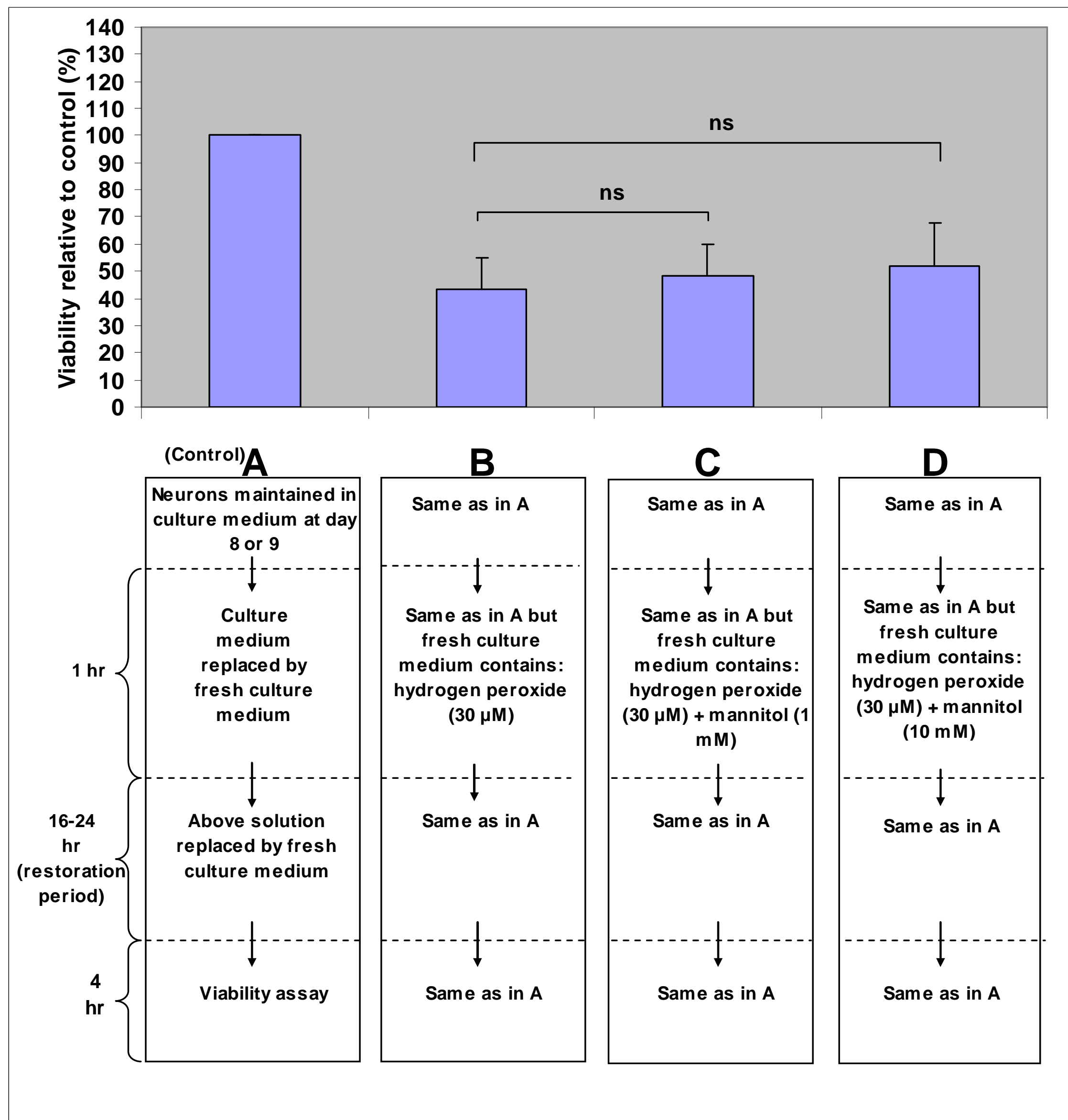


Figure 3-22: Effect of mannitol on the toxicity of hydrogen peroxide. It shows that this hydroxyl radical scavenger could not protect against hydrogen peroxide toxicity. ns: not significant. (n=3).

3.2.2.2 Antioxidants against X / XO toxicity

Since, as mentioned earlier, X / XO can directly generate hydrogen peroxide and superoxide, catalase (Cat.) and SOD-1 were tried against this type of toxicity. Catalase showed almost complete protection, but SOD-1 failed to show statistically significant protection. Also, since as mentioned in the Introduction that blocking either the site of X binding or the site of NADH binding on XO prevents the oxidation of X by XO, allopurinol (a blocker of the X binding site, the Mo site) and DPI (a blocker of the NADH binding site, the FAD site) were tried against X / XO toxicity, but they failed to show statistically significant protection. The effects of catalase, SOD, allopurinol, and DPI against X / XO toxicity are shown in Fig. 3-23. Catalase, SOD, allopurinol, and

DPI had no effect on cell viability when tested alone at the used concentrations and time interval (data not shown).

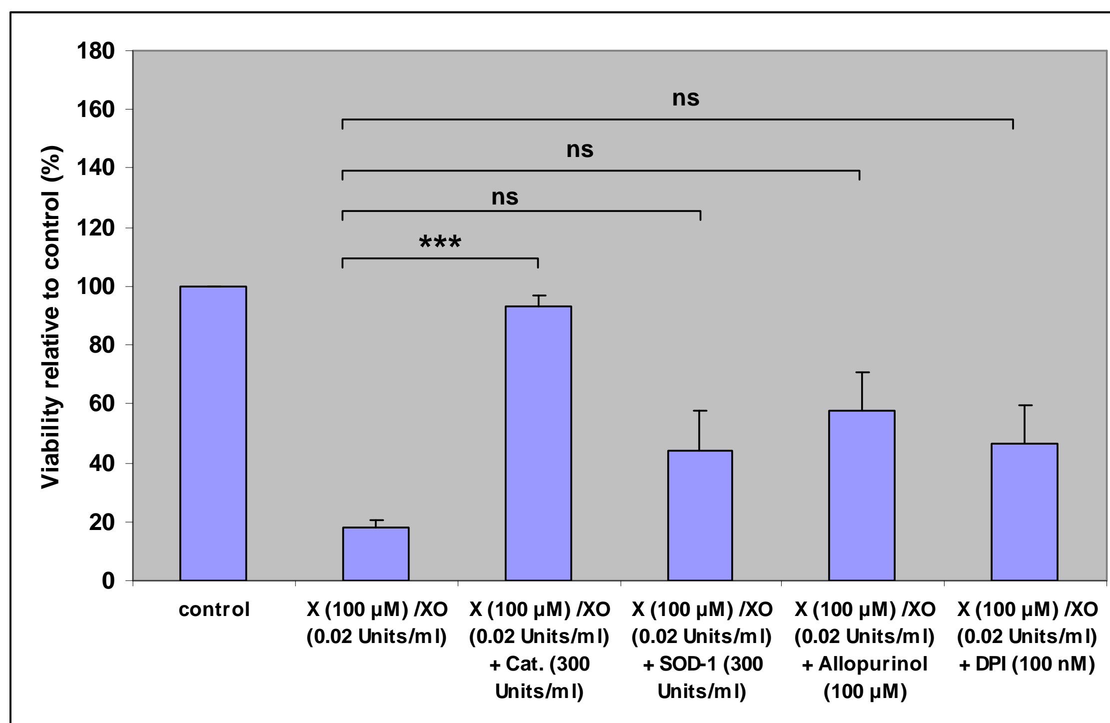


Figure 3-23: Effect of different antioxidants on X / XO toxicity. It shows that catalase, likely through deactivating hydrogen peroxide, protects almost completely against X / XO toxicity. Although SOD-1 (deactivator of superoxide), allopurinol (blocker of the Mo site on XO), and DPI (blocker of the FAD site on XO) seemed to protect against this toxicity, their effects were not statistically significant. The experiment was performed as follows: at day 8 or 9 after plating, the culture medium was replaced by fresh culture medium that does not contain (control) or contains the indicated test compounds, and left for 1 hour → the neurons in all groups (including control) were restored to fresh culture medium and left for 16-24 hours until the viability assay. ***$p<0.001$, ns: not significant. (n=5).

Since catalase showed almost complete protection, this suggests that hydrogen peroxide is a main toxic molecule responsible for X / XO toxicity, which makes this toxicity model somehow similar to the toxicity model of externally applying hydrogen peroxide. Since deferoxamine was protective against externally applied hydrogen peroxide, it was tried against X / XO toxicity. Deferoxamine at the concentration (and incubation time) that was very protective against externally applied hydrogen peroxide did not show protection against X (100 µM) / XO toxicity (Fig. 3-24). However, when X was used at 30 µM instead of 100 µM, deferoxamine showed a statistically significant protection against X / XO toxicity (Fig. 3-25), suggesting that deferoxamine did not protect in the experiment shown in Fig. 3-24 because the toxic insult was too severe for it to provide significant protection.

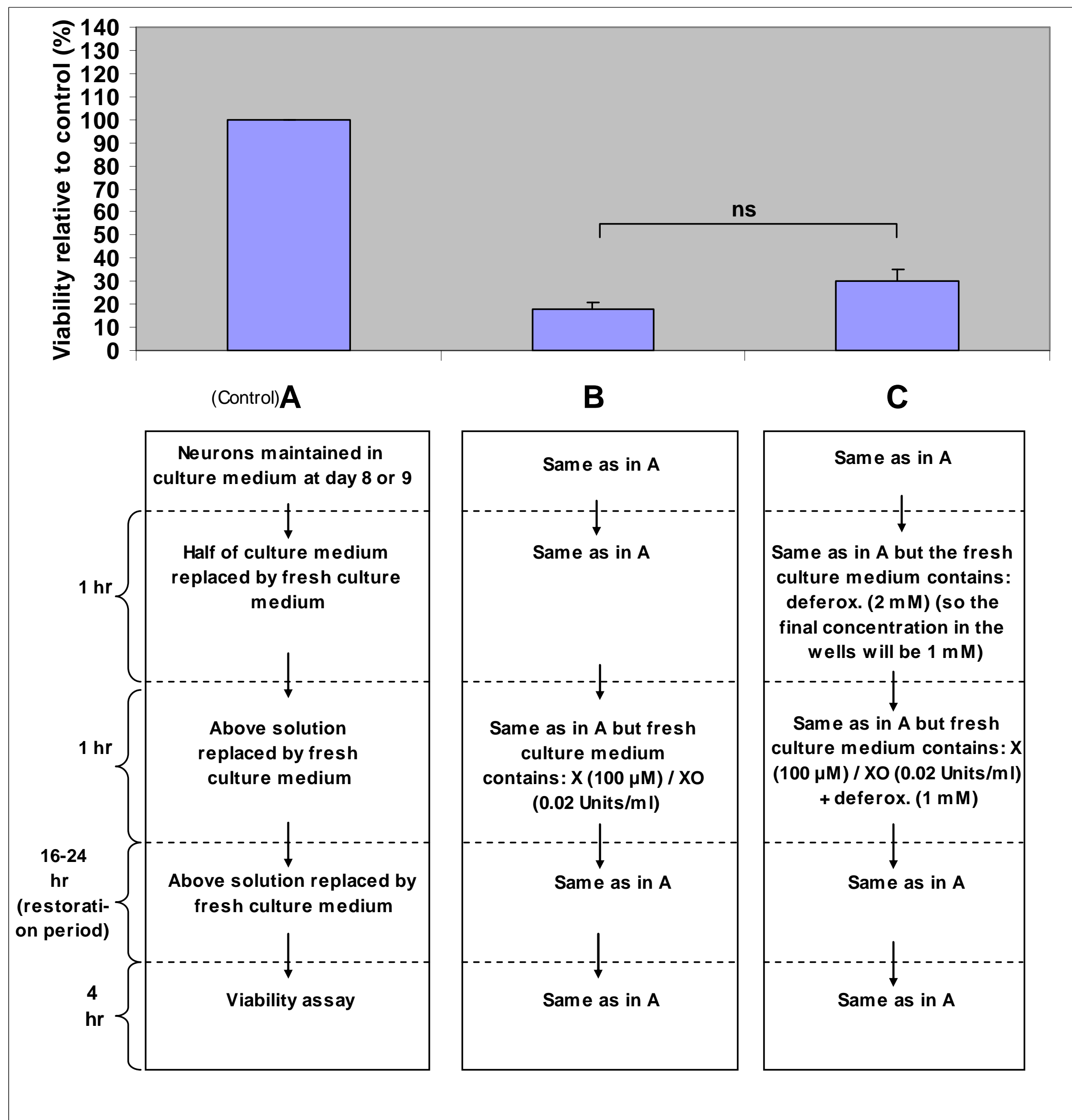


Figure 3-24: Effect of deferoxamine (deferox., 1mM) on the toxicity of X (100 µM) / XO. It shows that deferoxamine, at the concentration and incubation time that was very protective against externally applied hydrogen peroxide, could not protect against this toxicity level of X / XO combination. ns: not significant. (n=5).

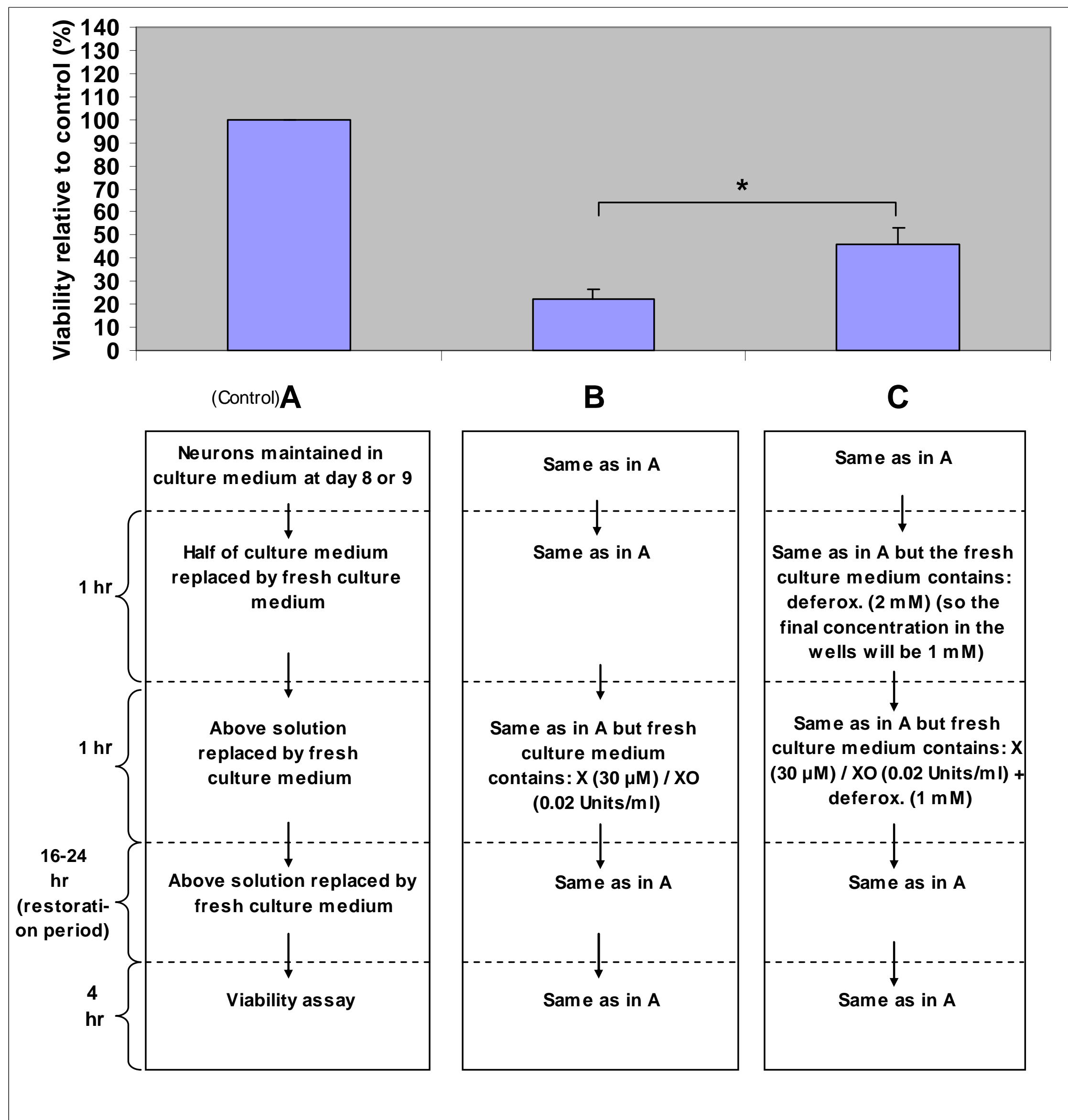


Figure 3-25: Effect of deferoxamine (deferox., 1 mM) on the toxicity of X (30 µM) / XO. It shows that when the toxicity level of X / XO (that was shown in Fig. 3-24) was reduced i.e. X was used at 30 instead of 100 µM, deferoxamine was able to protect against this toxicity. *p<0.05. (n=5).

Because deferoxamine is thought to be slow in getting inside the cells [*Porter et al., 1988*], the time of its pre-treatment (that was 1 hr in the experiments shown in Figures 3-24 and 3-25) was increased to see if more protection can be obtained with prolonged pre-treatment. A side experiment showed that although deferoxamine at 300 µM applied alone to the neurons for 6 hr had no effect on the neurons, deferoxamine at 1 mM applied the same way showed slight but statistically significant toxicity to CGNs cultures (Fig. 3-26). Therefore, it was decided not to try prolonged pre-treatment with 1 mM deferoxamine against X / XO toxicity, and a prolonged pre-treatment with 300 µM deferoxamine against this toxicity was tried instead. With 6 hr pre-treatment (in addition to the 1 hr co-treatment), deferoxamine at 300 µM protected against X (100 µM) / XO

(0.02 Units/ml) toxicity (Fig. 3-27). Notice that with only 1 hr pre-treatment (in addition to 1 hr co-treatment), deferoxamine at even 1 mM could not protect significantly against X (100 µM) / XO (0.02 Units/ml) toxicity (see again Fig. 3-24). This suggests that prolonged pre-treatment may bring more protection with deferoxamine, and this also suggests that the site of deferoxamine action is intracellular. Moreover, with 6 hr pre-treatment (in addition to 1 hr co-treatment), deferoxamine at 300 µM protected even more against X / XO toxicity when the toxic insult concentration was reduced i.e. when X concentration was reduced from 100 µM to 30 µM (Fig. 3-28).

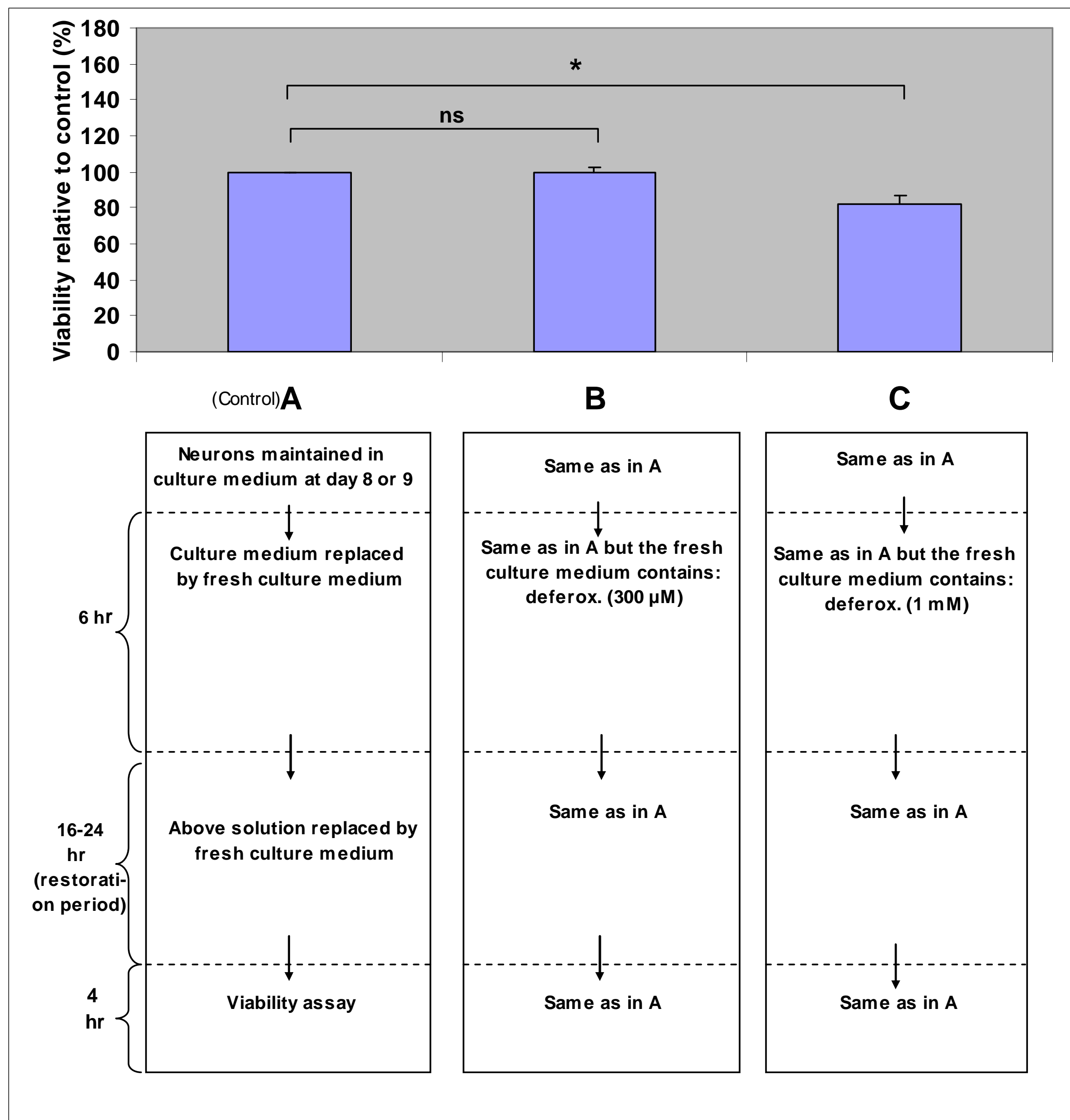


Figure 3-26: Effect of prolonged application of deferoxamine (deferox.) alone on cell viability. *$p<0.05$, ns: not significant. (n=3).

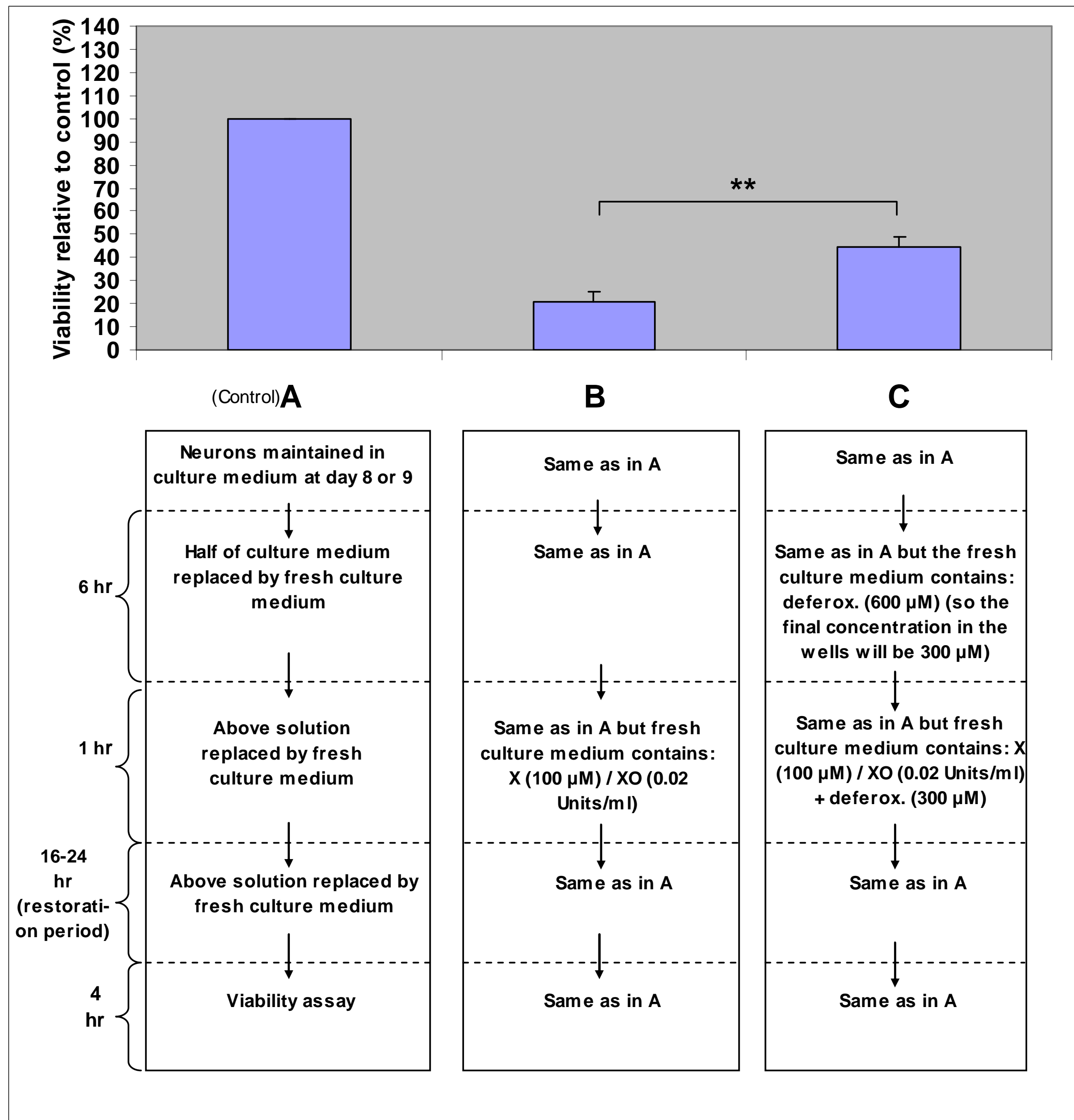


Figure 3-27: Effect of prolonged pre-treatment (in addition to the 1 hr co-treatment) of deferoxamine (deferox., 300 µM) on the toxicity of X (100 µM) / XO. It shows that by increasing its pre-treatment time up to 6 hr, deferoxamine at 300 µM was able to attenuate a toxicity level of X / XO that was not significantly attenuated by only 1 hr pre-treatment with deferoxamine at even 1 mM (see again Fig. 3-24). $^{**}p<0.01$. (n=5).

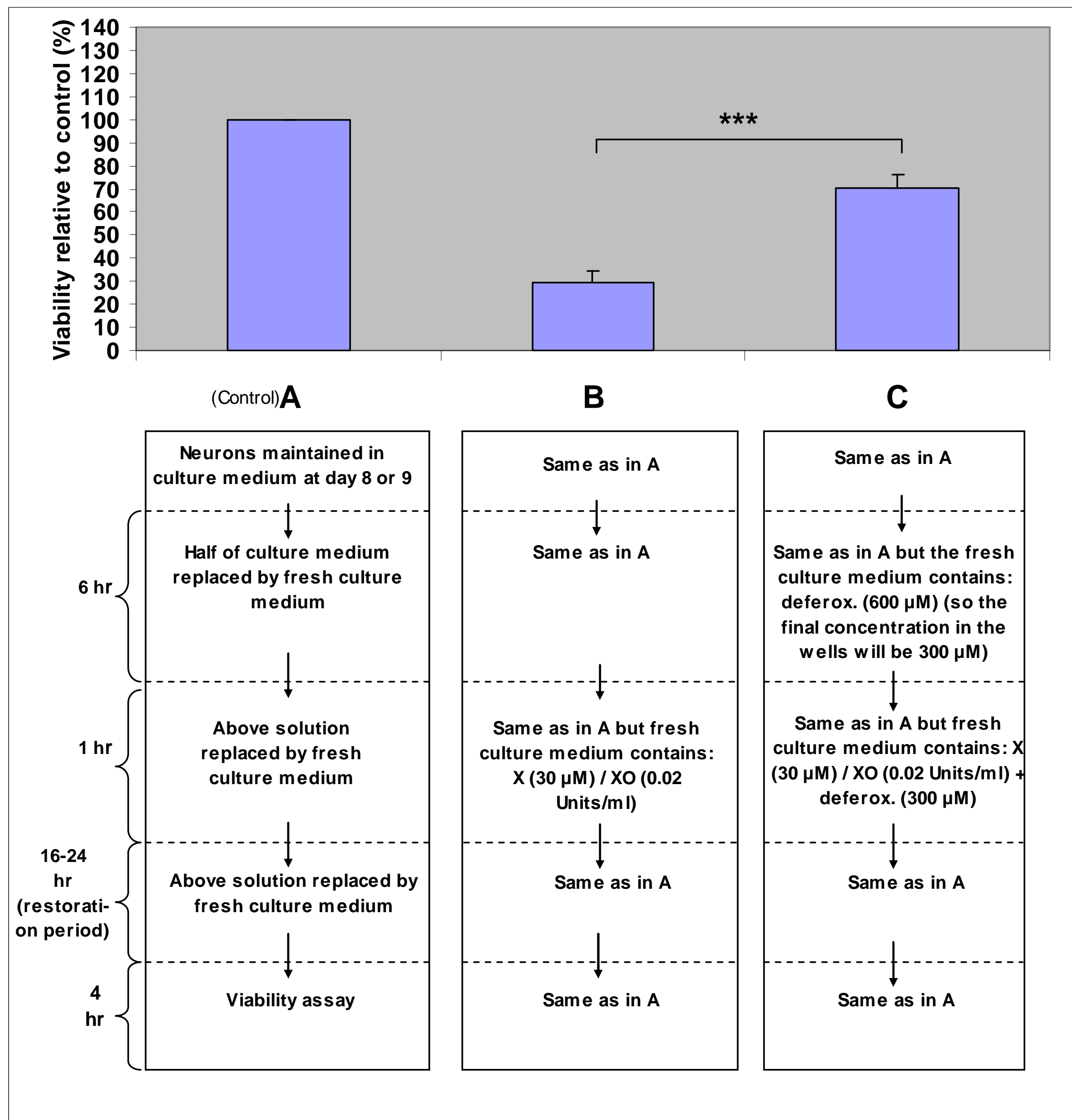


Figure 3-28: Effect of prolonged pre-treatment (in addition to the 1 hr co-treatment) of deferoxamine (300 µM) on the toxicity of X (30 µM) / XO. It shows that with both increasing the pre-treatment time up to 6 hr and reducing the toxic insult from X (100 µM) / XO to X (30 µM) / XO, deferoxamine brings even more and more protection. ***$p<0.001$. (n=5).

3.3 Oxidative stress experiments performed after solving the problem of fresh culture medium toxicity

After solving the problem of fresh culture medium toxicity, it was possible to conduct reliable experiments. In this stage of the project, it was possible to answer many of the questions stated earlier in the section on the Aim/Objectives in the Introduction. This section is divided into two main sections, the first is a comparison between X / XO and NADH / XO toxicities, and the second is a further investigation of X / XO toxicity.

In this stage, the solution used as a vehicle to add the compounds is (instead of fresh culture medium) a serum-free solution: either HEPES-sol or MEM-HEPES-sol., in order to avoid the excitotoxicity of fresh culture medium. There were two main reasons for trying two rather than only one serum-free solution as the treatment solution. First, obtaining the same effect of a compound by using two different treatment solutions adds more validity to the result (there were some cases where this was necessary, see Discussion). Second, since NADH alone at 2 mM was toxic in MEM-HEPES-sol (see later), this solution was not suitable for experiments in which there was investigation of the toxicity of XO / NADH (2 mM) combination. On the other hand, HEPES-sol was suitable for such experiments, since NADH alone at 2 mM was not toxic in this solution (see later).

Notice also that in this stage of the project, the medium to which the neurons were restored at the end of the treatment period was conditioned medium and not fresh culture medium, and this was also done to avoid the excitotoxicity of the latter.

3.3.1 Experimental check on the Alamar blue assay

As mentioned earlier, there was good correlation between the Alamar blue viability assay readings and the morphological examination under the microscope. Actually, this good correlation was the reason for believing that the protection of ethanol and DMSO against fresh culture medium toxicity was real and not due to an artefact in the Alamar blue viability assay, which led eventually to discovering the reason of fresh culture medium toxicity. Alamar blue was used previously in CGNs with an incubation time between 4-6 hr, where the viability results based on this incubation time were expected

to accurately reflect the viability of the cells [*White et al., 1996, Fatokun, 2006, Fatokun et al., 2007b*]. To add more validity to the assay, 4 and 6 hr incubation times were compared, to see if there is a difference between them, and if so, which one is more suitable to be used. In this experiment, there were three groups: control group, insult group, and insult with a protective compound group. The result shows that there was no statistically significant difference between the viabilities calculated at 4 hr and 6 hr Alamar blue incubation times (Fig. 3-29).

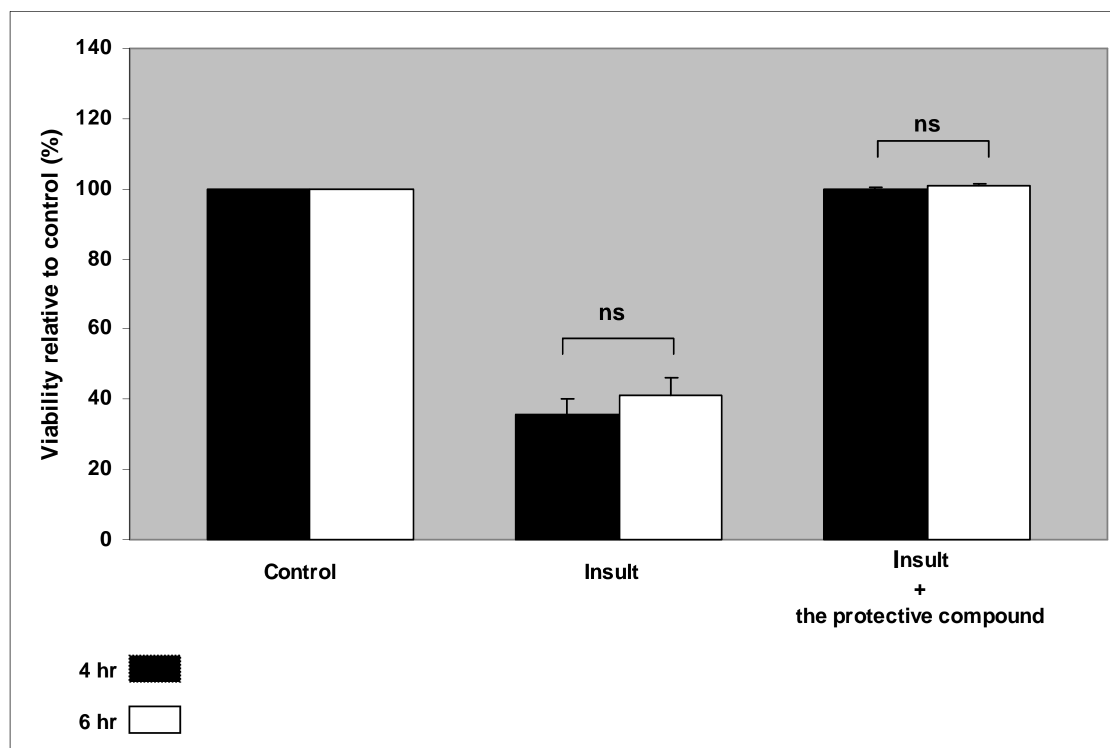


Figure 3-29: Comparison between viabilities calculated with 4 hr and 6 hr Alamar blue incubation times. The experiment was performed as follows: at day 8 or 9 after plating, the culture medium was replaced by HEPES-sol that does not contain (control) or contains the test compounds (either an insult compound or an insult compound with a protective compound), and left for 1 hour → the neurons in all groups (including control) were restored to conditioned medium and left for 16-24 hours → Alamar blue was added at 10% (V/V) and left for 4 hr → The Alamar blue readings were taken on a plate reader and the cultures were immediately returned to the incubator and left for additional two hours → The Alamar blue readings were taken again on the plate reader. ns: not significant. (n=3).

3.3.2 Comparison between X / XO and NADH / XO toxicities

Since, as mentioned in the Introduction, NADH / XO toxicity has rarely been investigated previously compared to X / XO toxicity, a series of experiments were conducted to compare these two toxicity models.

3.3.2.1 Establishing the toxicities of the X / XO and NADH / XO combinations

X was not toxic when tried alone in MEM-HEPES-sol at 30 or 100 μM, and it was also not toxic when tried alone in HEPES-sol at 15 or 30 μM (data not shown). NADH alone was not toxic when tried alone at 2 mM in HEPES-sol (see later on in Fig. 3-49). When tried in MEM-HEPES-sol, NADH alone was not toxic at 1 mM but was toxic at 2 mM (Fig. 3-30). XO was not toxic when tried alone at 0.02 Units/ml either in HEPES-sol (data not shown) or MEM-HEPES-sol (Fig. 3-31).

The Combination of XO and NADH tried in MEM-HEPES-sol was toxic only when the concentration of NADH was raised up to 1 mM (Fig. 3-31). Also, when tried in HEPES-sol, the combination of XO and NADH was toxic when NADH was used at 1 mM (see for example Fig. 3-33), 1.5 mM (see later on in Fig. 3-62), or 2 mM (see later on in Fig. 3-43 and Fig. 3-54). It is noticed that the toxicity of the NADH / XO combination tends to be more severe in HEPES-sol compared to MEM-HEPES-sol, though (as mentioned) NADH alone at 2 mM was toxic in MEM-HEPES-sol but was not toxic in HEPES-sol.

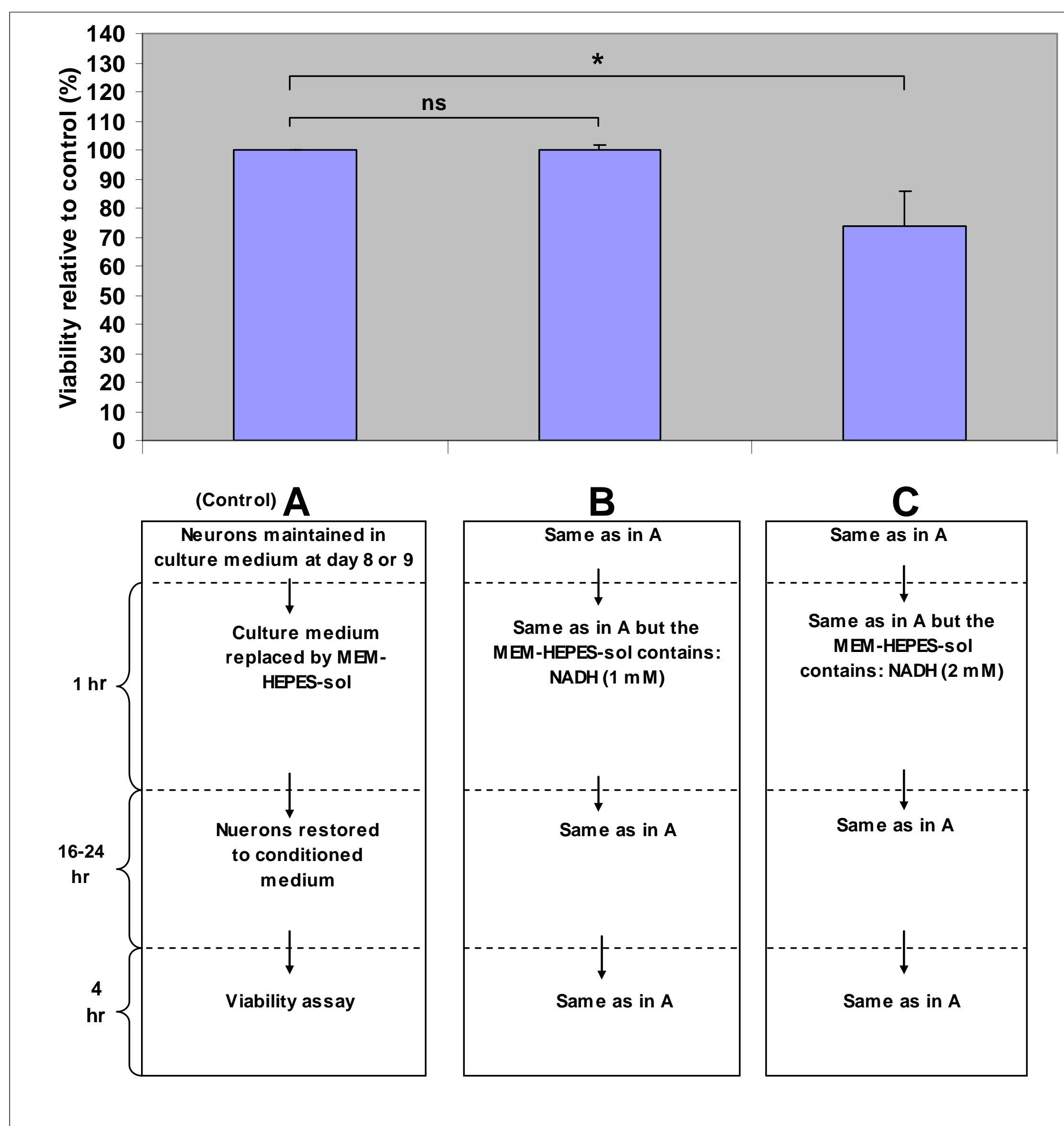


Figure 3-30: Effect of NADH alone on cell viability when applied in MEM-HEPES-sol. *$p<0.05$, ns: not significant. (n=5).

In MEM-HEPES-sol, the combinations of XO and X were toxic at X concentrations much lower than NADH, which is in agreement with previous cell-free experiments that showed that NADH is a much weaker substrate than X for bovine milk XO [*Gilbert, 1963, Liochev et al., 1989, Nakamura, 1991*]. The X / XO combinations were toxic when X was used at either 30 or 100 µM. Although these results were consistent throughout the months, there were some variations. For example, in some cases the toxicity of X (100 µM) / XO (0.02 Units/ml) gave a lower toxicity level (see for example Fig. 3-36) than the toxicity level of X (30 µM) / XO (0.02 Units/ml) combination (see for example Fig. 3-42) despite the fact that all treatment conditions (apart from the lower X concentration in the latter) were the same in the two experiments. However, this observed variation was usually between experiments, not within them, so it should not

affect the validity of the comparisons between groups within an experiment. Also this observed variation between experiments was usually between those experiments where one experiment was conducted several months after completing the other (i.e. separated by wide intervals during the year).

On the other hand, when tried in HEPES-sol, two things were noticed about the toxicities of X / XO combinations. First, these toxicities were more consistent than the toxicities of X / XO combinations tried in MEM-HEPES-sol e.g. in HEPES-sol, a combination of X (15 μM) / XO (0.02 Units/ml) always produced a consistent toxicity level between 50 and 75% cell damage (see all figures where this combination was used at this concentration in HEPES-sol). Second, the toxicity of these combinations tended always to be more severe than when tried in MEM-HEPES-sol e.g. as mentioned above, the cell damage induced by only X (15 μM) / XO (0.02 Units/ml) combination in HEPES-sol was never less than 50%.

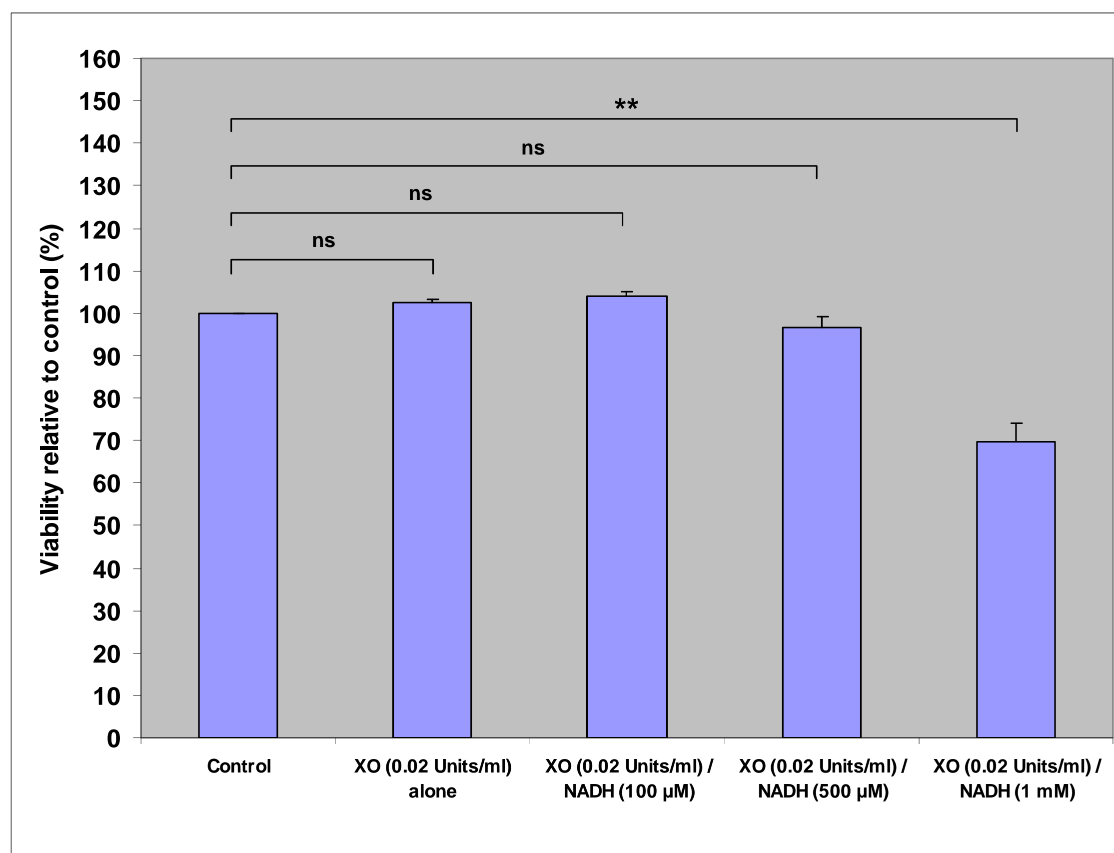


Figure 3-31: Dose response of NADH / XO toxicity in MEM-HEPES-sol. The experiment was performed as follows: at day 8 or 9 after plating, the culture medium was replaced by MEM-HEPES-sol that does not contain (control) or contains the indicated test compounds, and left for 1 hour → the neurons in all groups (including control) were restored to conditioned medium and left for 16-24 hours until the viability assay. **p<0.01, ns: not significant. (n=5).

3.3.2.2 Effects of using XO inhibitors on X / XO and NADH / XO toxicities

There are two blockable sites on XO, the X binding site (the Mo site) and the NADH binding site (the FAD site) (see Introduction). The main point behind trying XO inhibitors was, using viability studies, to reproduce previous cell-free experiments that showed that blocking the Mo site does not prevent NADH oxidation, which may have important implications in interpreting the results of studies that sought a role of XOR in some diseases. The other point behind using the enzyme inhibitors was to prove that the toxicity of X / XO and NADH / XO combinations was indeed due to the enzymatic oxidation of the substrates and not merely due to non-specific interactions between the substrates and the enzyme.

Allopurinol (100 μM), a blocker of the Mo site on XO, when tried in MEM-HEPES-sol, failed to prevent NADH / XO toxicity (Fig. 3-32). This failure of allopurinol was also obtained when the experiment was repeated using HEPES-sol instead of MEM-HEPES-sol (Fig. 3-33). Allopurinol alone was not toxic either in MEM-HEPES-sol or HEPES-sol (data not shown).

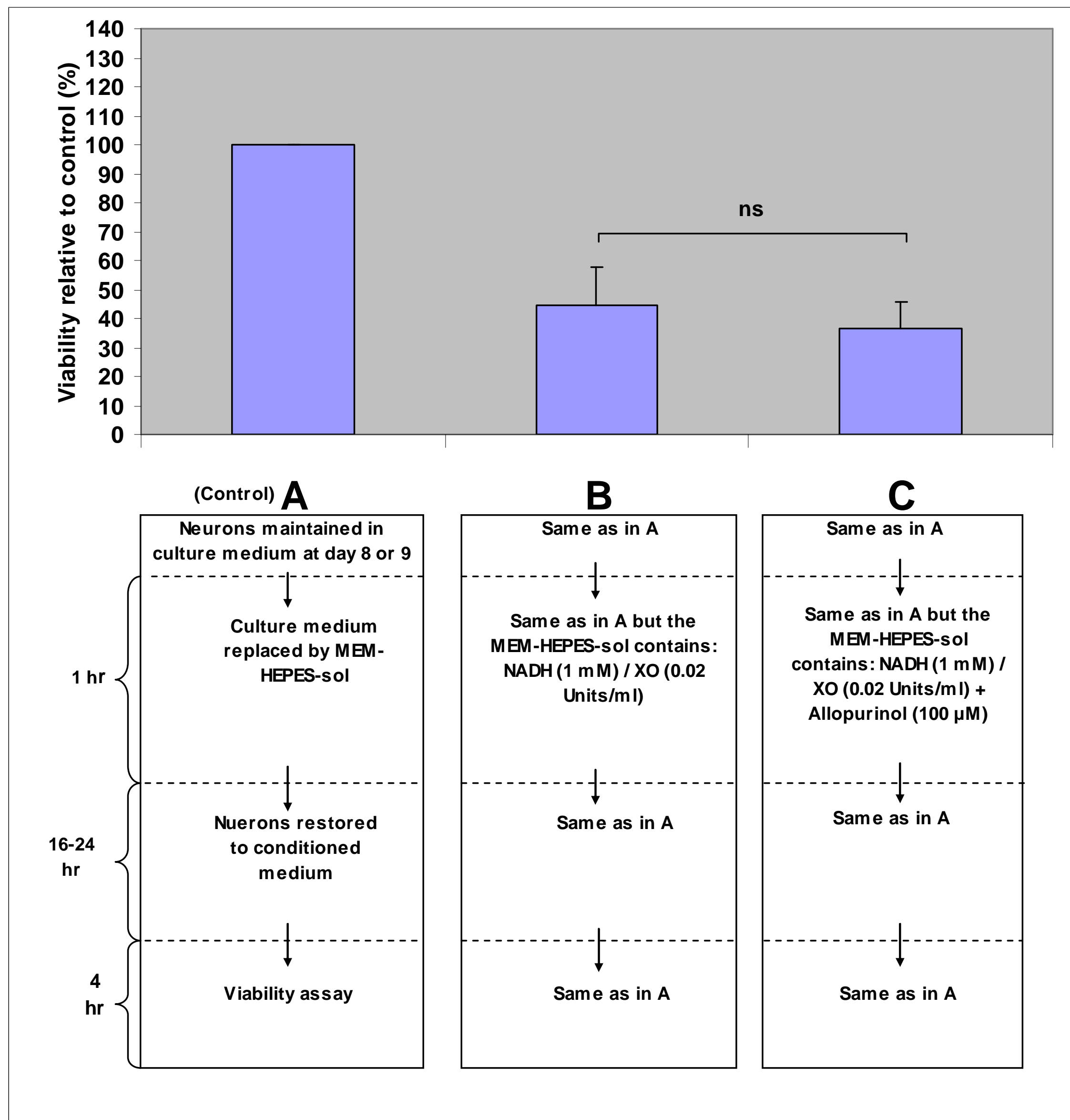


Figure 3-32: Effect of allopurinol on the toxicity of NADH / XO combination in MEM-HEPES-sol. It shows that blocking the Mo site on XO does not prevent NADH / XO toxicity. ns: not significant. (n=5).

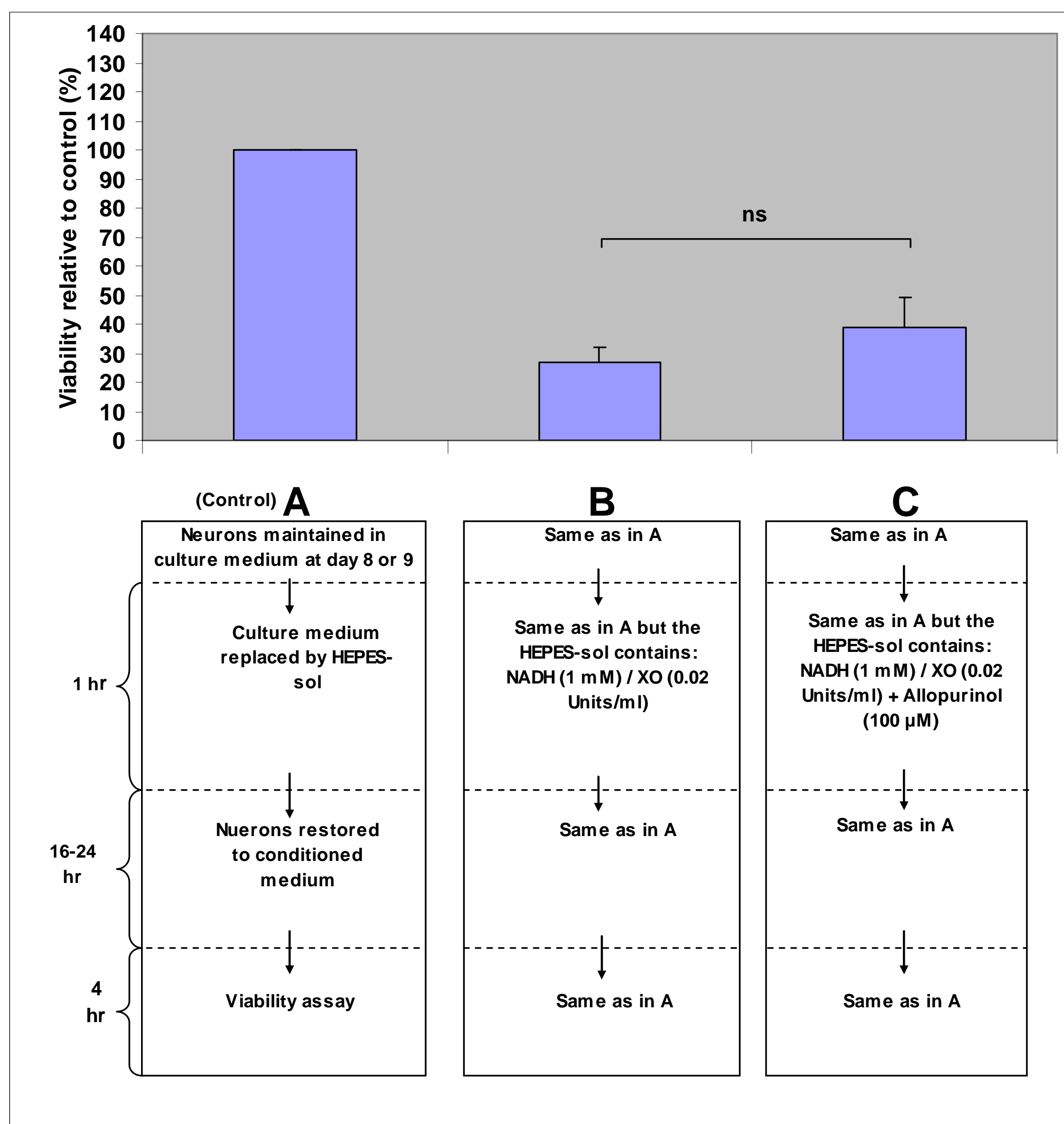


Figure 3-33: Effect of allopurinol on the toxicity of NADH / XO combination in HEPES-sol. It shows that also when the experiment was carried out in HEPES-sol instead of MEM-HEPES-sol, allopurinol could not protect against NADH / XO toxicity. ns: not significant. (n=5).

On the other hand, DPI (100 nM), a blocker of the FAD site on XO, when tried in MEM-HEPES-sol, prevented the toxicity of the NADH / XO combination (Fig. 3-34), which is expected since blocking this site does indeed prevent NADH oxidation by XO (see Introduction). This protective effect of DPI was also obtained when the experiment was repeated using HEPES-sol instead of MEM-HEPES-sol (Fig. 3-35). DPI alone was not toxic either in MEM-HEPES-sol or HEPES-sol (data not shown).

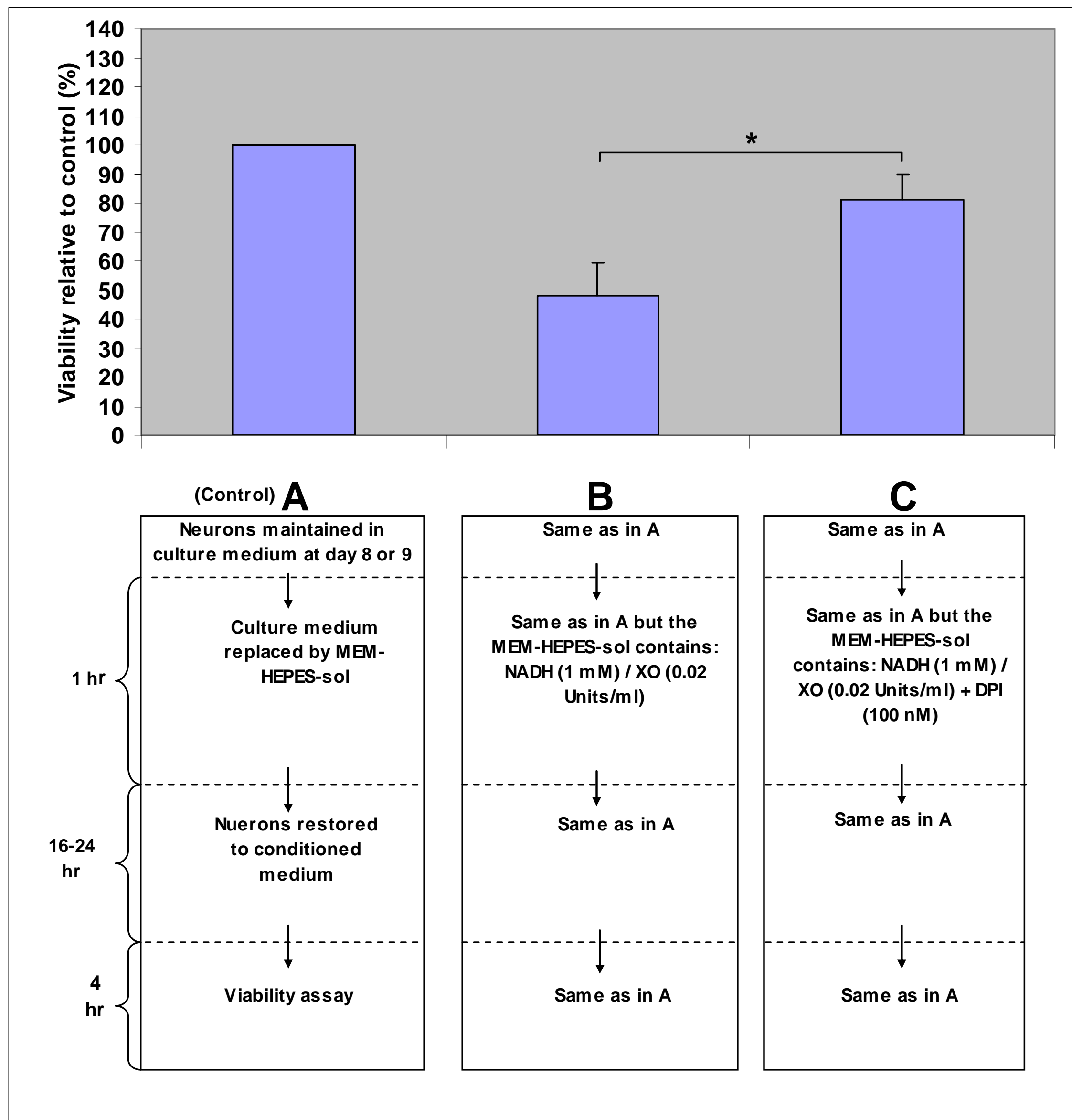


Figure 3-34: Effect of DPI on the toxicity of NADH / XO combination in MEM-HEPES-sol. It shows that blocking the FAD site on XO with DPI prevents NADH / XO toxicity. *$p<0.05$. ($n=5$).

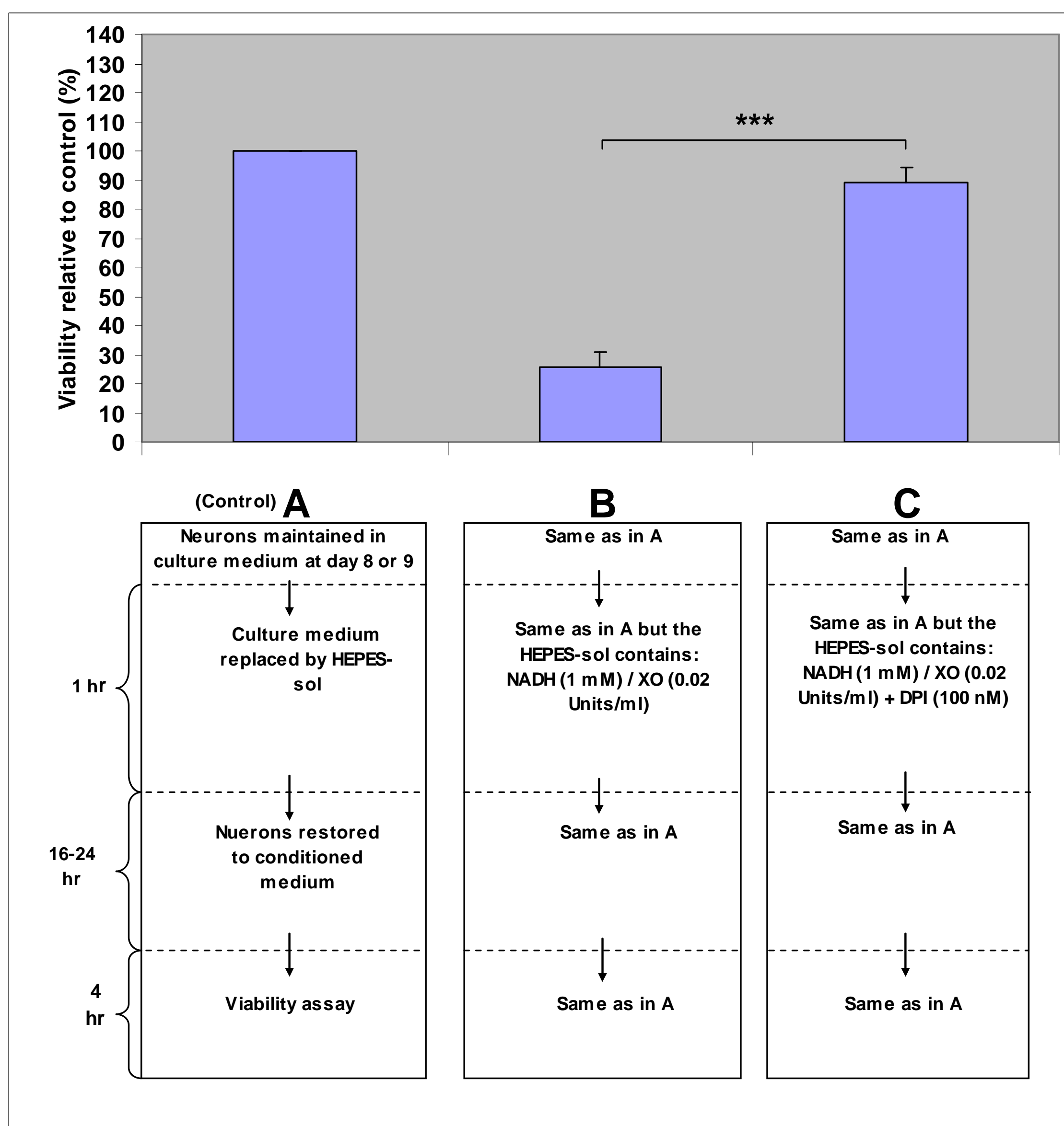


Figure 3-35: Effect of DPI on NADH / XO toxicity in HEPES-sol. It shows that also when the experiment was carried out in HEPES-sol instead of MEM-HEPES-sol, DPI protected against NADH / XO toxicity. ***p<0.001. (n=5).

The failure of allopurinol to prevent NADH / XO toxicity was not due to the failure of this compound to block the Mo site, since it was able to prevent the toxicity of the X / XO combination applied in either MEM-HEPES-sol (Fig. 3-36) or HEPES-sol (Fig. 3-37).

DPI when tried in MEM-HEPES-sol, failed to show statistically significant protection against X / XO toxicity (Fig. 3-38). However, when the experiment was repeated using HEPES-sol instead of MEM-HEPES-sol, DPI showed clear, substantial, and statistically significant protection against X / XO toxicity (Fig. 3-39).

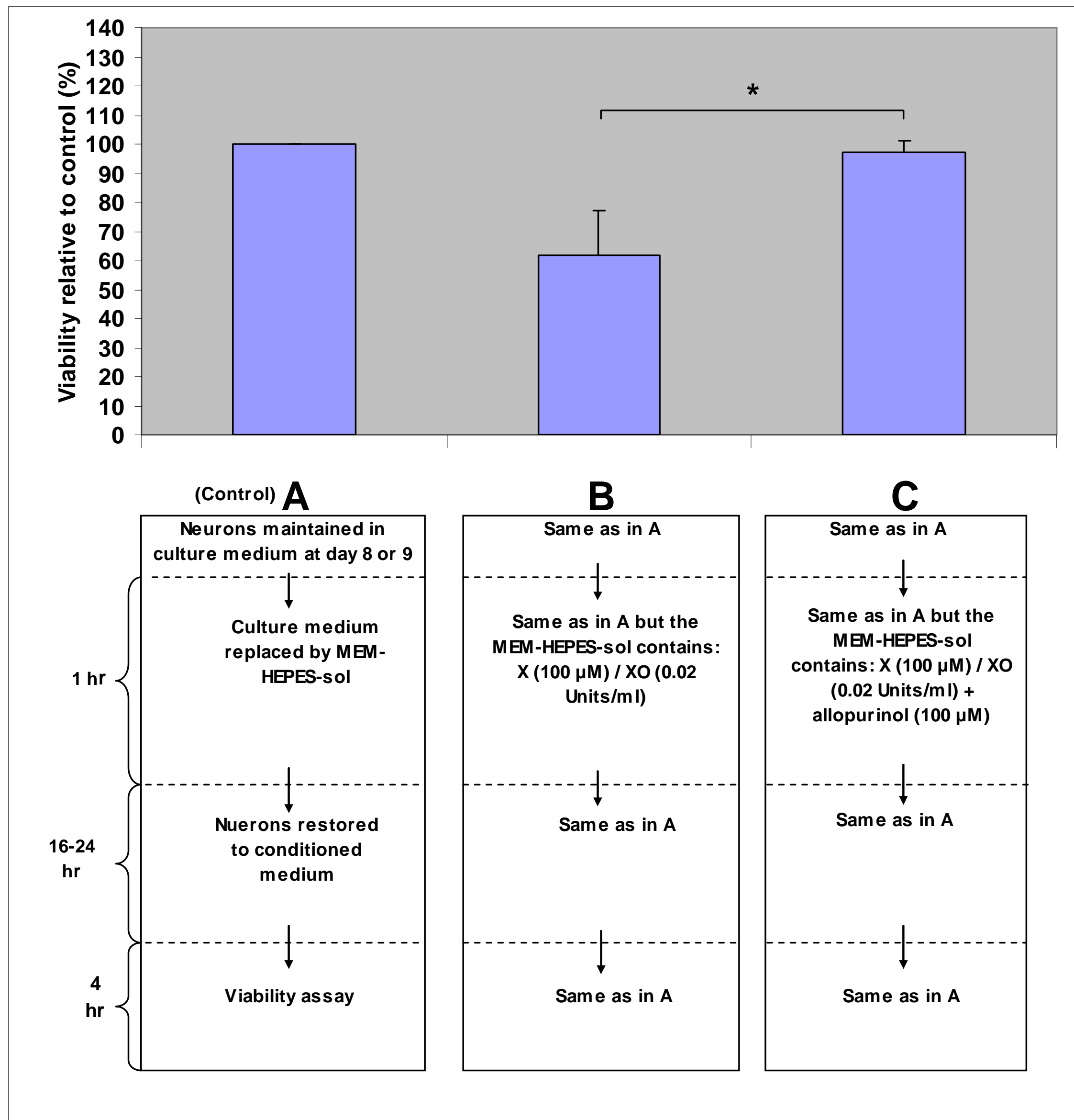


Figure 3-36: Effect of allopurinol on X / XO toxicity in MEM-HEPES-sol. It shows that blocking the Mo site on XO with allopurinol prevents X / XO toxicity. (n=5).

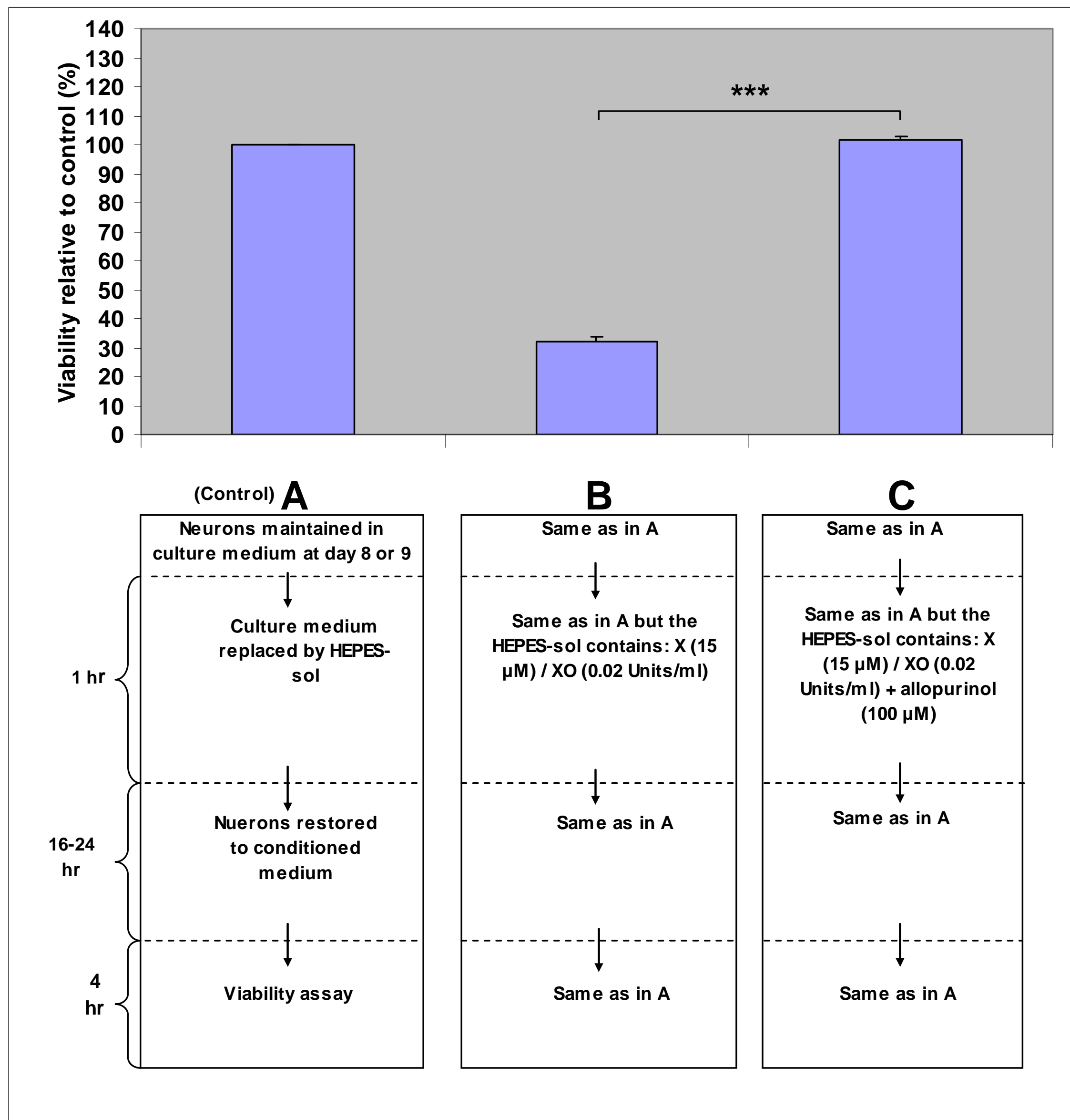


Figure 3-37: Effect of allopurinol on X / XO toxicity in HEPES-sol. It shows that also when the experiment was carried out in HEPES-sol instead of MEM-HEPES-sol, allopurinol prevented X / XO toxicity. ***$p<0.001$. (n=5).

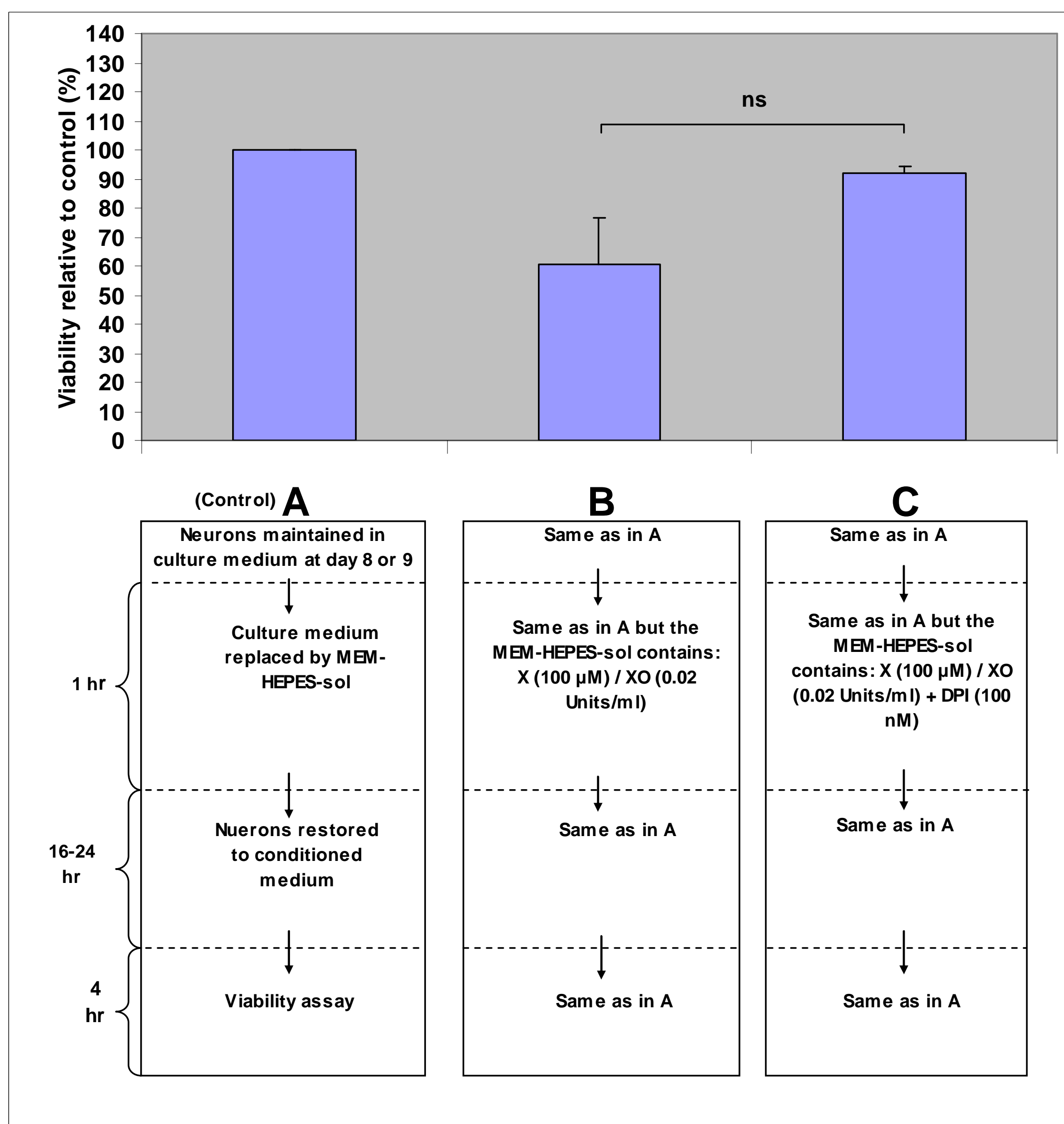


Figure 3-38: Effect of DPI on X / XO toxicity in MEM-HEPES-sol. It shows that blocking the FAD site on XO with DPI, although tended to produce protection against the toxicity of X / XO combination applied in this treatment solution, could not produce statistically significant protection. ns: not significant. (n=5).

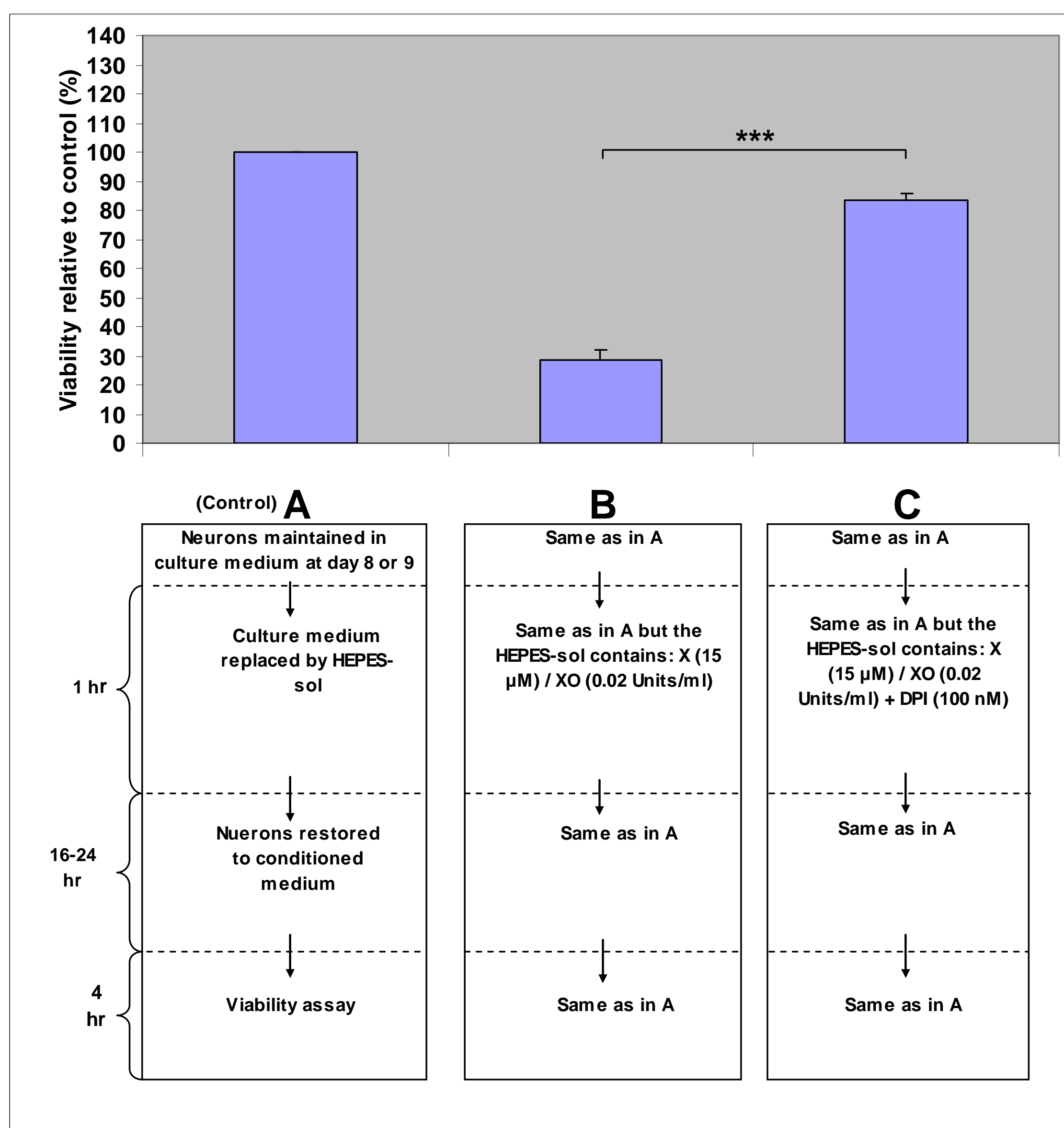


Figure 3-39: Effect of DPI on X / XO toxicity in HEPES-sol. It shows that when the experiment was performed in HEPES-sol instead of MEM-HEPES-sol, DPI produced substantial and statistically significant protection against X / XO toxicity. ***p<0.001. (n=5).

3.3.2.3 Effects of SOD against X / XO and NADH / XO toxicities

Since superoxide is known to be directly generated from X / XO and NADH / XO combinations (see Introduction), these two systems of toxicity were compared in their responses to treatment with SOD-1 (Copper,Zinc-SOD) (which deactivates superoxide by converting two molecules of it into one molecule of hydrogen peroxide plus oxygen). SOD-1 alone at 300 Units/ml was not toxic either in HEPES-sol or MEM-HEPES-sol (data not shown).

When tried in HEPES-sol, SOD-1 (300 Units/ml) failed to protect against X / XO toxicity (Fig. 3-40). Also, when tried in HEPES-sol, Tiron, a known superoxide scavenger [*Greenstock and Miller, 1975, Hassan et al., 1980*], failed to protect against X / XO toxicity (Fig. 3-41). Tiron alone was not toxic (data not shown). The failure of SOD-1 was also obtained when the experiment was performed in MEM-HEPES-sol instead of HEPES-sol (Fig. 3-42).

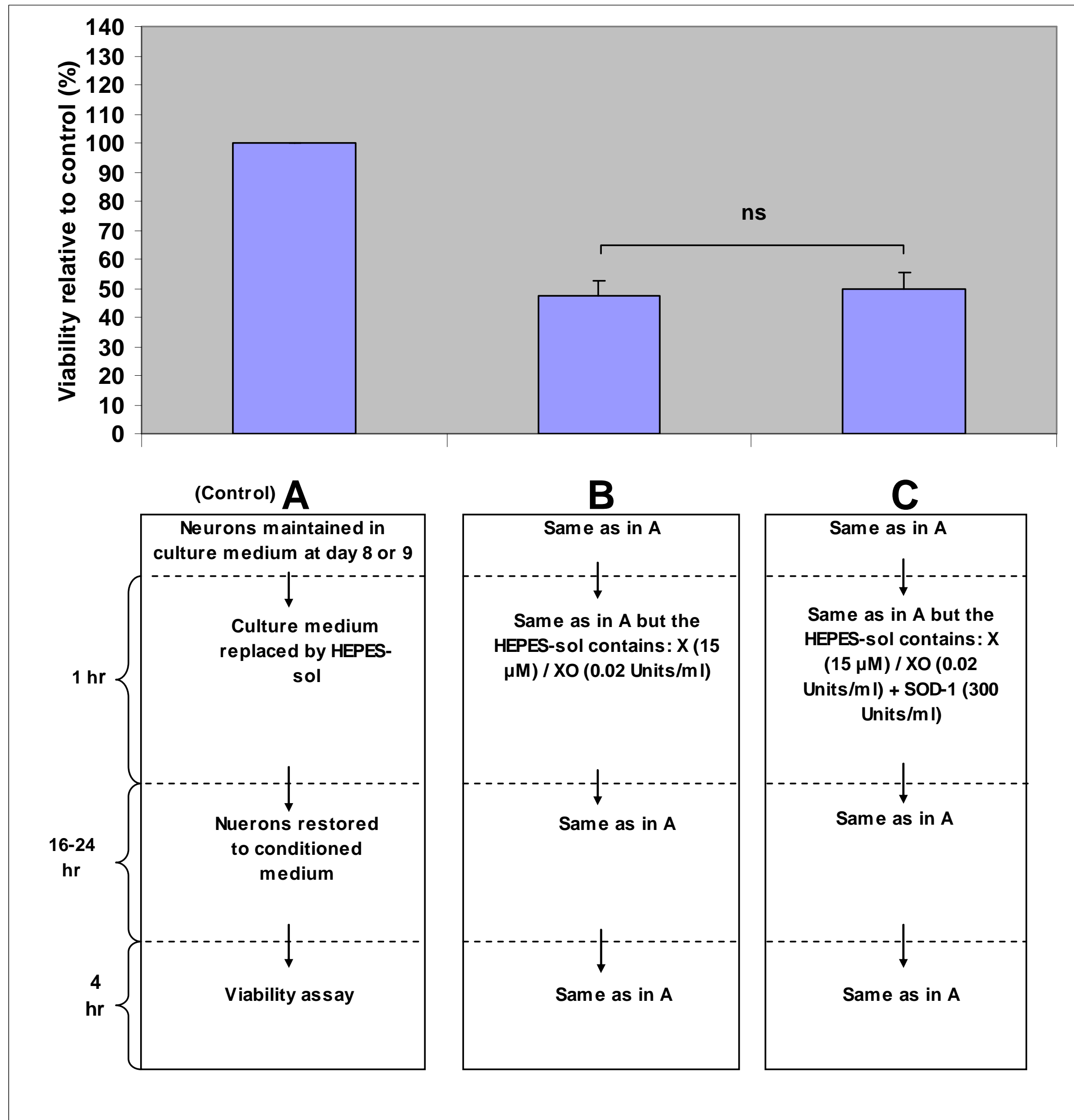


Figure 3-40: Effect of SOD-1 on X / XO toxicity in HEPES-sol. ns: not significant. (n=5).

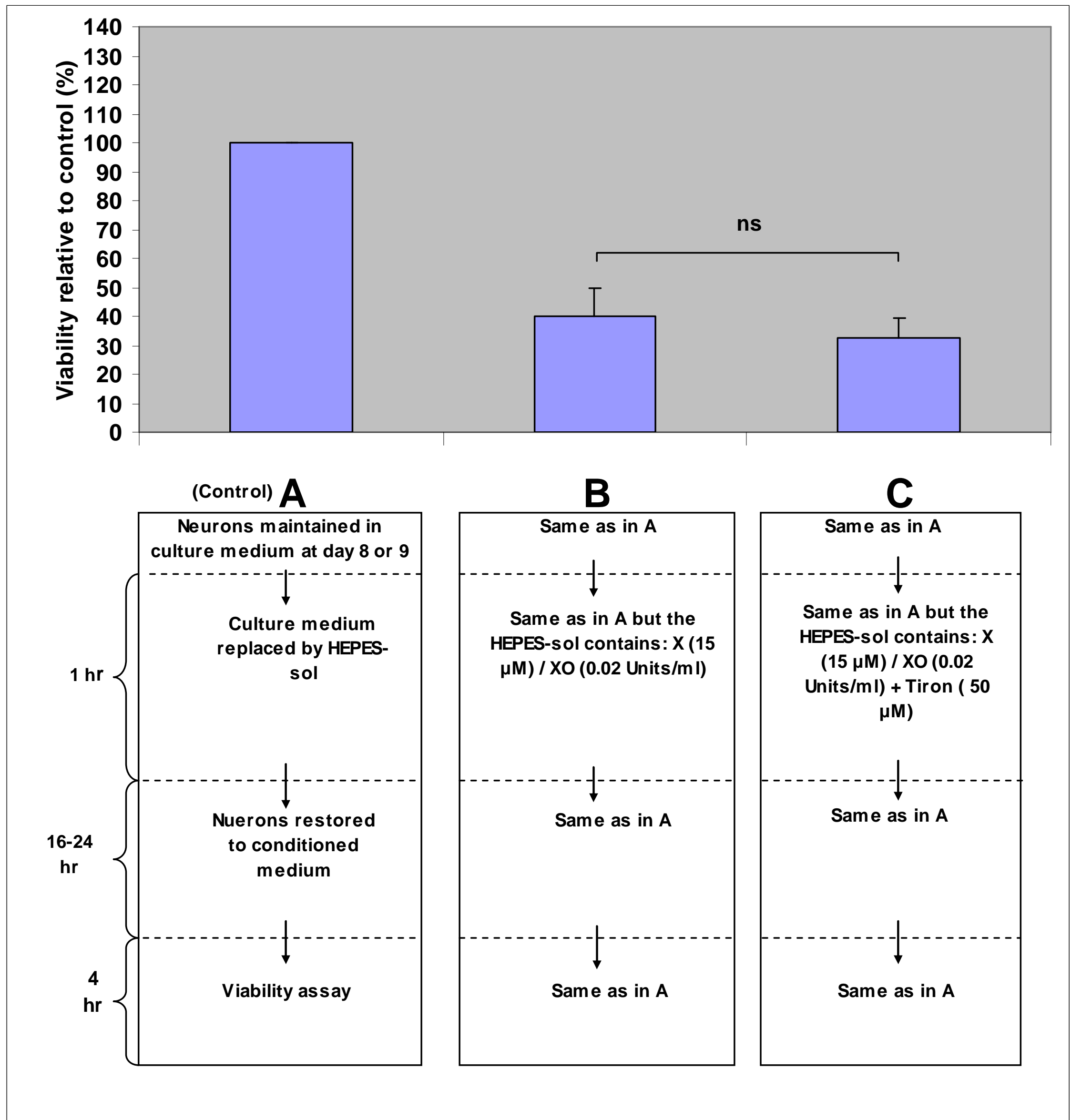


Figure 3-41: Effect of Tiron on X / XO toxicity in HEPES-sol. It shows that like SOD-1, the superoxide scavenger, Tiron, has no effect on X / XO toxicity. ns: not significant. (n=3).

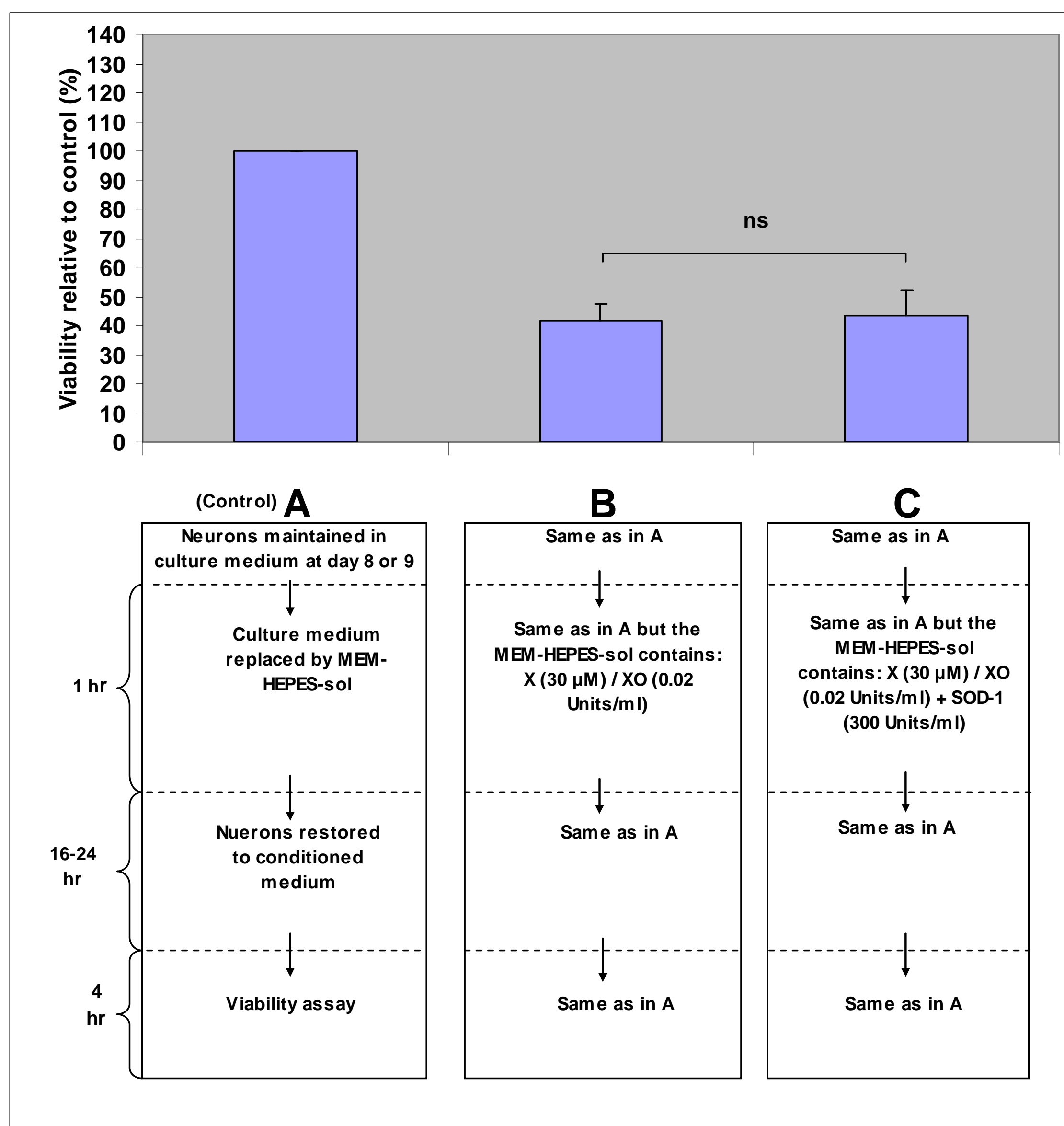


Figure 3-42: Effect of SOD-1 on X / XO toxicity in MEM-HEPES-sol. It shows that also when the experiment was performed in MEM-HEPES-sol instead of HEPES-sol, SOD-1 had no effect on X / XO toxicity. ns: not significant. (n=5).

When tried in HEPES-sol, SOD-1 at either 300 (data not shown) or only 3 Units/ml (Fig. 3-43) was substantially protective against NADH / XO toxicity. This was interesting because, as mentioned above, most previous cell cultures studies found no protection with SOD-1 co-treatment against X / XO toxicity. Also when tried in MEM-HEPES-sol instead of HEPES-sol, SOD-1 at 300 Units/ml (it was not tried at 3 Units/ml in this solution) was protective against NADH / XO toxicity (Fig. 3-44).

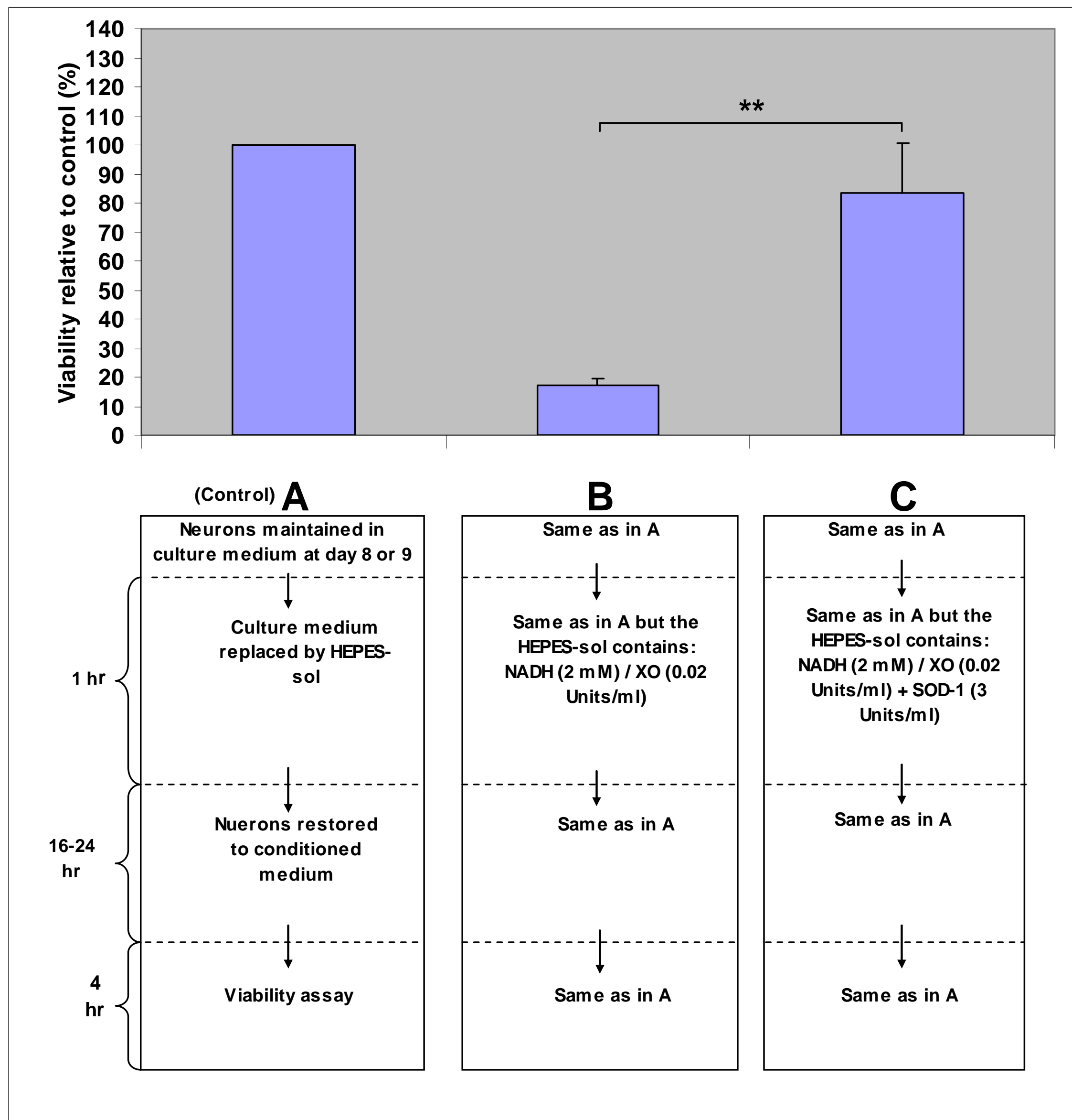


Figure 3-43: Effect of SOD-1 on NADH / XO toxicity in HEPES-sol. It shows that, although failed to protect against X / XO toxicity, SOD-1 substantially protected against NADH / XO toxicity. **$p<0.01$. (n=5).

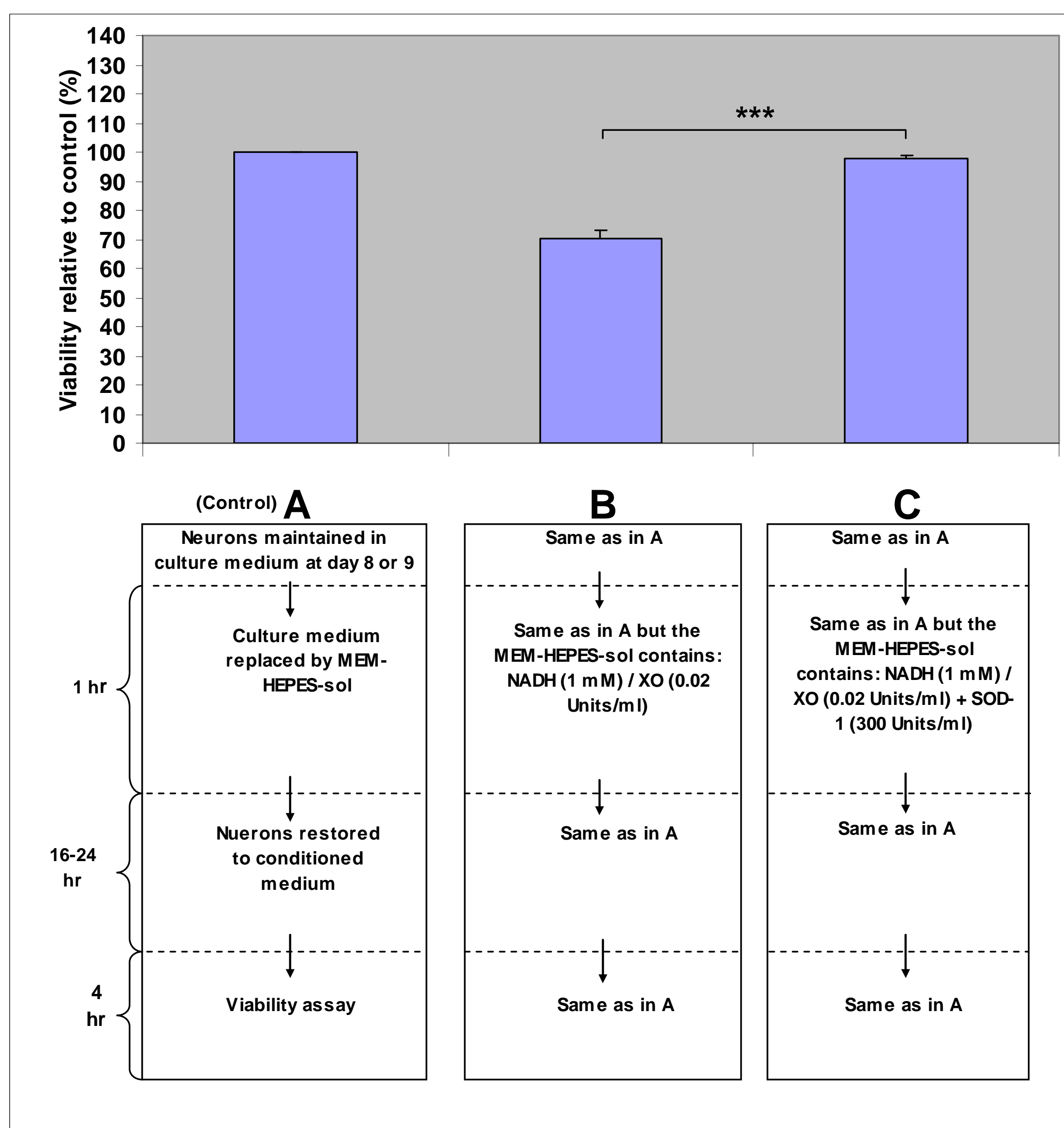


Figure 3-44: Effect of SOD-1 on NADH / XO toxicity in MEM-HEPES-sol. It shows that also when the experiment was performed in MEM-HEPES-sol instead of HEPES-sol, SOD-1 protected against NADH / XO toxicity. ***p<0.001. (n=5).

To make sure that the protection with SOD-1 against NADH / XO toxicity was due to its elimination (dismutation) of superoxide and not due to any of the known non-specific actions of SOD-1 (see Discussion), two additional experiments were conducted. In the first experiment, Mn-SOD, which is known to be free of at least some of the known non-specific actions of SOD-1 [*Sankarapandi and Zweier, 1999, Liochev and Fridovich, 2000*], was tried at 3 Units/ml against NADH / XO toxicity, where it was as protective as SOD-1 (data not shown). In the second experiment, Tiron, which is (as mentioned above) a known superoxide scavenger, was tried in HEPES-sol, and showed substantial protection against NADH / XO toxicity (Fig. 3-45) (though Tiron is also known to be (among other actions) an effective chelator of some metals including iron

and molybdenum [*Fridovich and Handler, 1962*], an activity that can not be ruled out as the reason for its protection).

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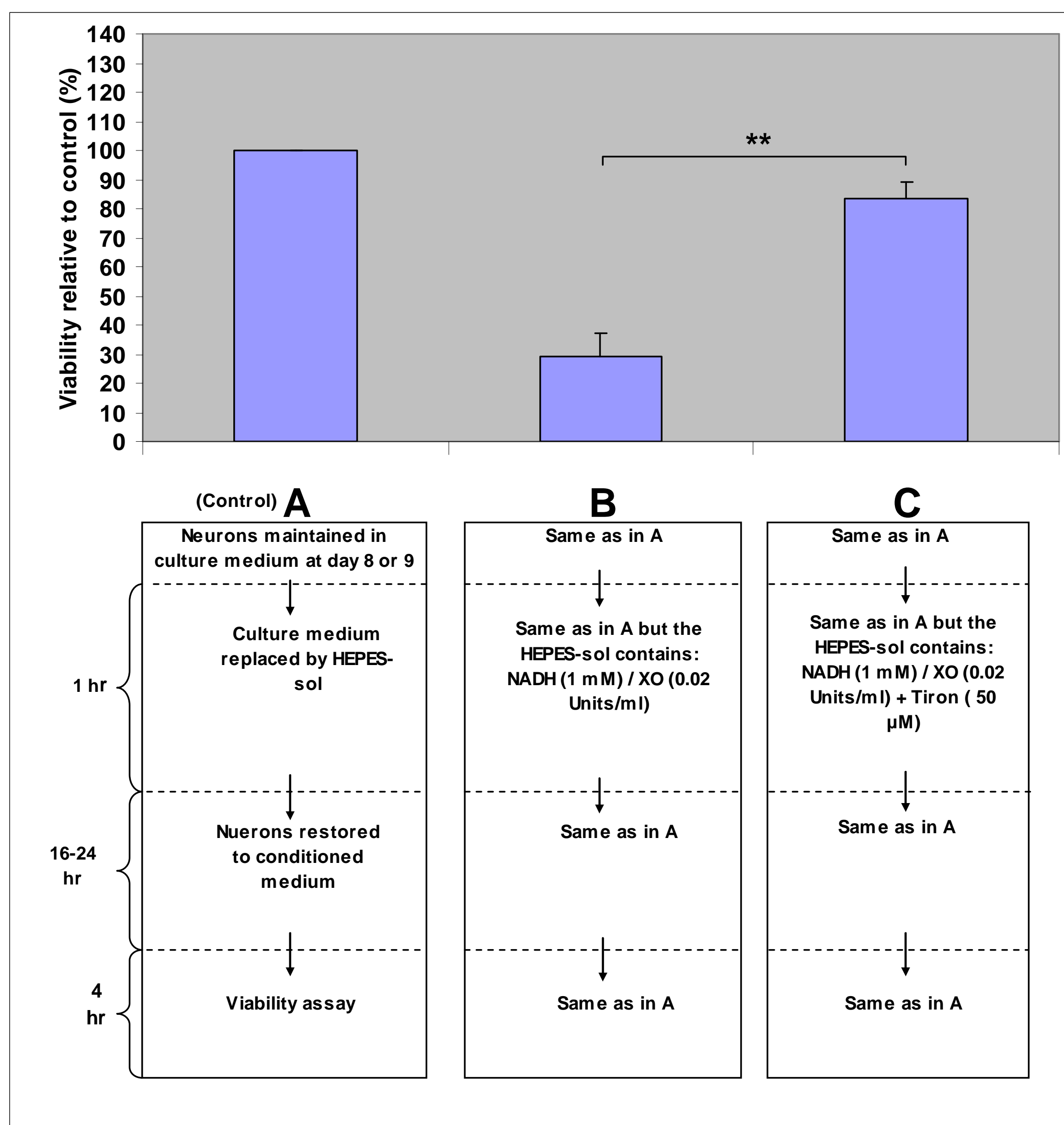

Figure 3-45: Effect of Tiron on NADH / XO toxicity in HEPES-sol. It shows that like SOD-1, although failed to protect against X / XO toxicity, Tiron substantially protected against NADH / XO toxicity. **p<0.01. (n=3).

The failure of SOD-1 and Tiron to protect against X / XO toxicity is unlikely to be because superoxide was not generated from the X / XO combination, since (as mentioned before) this radical is known to be directly generated from the X / XO combination. Also, in a cell-free experiment where Cytochrome c was used as a detection molecule (see Materials and Methods), X / XO combination generated superoxide, whereas this radical was barely detected in the presence of SOD-1 (Fig. 3-

46) (also see later on in Fig. 3-50 and Fig. 3-51 where the time course of superoxide generation is shown).

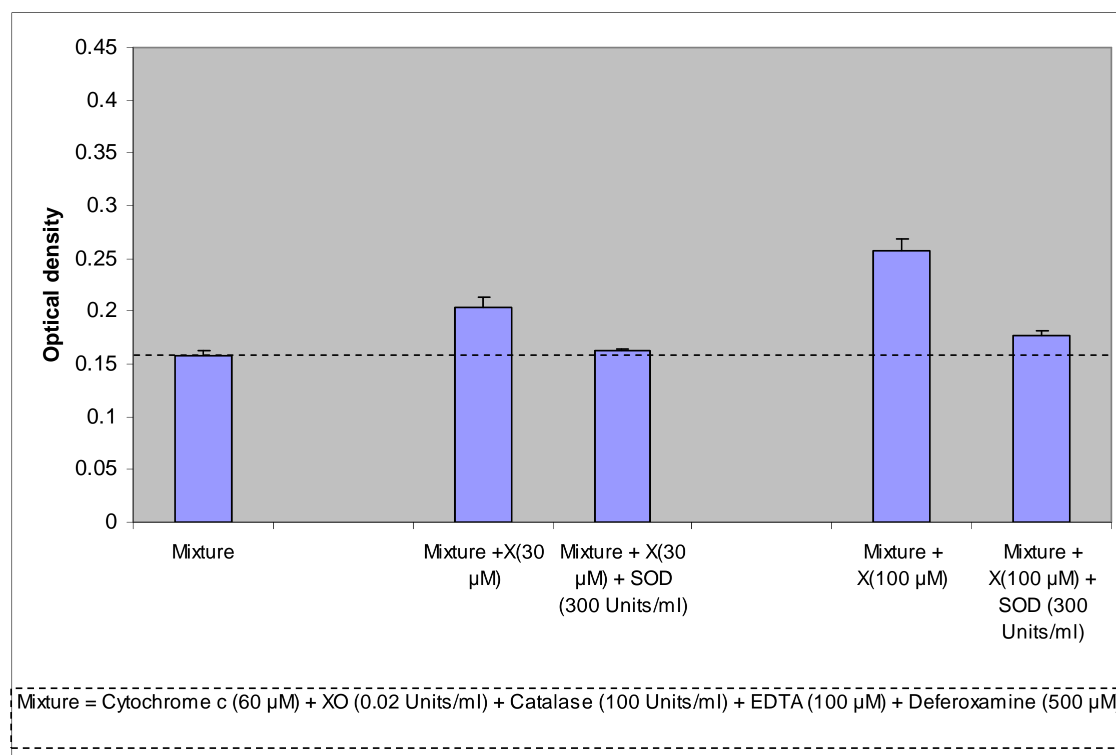

Figure 3-46: Cell-free detection (using Cytochrome c reduction method) of superoxide generation by the X / XO combination in the HEPES-sol. It shows that, as expected, the X / XO combination generates superoxide. The experiment was performed as follows: test cell-free wells contained the treatment groups in HEPES-sol and left for 1 hr. After that, the optical density at 550 nm was recorded on a plate reader. Deferoxamine and EDTA were included in the mixture to suppress any unwanted reactions of possibly contaminating traces of metals. Catalase is included because hydrogen peroxide, which will be produced in the system, interferes with the assay. The signal observed in the first group (the mixture only group) is expected, since oxidized Cytochrome c should give such a signal. (n=3).

The protection of SOD-1, Mn-SOD, and Tiron against NADH / XO toxicity suggests that superoxide generated from this combination plays a role in the toxicity. Superoxide, as mentioned above, does indeed get generated directly from the NADH / XO combination. Unfortunately, it was not possible to confirm this generation in cell-free experiments, because NADH added alone interfered with the two cell-free detection assays of superoxide that were tried here. So, NADH added alone (without XO) caused increase in Cytochrome c signal. This reduction of Cytochrome c was partially inhibited by SOD-1 (Fig. 3-47). This suggests either that superoxide was generated spontaneously from NADH in the HEPES-sol and was then detected by Cytochrome c or that

Cytochrome c was interacting with NADH where superoxide was generated from such interaction, which was then detected by Cytochrome c.

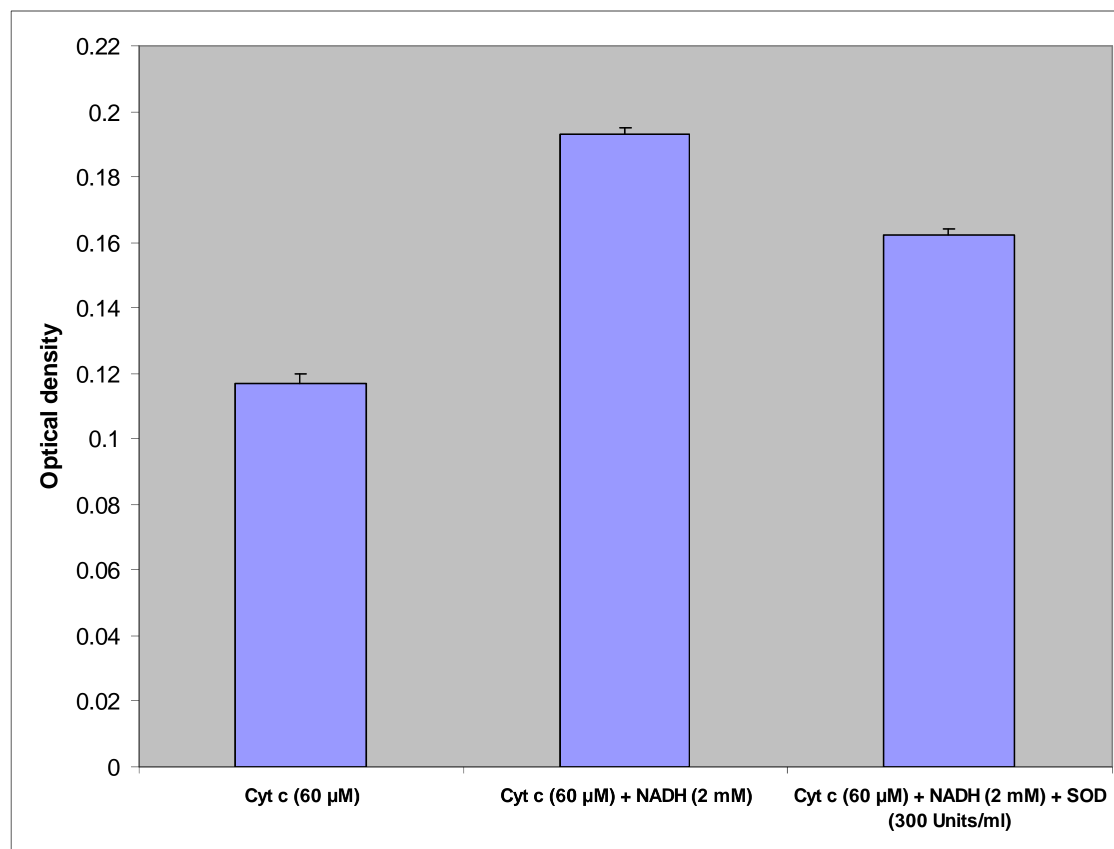

Figure 3-47: Cell-free experiment showing the effect of NADH alone (without XO) on Cytochrome c (Cyt c) signal in the HEPES-sol. The experiment was performed as follows: test cell-free wells contained the treatment groups in HEPES-sol and left for 1 hr. After that, the optical density at 550 nm was recorded on a plate reader. (n=3).

Also, when another cell-free detection method of superoxide was tried, the reduction of XTT (see Materials and Methods), NADH added alone (without XO) caused substantial reduction of XTT. This reduction of XTT was completely prevented in the presence of SOD-1 (Fig. 3-48). Again, this suggests either that superoxide was generated spontaneously from NADH in the HEPES-sol and was then detected by XTT or that XTT was interacting with NADH where superoxide was generated from such interaction, which was then detected by XTT. One way to test for that was to see the effect of adding XTT and NADH to cells. In a viability experiment, it was clear that XTT was not acting as just an innocent detector of superoxide, but rather was interacting with NADH to cause substantial damage, likely through generating ROS, to the neurons (Fig. 3-49) (notice that in this viability experiment, XTT or NADH were

not toxic when added alone, but when added together, they caused severe damage to the neurons).

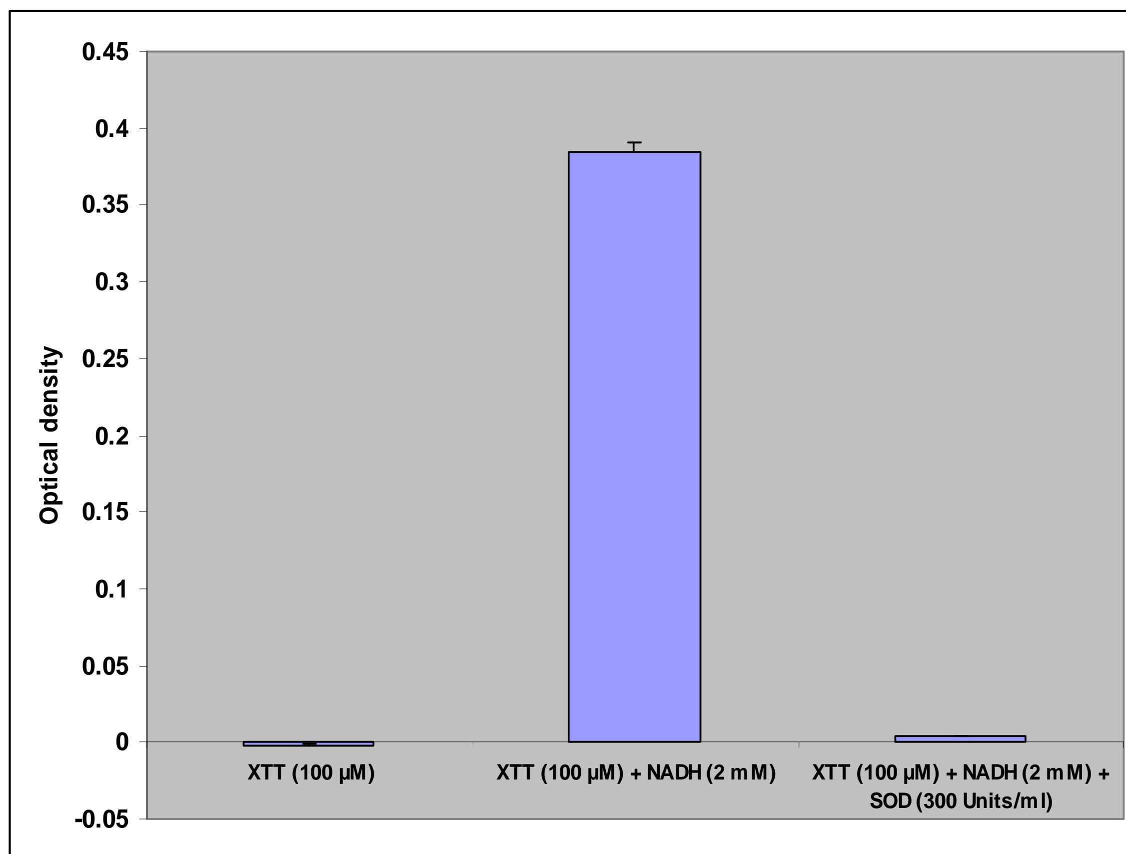

Figure 3-48: Cell-free experiment showing the reduction of XTT by NADH alone (without XO) in HEPES-sol. The experiment was performed as follows: test cell-free wells contained the treatment groups in HEPES-sol and left for 1 hr. After that, the optical density at 450 nm was recorded on a plate reader. (n=3).

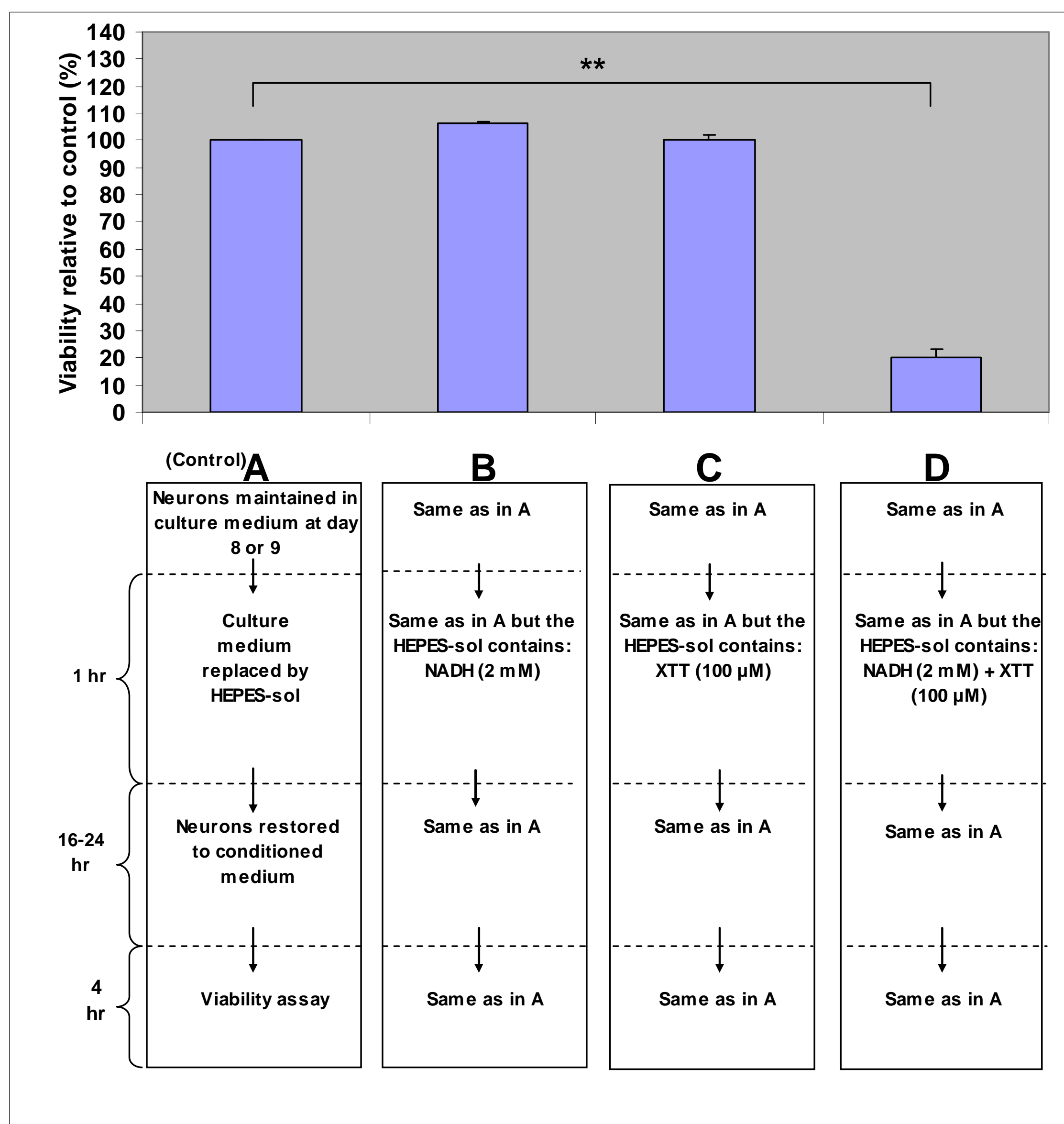

Figure 3-49: Effect of NADH and XTT applied alone or in combination in HEPES-sol on the cell viability. It shows that the superoxide detector, XTT, interacts with NADH to cause, probably through generating ROS, severe damage to the neurons. $^{**}p<0.01$. (n=5).

3.3.2.4 Effects of catalase against X / XO and NADH / XO toxicities

Since hydrogen peroxide is known to be directly generated from X / XO and NADH / XO combinations (see Introduction), these two systems of toxicity were compared in their responses to treatment with catalase (which deactivates hydrogen peroxide by converting it into water and oxygen). Catalase alone at 300 Units/ml was not toxic to neurons either in HEPES-sol or MEM-HEPES-sol (data not shown).

In the early viability experiments with catalase, it was used at 300 Units/ml. however, some reports in the literature showed that some commercial preparations of catalase are

contaminated with significant amount of SOD [*Halliwell, 1973, Liochev and Fridovich, 1989*], so it was necessary to check if the preparation of catalase used here is also contaminated with SOD activity. Using the cell-free XTT reduction as a detection method of superoxide generation in X / XO system, catalase at 3 Units/ml did not seem to contain significant contamination of SOD activity. However, catalase at 300 or 1000 Units/ml seemed to contain significant contamination of SOD activity (Fig. 3-50). The contamination of catalase (1000 Units/ml) by SOD activity was also confirmed by the other detection method of superoxide, the Cytochrome c reduction method (Fig. 3-51) (notice that contamination of catalase at 300 Units/ml by SOD activity could not be verified in this Cytochrome c experiment because catalase at 300 Units/ml was an essential component of the reaction mixture used to prevent interference of hydrogen peroxide with the assay (see Materials and Methods)). Therefore, only those viability experiments where catalase was used at no more than 3 Units/ml will be presented in this section.

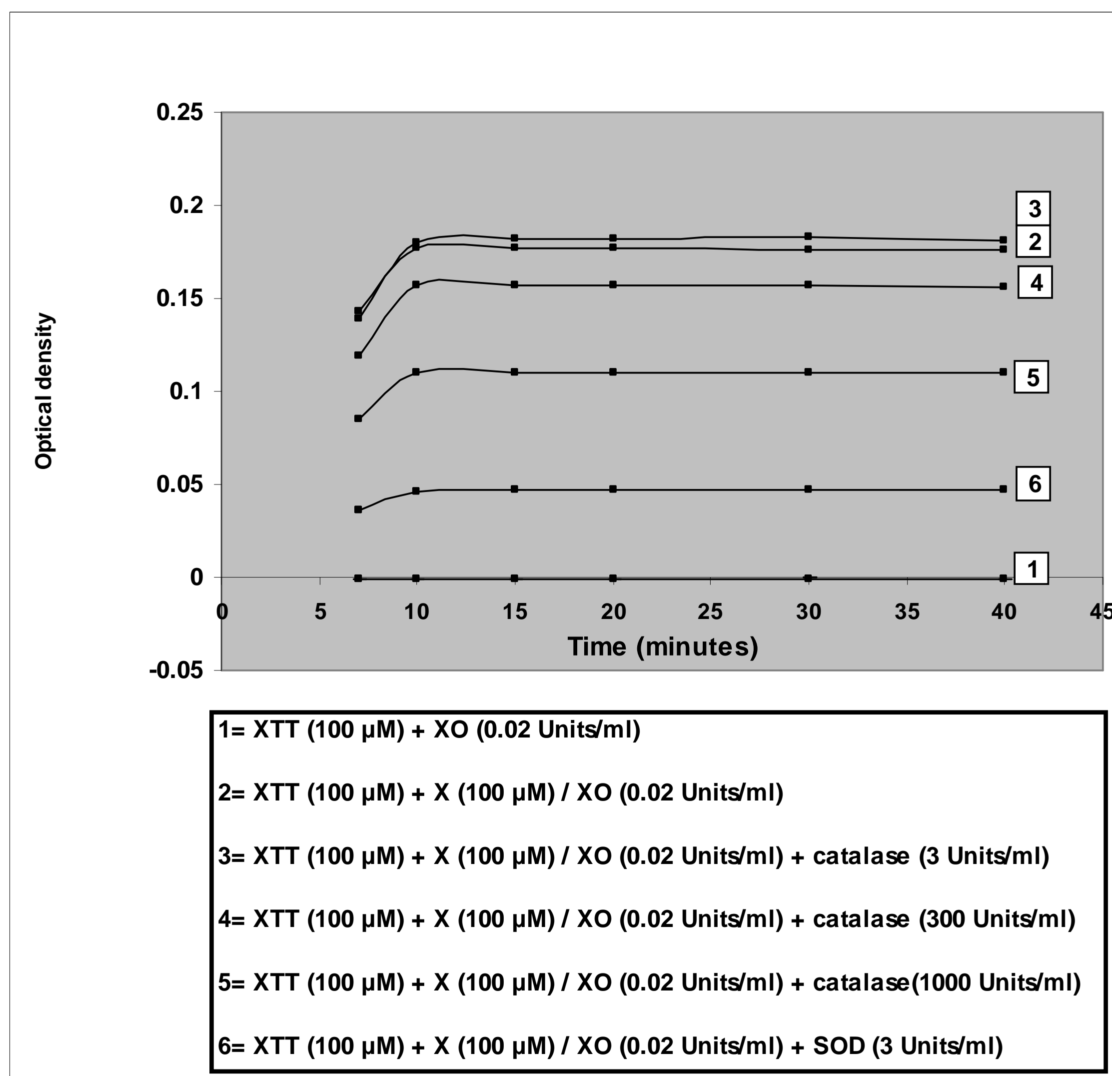

Figure 3-50: Cell-free XTT reduction assay showing the time course of superoxide production by the X / XO combination in HEPES-sol. It shows the contamination of catalase with some SOD activity. The experiment was performed as follows: test cell-free wells contained the treatment groups and the optical density was recorded on a plate reader at the indicated time points. Notice that by 10 minutes after starting the reactions, superoxide generation ceased. The readings are the averages of two repeats of the experiment (every repeat conducted in a different day), where both of the repeats gave very similar readings. Notice that (unfortunately) the first readings were taken after 7 minutes of starting the reaction, by which the superoxide production was approaching its completion. This delay was due to the time required (after starting the reactions in test tubes) for aliqouting the mixture solutions into a 96-well plate, and then taking the readings on the plate reader. To observe the initial enzyme kinetics, the groups can be measured individually in a spectrophotometer with a single cuvette.

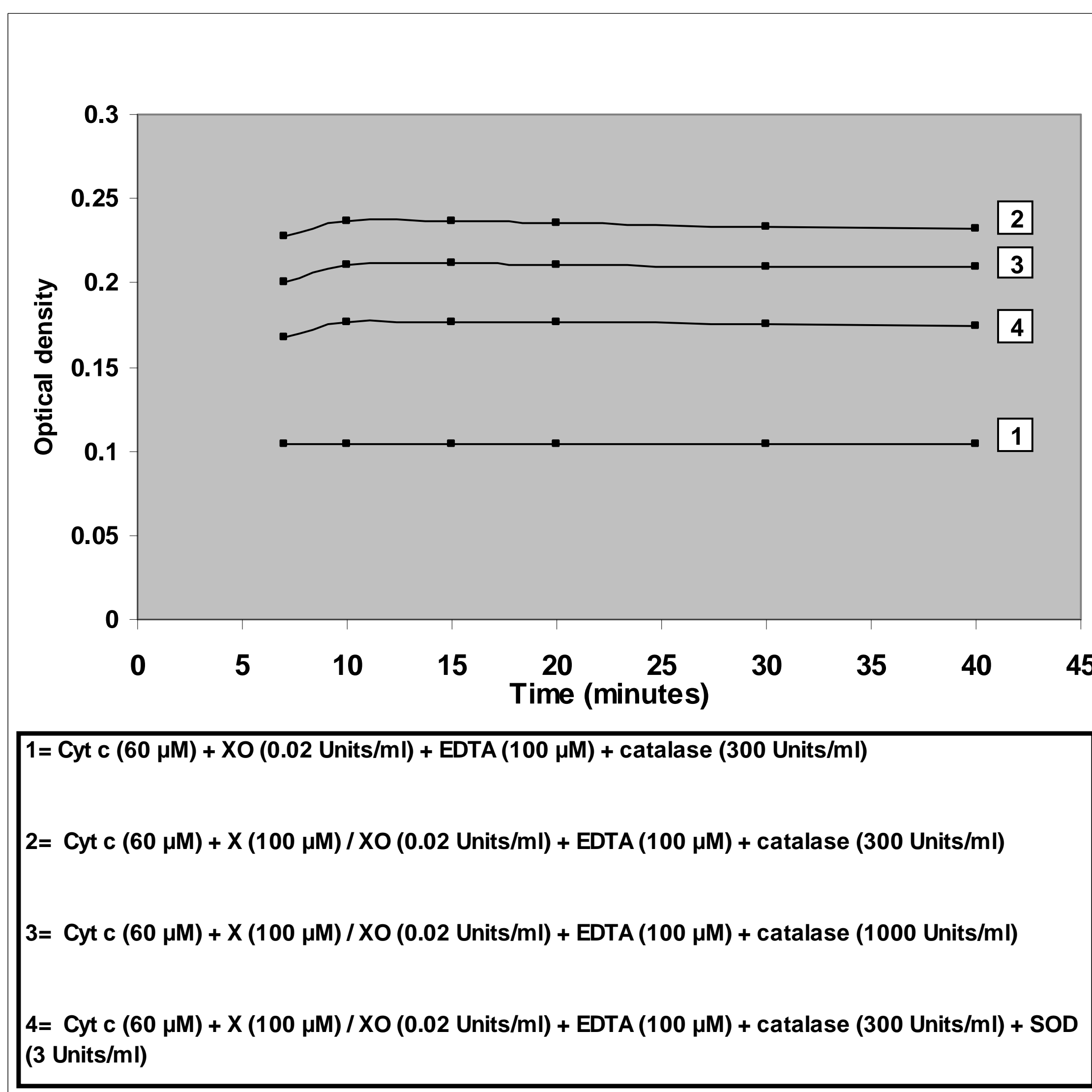

Figure 3-51: Cell-free Cytochrome c reduction assay showing the time course of superoxide production by the X / XO combination in HEPES-sol. It shows the contamination of catalase (1000 Units/ml) with some SOD activity. The experiment was performed as follows: test cell-free wells contained the treatment groups and the optical density was recorded on a plate reader at the indicated time points. The signal at trace 1 is expected, since oxidized Cytochrome c should give such a signal. Notice that by 10 minutes after starting the reactions, as it was the case in the XTT reduction assay shown in Fig. 3-50, superoxide generation ceased. The readings are the averages of two repeats of the experiment (every repeat conducted in a different day), where both of the repeats gave very similar readings. For the explanation for why the first readings were not taken until 7 minuters of starting the reactions, please see Fig. 3-50.

In viability experiments, when tried in HEPES-sol, catalase (3 Units/ml) offered complete protection against X / XO toxicity (Fig. 3-52). Also, when tried in MEM-HEPES-sol, catalase (up to 3 Units/ml) showed protection against X / XO toxicity in a dose dependent manner (Fig. 3-53). When tried in HEPES-sol, catalase (3 Units/ml) offered complete protection against NADH / XO toxicity (Fig. 3-54) (catalase was not tried in MEM-HEPES-sol against NADH / XO toxicity).

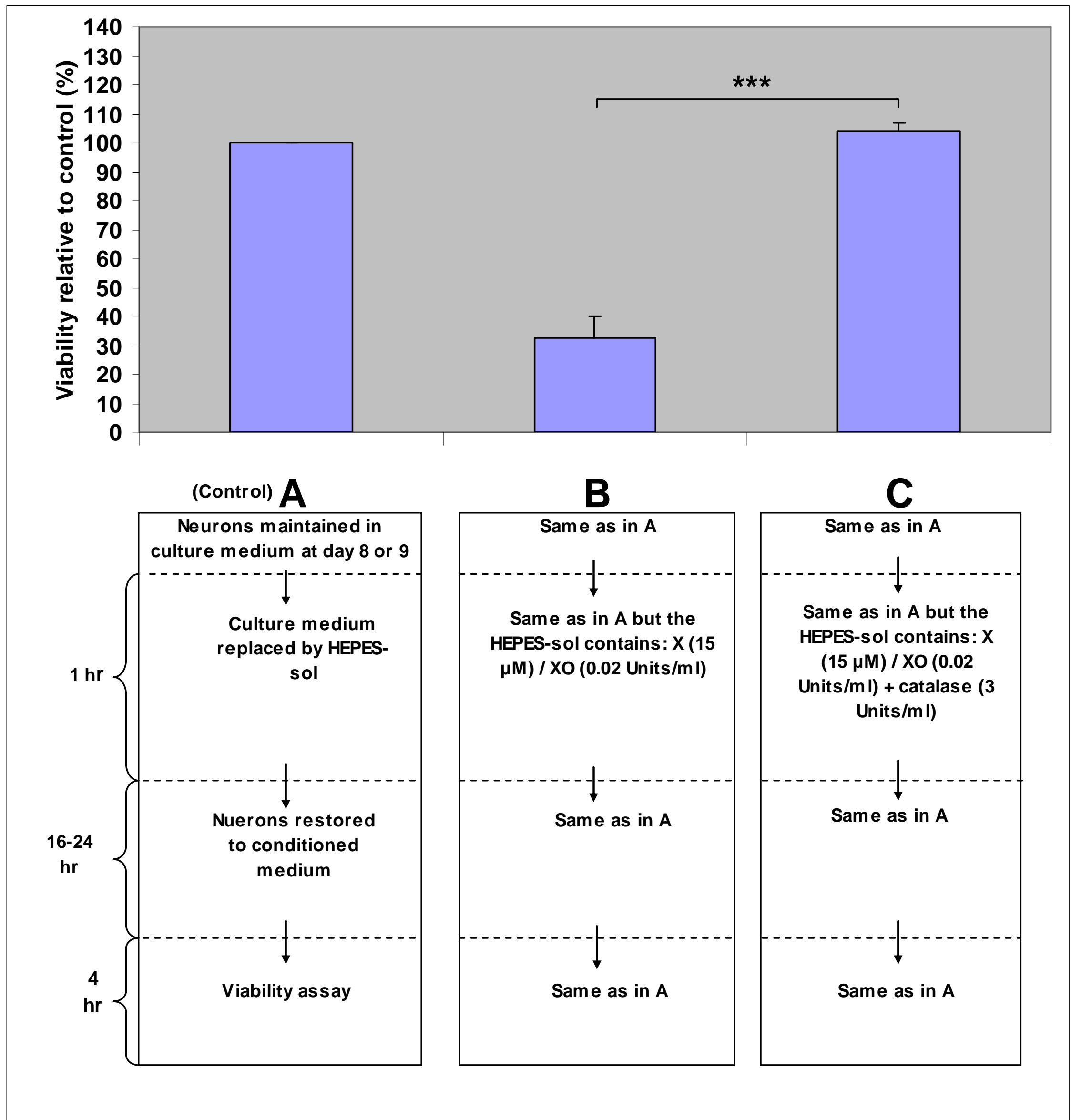

Figure 3-52: Effect of catalase on X / XO toxicity in HEPES-sol. ***p<0.01. (n=4).

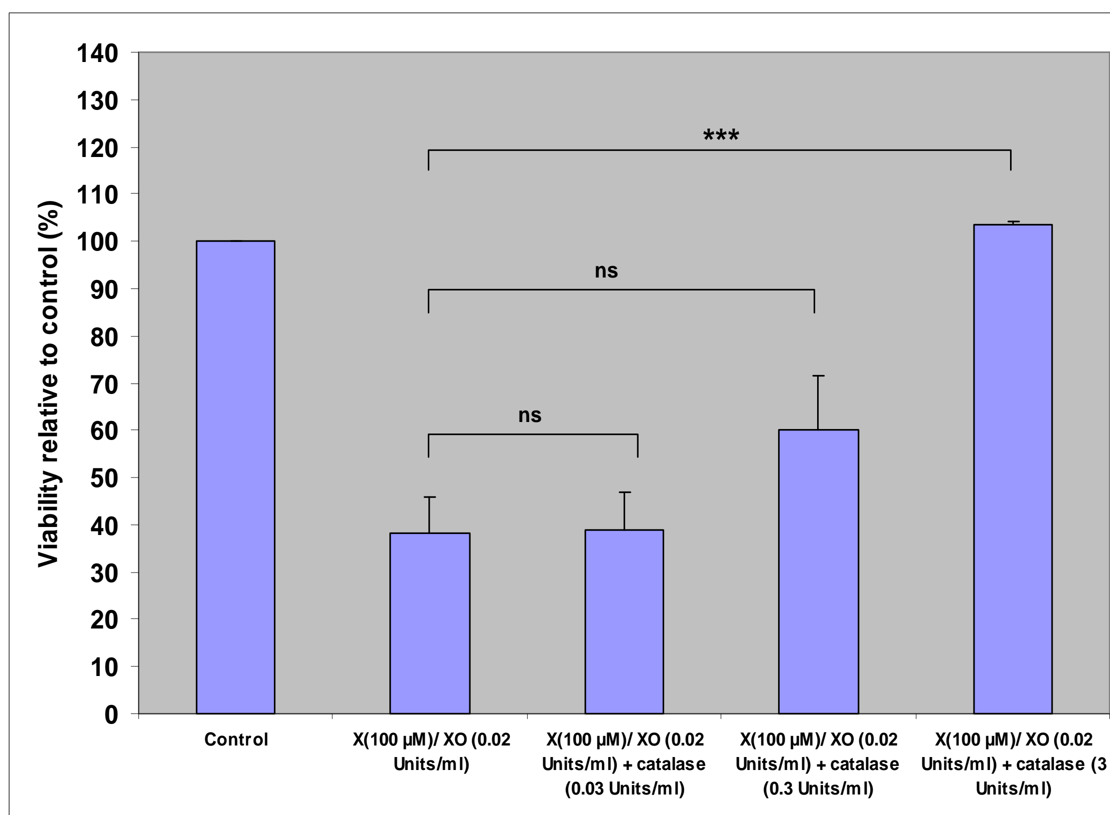

Figure 3-53: Effect of catalase on X / XO toxicity in MEM-HEPES-sol. The experiment was performed as follows: at day 8 or 9 after plating, the culture medium was replaced by MEM-HEPES-sol that does not contain (control) or contains the indicated test compounds, and left for 1 hour → the neurons in all groups (including control) were restored to conditioned medium and left for 16-24 hours until the viability assay. ***$p<0.001$, ns: not significant. (n=5).

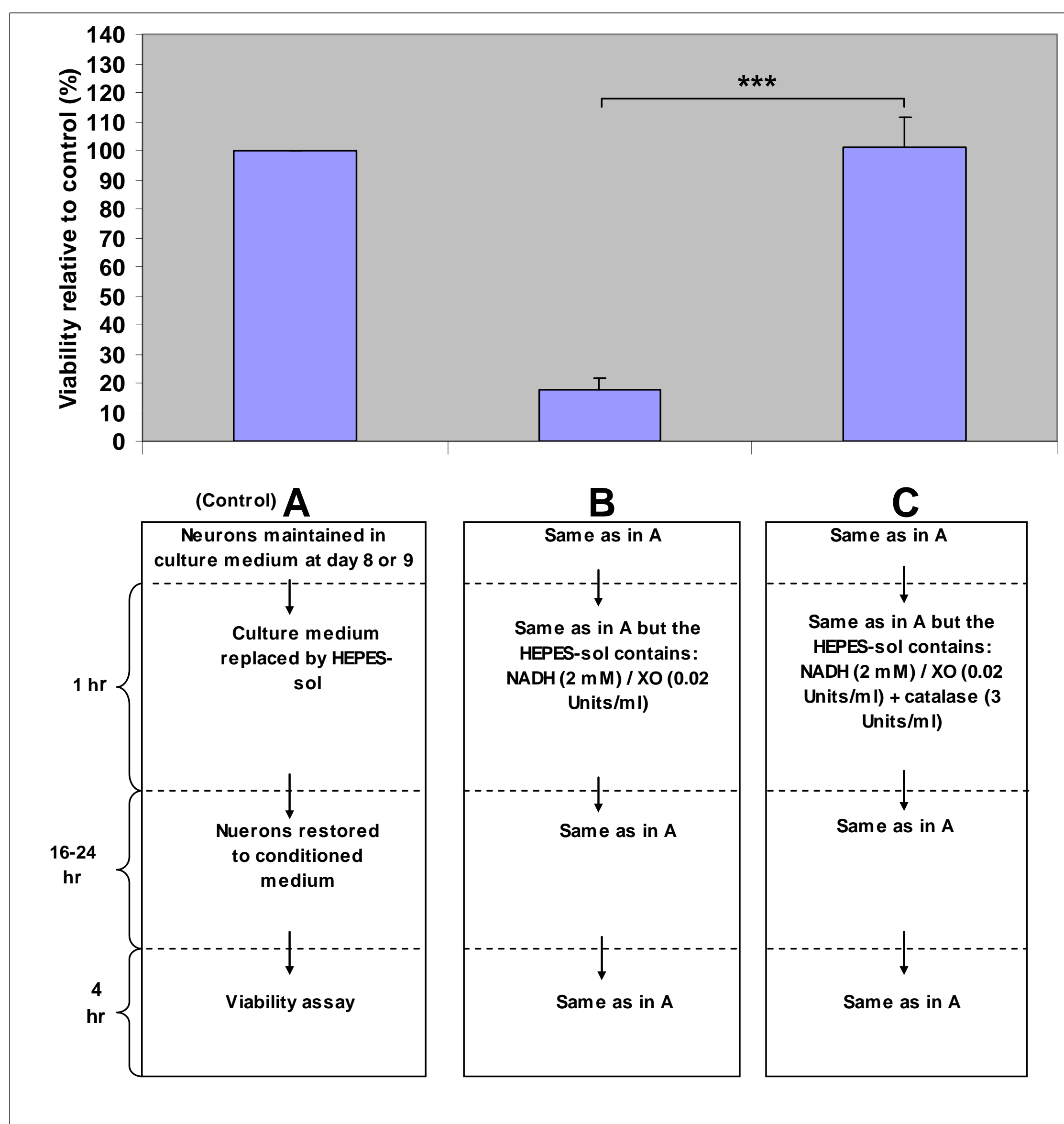

Figure 3-54: Effect of catalase on NADH / XO toxicity in HEPES-sol. ***p<0.001. (n=3).

As seen, the viability experiments with catalase show its ability to offer complete protection against both of the toxicity systems i.e. X / XO and NADH / XO toxicities. This suggests that hydrogen peroxide plays a central role in both of the toxicity systems. Hydrogen peroxide does indeed get generated directly from X / XO and NADH / XO combinations, and should also be produced from the spontaneous dismutation of superoxide which is directly generated from these combinations (see Introduction).

To confirm the production of hydrogen peroxide, some cell-free experiments were conducted. There was also another reason for measuring hydrogen peroxide production/accumulation in the systems. In the case of NADH / XO toxicity, since

catalase, likely through deactivating hydrogen peroxide, provided complete protection against the toxicity, it was expected that SOD-1 and Mn-SOD (which convert superoxide to hydrogen peroxide) should potentiate the toxicity (or at least have no effect) rather than preventing it as observed. A possible explanation for this paradox is that the NADH / XO toxicity was due to a superoxide-dependent hydrogen peroxide-accumulating free radical chain reaction where adding SOD to such a reaction can, although by converting superoxide to hydrogen peroxide, block the hydrogen peroxide-accumulating chain reaction from the start, and hence prevent much larger and toxic production/accumulation of hydrogen peroxide in the system (see Discussion). In a cell-free experiment conducted to detect hydrogen peroxide production/accumulation in the NADH / XO system, adding SOD-1 (3 Units/ml) seemed to potently inhibit hydrogen peroxide production/accumulation in the system (Fig. 3-55), which gives support to the proposed explanation for the above mentioned paradox. Using the same assay, SOD-1 at either 3 or 300 Units/ml did not seem to influence hydrogen peroxide production/accumulation in X / XO system, whereas allopurinol (used to confirm the enzymatic oxidation of X) potently inhibited this production/accumulation (Fig. 3-56). Almost exactly the same degree of inhibition of hydrogen peroxide production/accumulation in the NADH / XO system by SOD-1 was also observed when the reaction was carried out in a HEPES-free solution (Dulbecco's Phosphate-Buffered Saline (DPBS)) instead of HEPES-sol (data not shown).

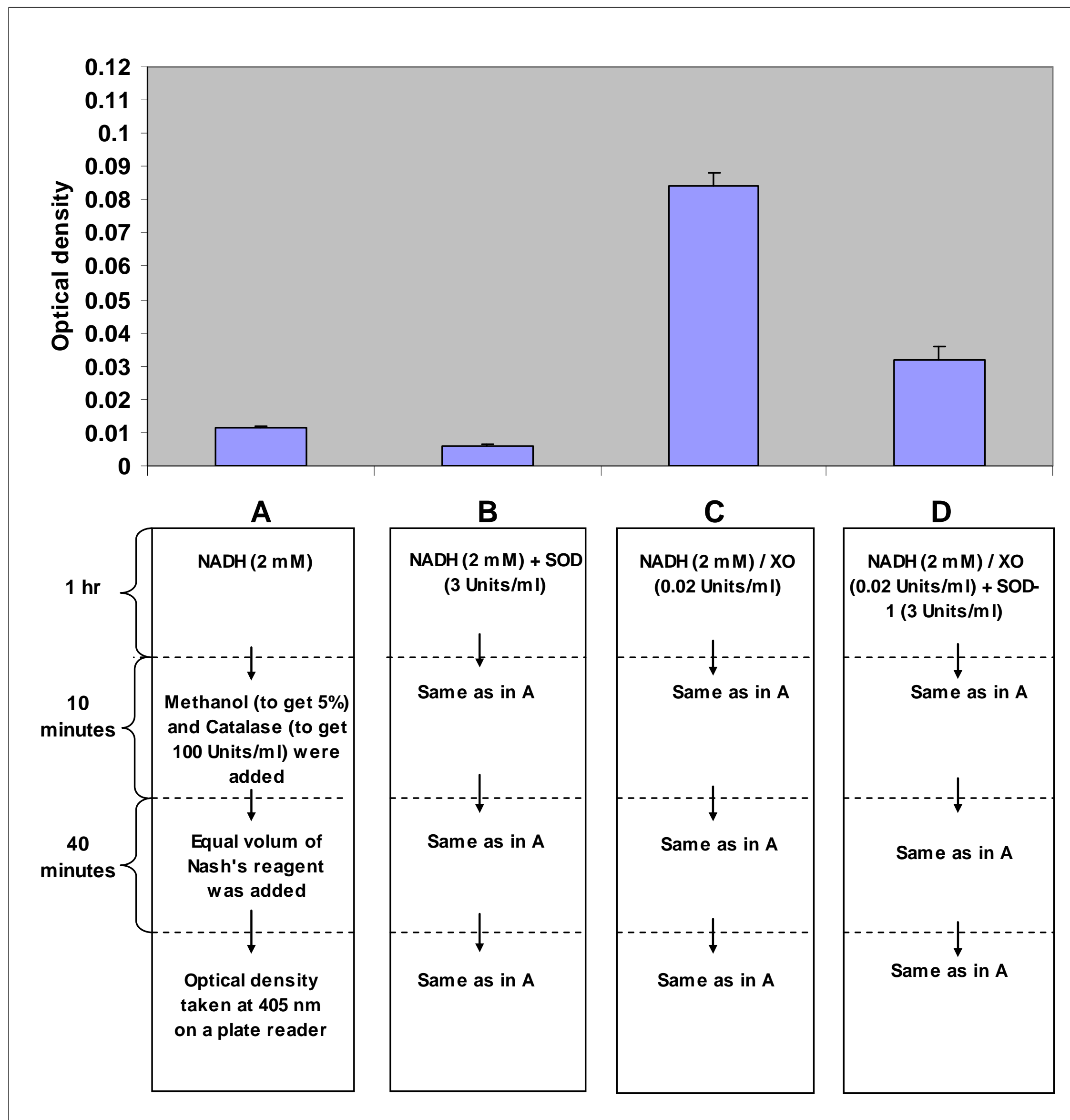

Figure 3-55: Cell-free catalase-based assay of hydrogen peroxide production/accumulation from the NADH / XO combination. It shows that SOD-1 decreases hydrogen peroxide accumulation in NADH / XO system. The reactions were carried in HEPES-sol. (n=3).

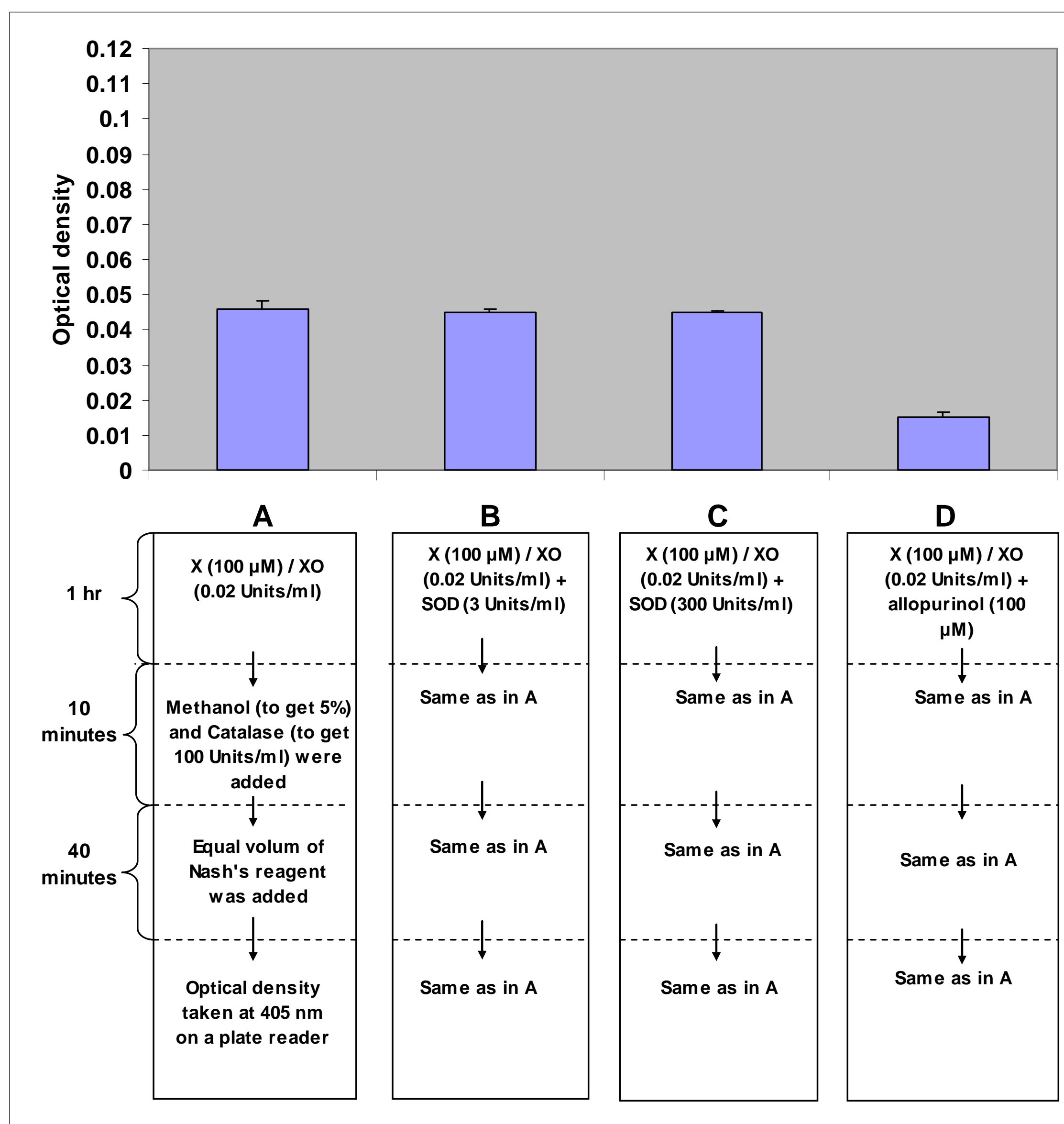

Figure 3-56: Cell-free catalase-based assay of hydrogen peroxide production/accumulation from the X / XO combination. It shows that SOD-1 does not influence hydrogen peroxide production/accumulation in X / XO system, whereas allopurinol (used to confirm the enzymatic oxidation of X) potently inhibited this production/accumulation of hydrogen peroxide. The reactions were carried in HEPES-sol. (n=3).

3.3.2.5 Role of metals in X / XO and NADH / XO toxicities

Since the viability experiments indicated the involvement of hydrogen peroxide in X / XO and NADH / XO toxicities and indicated the involvement of superoxide in NADH / XO toxicity, and since hydrogen peroxide and superoxide can exert their toxic effects through interacting with some metals (see Introduction), a series of experiments were conducted in order to investigate the involvement of metals in the studied toxicity systems.

Deferoxamine at 300 µM was not toxic to CGNs when applied alone in MEM-HEPES-sol as a 3 hr pre-treatment (data not shown). In MEM-HEPES-sol, when deferoxamine was applied to CGNs as pre-treatment for 3 hr, removed, and replaced by X / XO combination, it provided protection against the toxicity of this combination (Fig. 3-57). When the experiment was repeated but with only 15 minutes deferoxamine pre-treatment instead of 3 hr, it failed to protect (data not shown). This suggests that deferoxamine was exerting its protective effect intracellularly and also suggests that this chelator, as expected, needs relatively long pre-treatment time to get inside the cells.

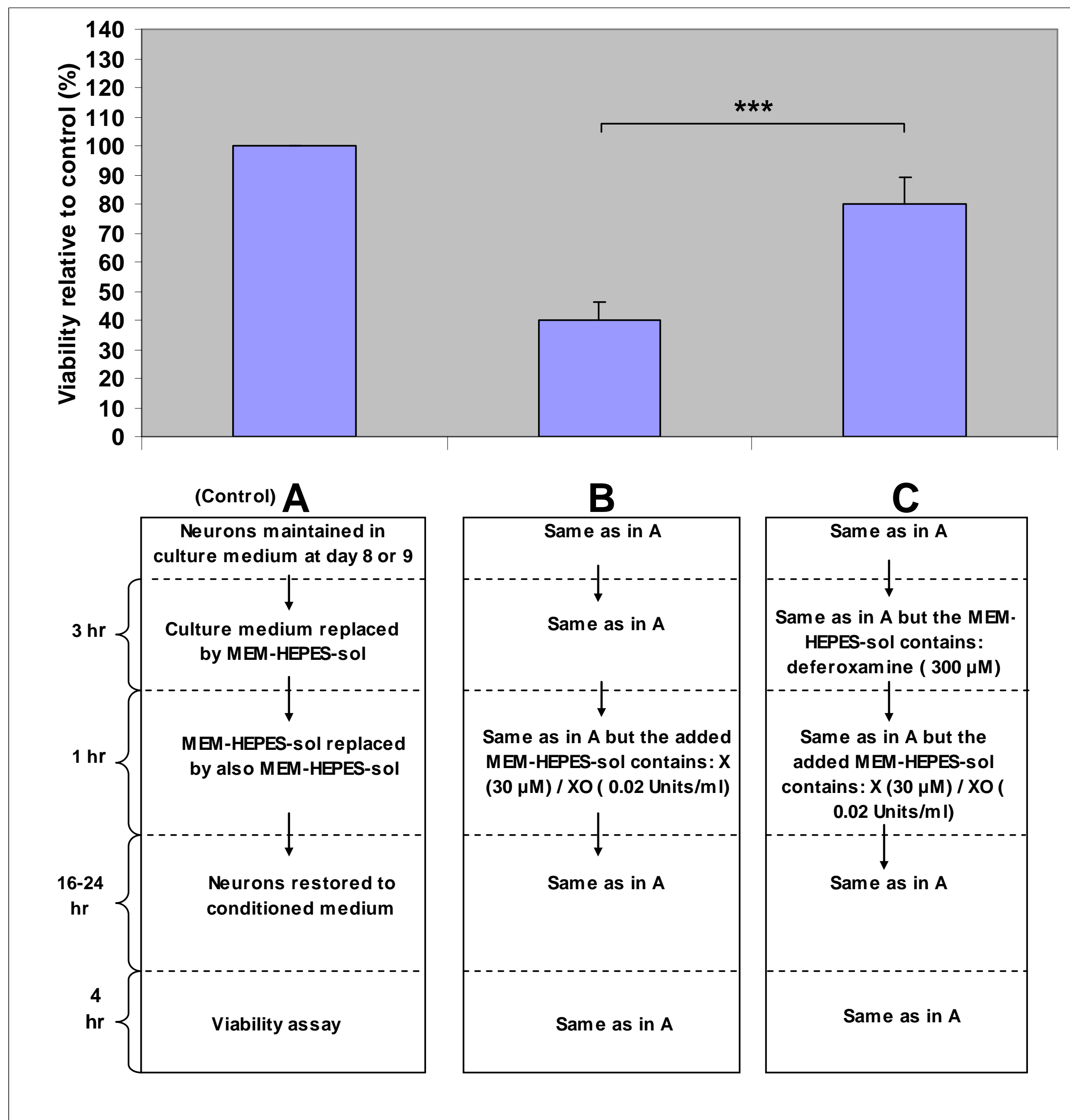

Figure 3-57: Effect of deferoxamine pre-treatment on X / XO toxicity in MEM-HEPES-sol. Notice that deferoxamine was removed before applying the toxic insult. ***p<0.001. (n=5).

Although the above experiment with deferoxamine involved pre-treating neurons for 3 hr and then treating for 1 hr in a serum-free medium, it is unlikely that withdrawing the serum from the neurons for 4 hr per se was causing damage because the neurons in the control group were looking healthy. Also, in a separate experiment, serum withdrawal for 4 hr did not cause statistically significant damage to the neurons (the viability of neurons withdrawn from serum for 4 hr was 93.5% ± 3 of the control neurons (i.e. not withdrawn from serum), $p>0.05$, $n = 4$). When serum was withdrawn for 24 hr, there was a small but statistically significant damage to the neurons (the viability of neurons withdrawn from serum for 24 hr was 84.4% ± 4.3 of the control neurons (i.e. not withdrawn from serum), $p<0.05$, $n = 5$).

When the experiment with deferoxamine pre-treatment for 3 hr was repeated but with the toxic insult applied in HEPES-sol instead of MEM-HEPES-sol, deferoxamine also protected against X / XO toxicity (Fig. 3-58). Notice that in this experiment, deferoxamine was applied in MEM-HEPES-sol as pre-treatment before removing it and replacing it by the toxic insult applied in HEPES-sol. The reason for using MEM-HEPES-sol as the pre-treatment solution is that HEPES-sol contains low potassium concentration, and low potassium solutions might cause damage to the cells if applied to them for a relatively prolonged time as for the pre-treatment time in this experiment. MEM-HEPES-sol was therefore preferred as the pre-treatment solution in this and other experiments.

When the neurons were pre-treated for 3 hr with deferoxamine at 300 µM, it failed to protect against NADH / XO toxicity (data not shown). However, when the neurons were pre-treated for 3 hr with deferoxamine at 1 mM, it protected against NADH / XO toxicity (Fig. 3-59).

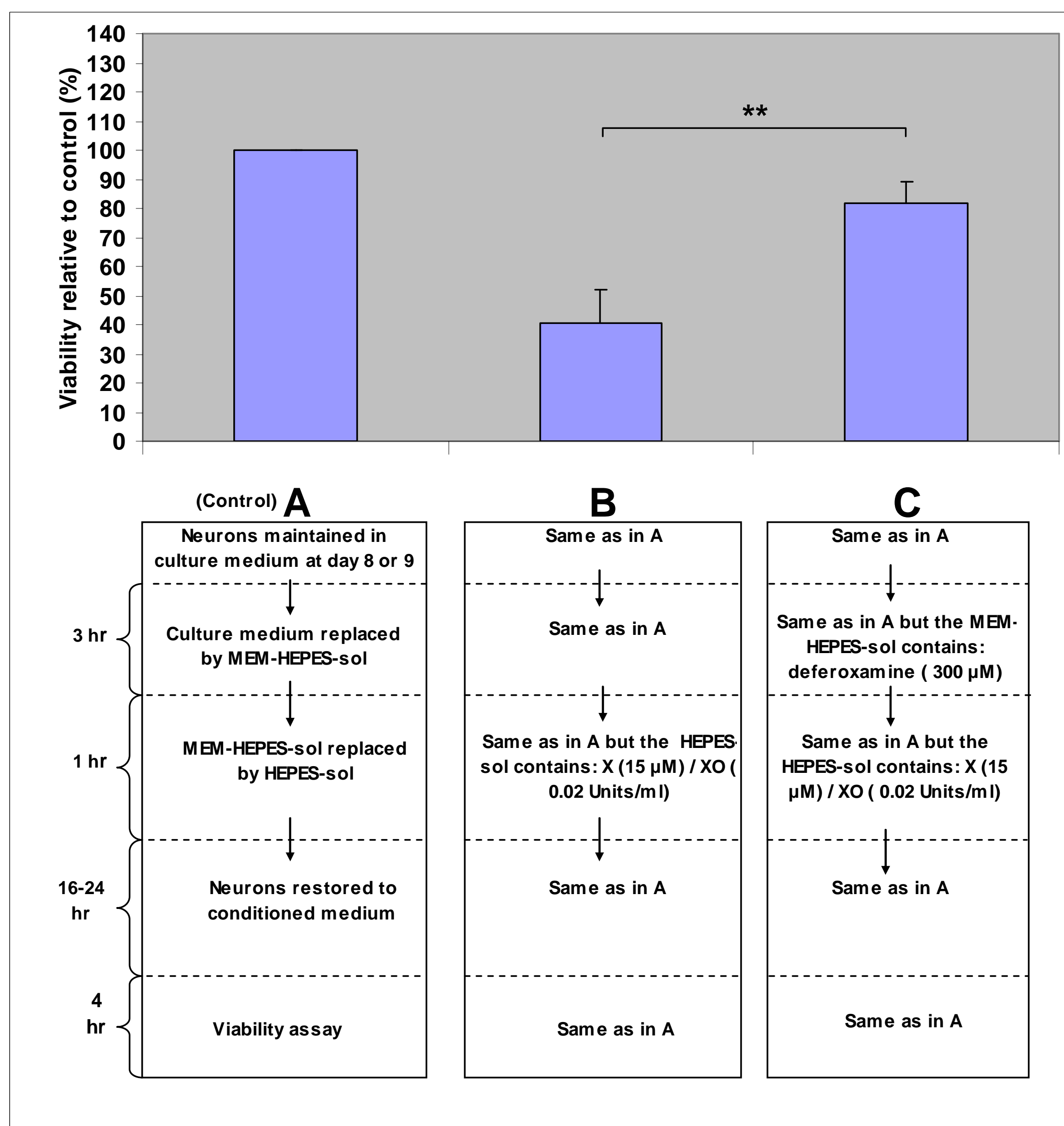

Figure 3-58: Effect of deferoxamine pre-treatment (in MEM-HEPES-sol) on the toxicity of X / XO combination applied in HEPES-sol. Notice that deferoxamine was removed before applying the toxic insult. **p<0.01. (n=5).

The foregoing results show that deferoxamine, if applied as pre-treatment at the appropriate concentration and time interval, can protect against X / XO toxicity and also against NADH / XO toxicity, which suggests that intracellular iron ion plays a role in the damage observed in these two systems. Alternatively, deferoxamine pre-treatment may have been protective by blocking intracellular peroxynitrite-mediated effects [*Bartesaghi et al., 2004*], where peroxynitrite can be produced from reaction between superoxide (produced by XO or other sources) and nitric oxide produced by endogenous nitric oxide synthase. To test this possibility, L-NAME (1 mM), a nitric oxide synthase inhibitor [*Patel et al., 1996, Gunasekar et al., 1995*], was used as pre-treatment for 1 hr to inhibit intracellular nitric oxide-mediated peroxynitrite production, but it failed to offer

protection against either NADH / XO or X / XO toxicities. So, in NADH / XO toxicity, the viability of neurons insulted by NADH (1 mM) / XO (0.02 Units/ml) was 39.7% ± 3.8 of the control neurons, and the viability of neurons pre-treated with L-NAME (1 mM) before applying the insult was 41% ± 1.2 of the control neurons; the P value for the difference between the insult and the insult with L-NAME pre-treatment was > 0.05; n = 3. In X / XO toxicity, the viability of neurons insulted by X (15 μM) / XO (0.02 Units/ml) was 36.3% ± 9.3 of the control neurons, and the viability of neurons pre-treated with L-NAME (1 mM) before applying the insult was 32.3% ± 6.1 of the control neurons; the P value for the difference between the insult and the insult with L-NAME pre-treatment was > 0.05; n = 3. L-NAME alone was not toxic (data not shown). Therefore, the failure of L-NAME pre-treatment to protect against either NADH / XO or X / XO toxicities argues against the blockade of peroxynitrite-mediated effects as the mechanism of protection by deferoxamine pre-treatment.

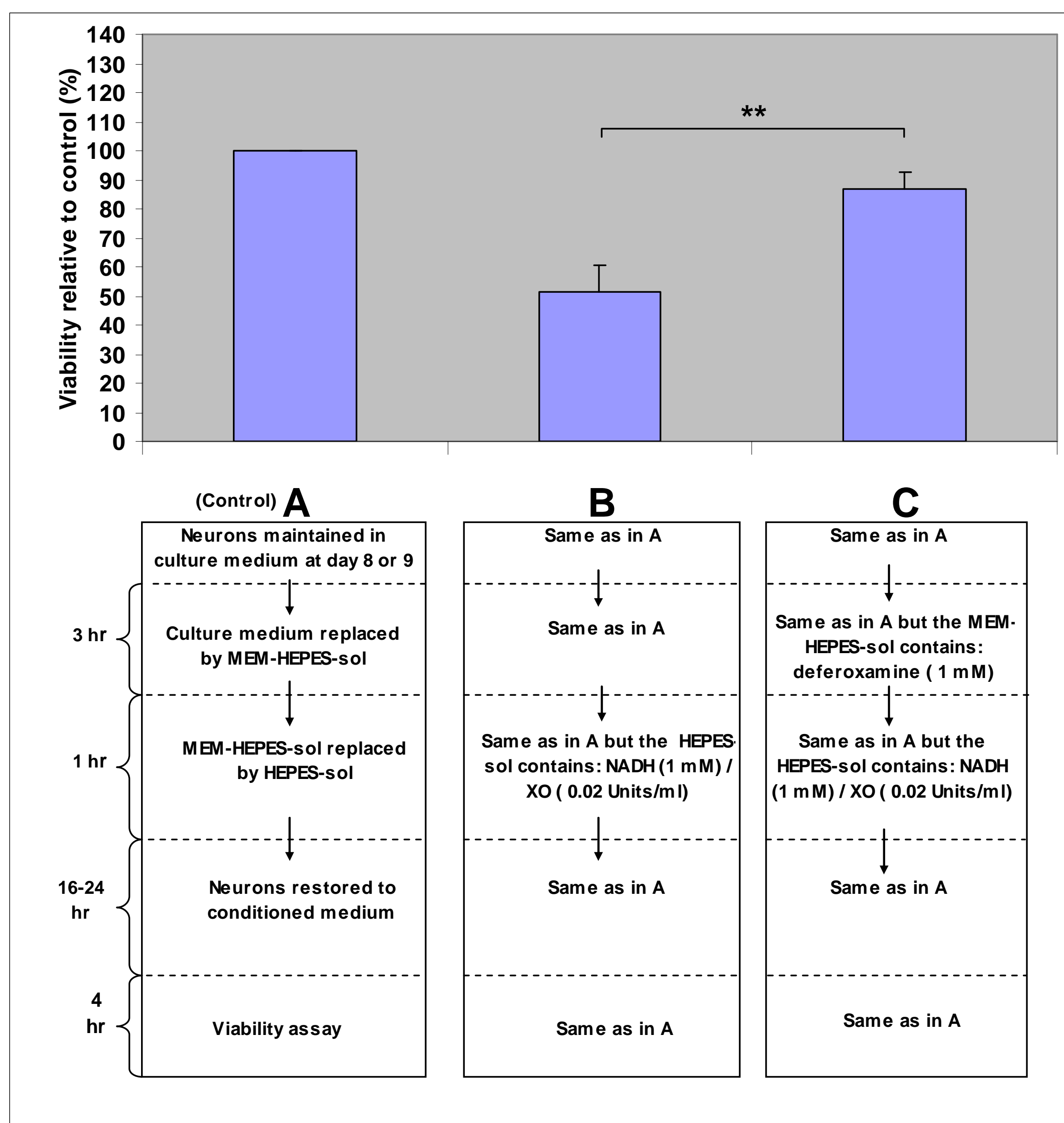

Figure 3-59: Effect of deferoxamine pre-treatment (in MEM-HEPES-sol) on the toxicity of the NADH / XO combination applied in HEPES-sol. Notice that deferoxamine was removed before applying the toxic insult. **p<0.01. (n=5).

It is commonly observed that traces of metals do often contaminate solutions used in cell culture studies. Also, some commercial preparations of XO are known to be contaminated with iron ion (which is different from iron that is an integral part of XO) [*Britigan et al., 1990, Vile and Winterbourn, 1986*]. Thus, a series of experiments were conducted in order to investigate the involvement of metal contaminants in the extracellular solutions (or contaminating XO) in the toxicity of X / XO and NADH / XO combinations. In the case of NADH / XO toxicity in particular, there was another reason for suspecting the involvement of an extracellular metal contaminant in the toxicity, which was the observed protection by SOD. Since SOD-1 and Mn-SOD were likely producing their protective effects through an extracellular action, and since superoxide can exert its toxicity through some sort of a reaction with metals (see Introduction), an extracellular toxic interaction between superoxide (generated extracellularly from NADH / XO combination) and an extracellular metal contaminant was suspected. Two metal chelators were tried, ethylenediaminetetraacetate (EDTA, a non specific cation chelator [*Hutcheson et al., 2004*]) and deferoxamine.

Deferoxamine (300 μM) was pre-incubated with XO for 3 hr in HEPES-sol in a test tube (this pre-incubation time was without any contact with CGNs). After the 3 hr, NADH was added to the solution in the test tube (to start the reaction) and the whole mixture (which contains NADH, XO, and deferoxamine) was applied to CGNs. Deferoxamine, when tried this way, failed to protect against NADH / XO toxicity (Fig. 3-60) (notice that the volume of HEPES-sol containing deferoxamine and XO before adding NADH was 95% of its volume after adding NADH).

When the experiment was repeated exactly but with using EDTA (at 2 μM (Fig. 3-61), 20 μM (Fig. 3-62), or 200 μM (data not shown)) instead of deferoxamine, it protected against NADH / XO toxicity. Interestingly, EDTA lost its protective effect when XO was omitted from the solution that was pre-incubated for 3 hr with EDTA. In other words, EDTA (20 μM) was pre-incubated for 3 hr in HEPES-sol (without XO) in a test tube (again, this pre-incubation time was without any contact with CGNs). After the 3 hr, both XO and NADH were added to the solution in the test tube (to start the reaction) and the whole mixture (which contains NADH, XO, and EDTA) was applied to CGNs. With this protocol, EDTA no longer protected against NADH / XO toxicity (Fig. 3-63) (notice that the volume of the EDTA-containing HEPES-sol before adding XO and NADH was more than 90% of its volume after adding XO and NADH).

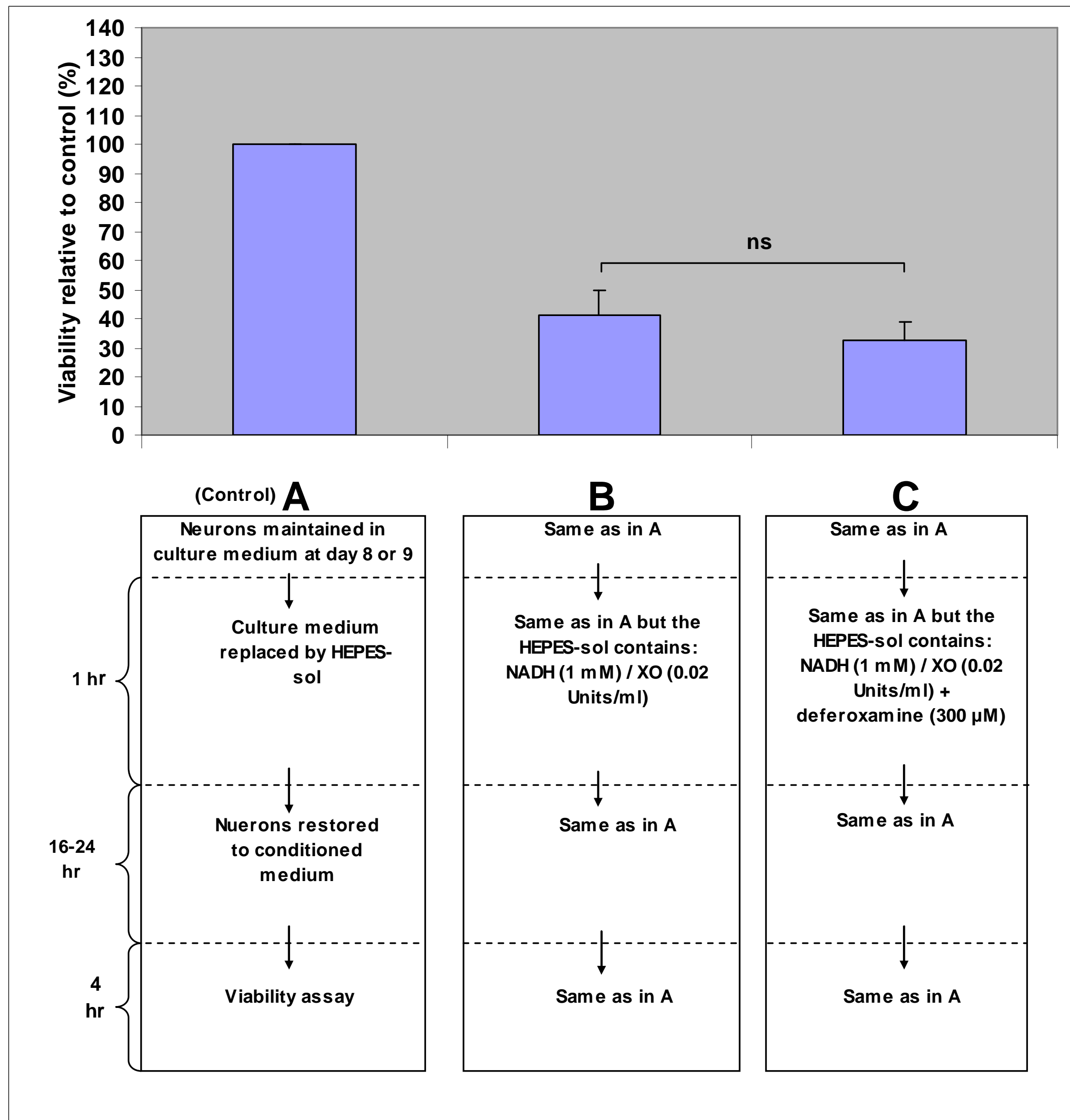

Figure 3-60: Effect of deferoxamine co-treatment on NADH / XO toxicity. Notice: in column C, HEPES-sol including XO was pre-incubated with deferoxamine for 3 hr (notice that this 3 hr pre-incubation was in a test tube without any contact with the CGNs cultures) and then NADH was added to the solution (to initiate the reaction), and then the mixture (including deferoxamine, NADH, and XO) was applied to the neurons as shown in the figure (notice that the volume of HEPES-sol containing deferoxamine and XO before adding NADH was 95% of its volume after adding NADH). The same was done to column B but without deferoxamine, and the same was done to column A but without either deferoxamine or the toxic insult. ns: not significant. (n=5).

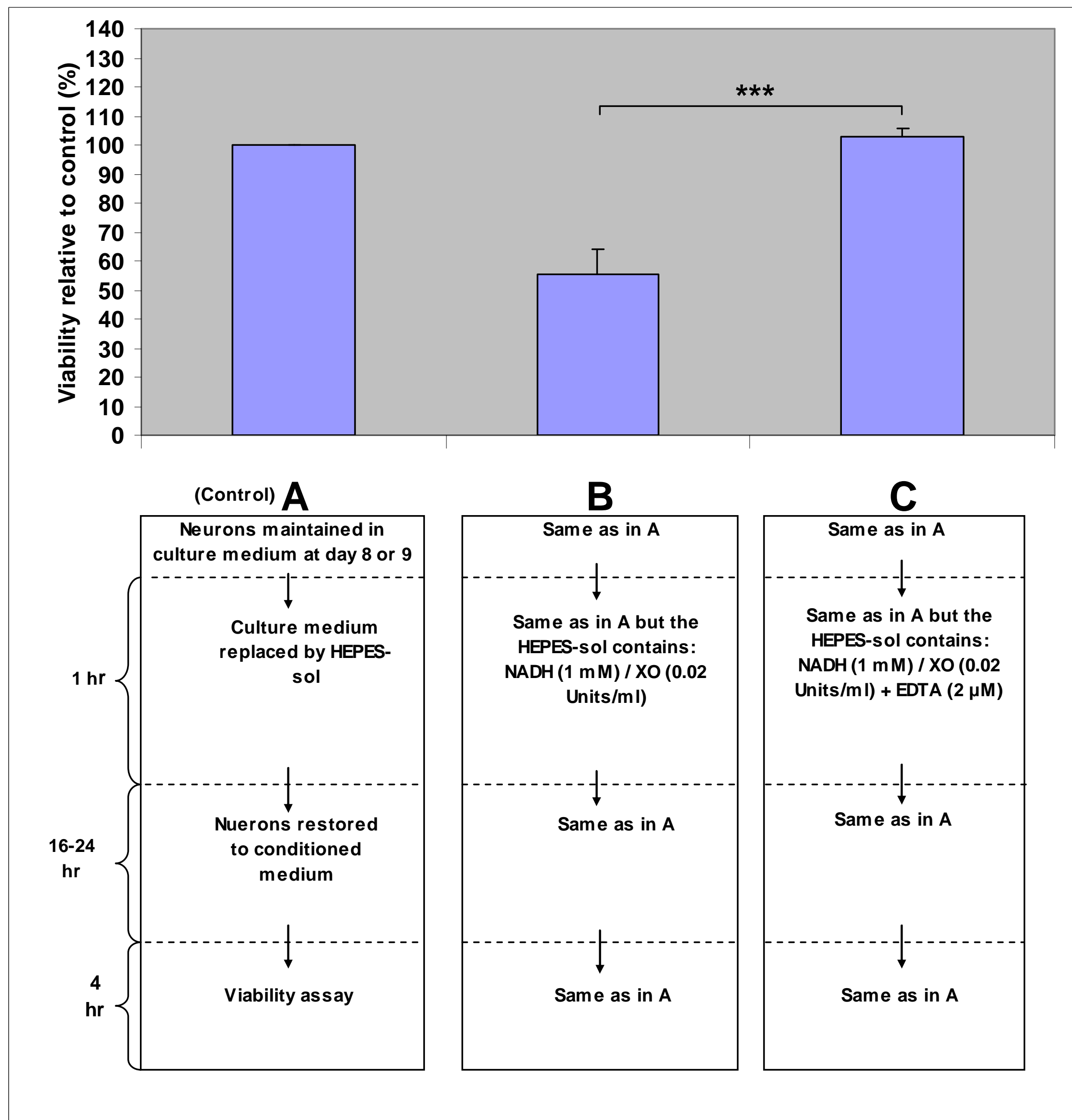

Figure 3-61: Effect of EDTA (2 µM) co-treatment on NADH / XO toxicity. Notice: in column C, HEPES-sol including XO was pre-incubated with EDTA for 3 hr (notice that this 3 hr pre-incubation was in a test tube without any contact with the CGNs cultures) and then NADH was added to the solution (to initiate the reaction), and then the mixture (including EDTA, NADH, and XO) was applied to the neurons as shown in the figure. The same was done to column B but without EDTA, and the same was done to column A but without either EDTA or the toxic insult. ***$p<0.001$. (n=5).

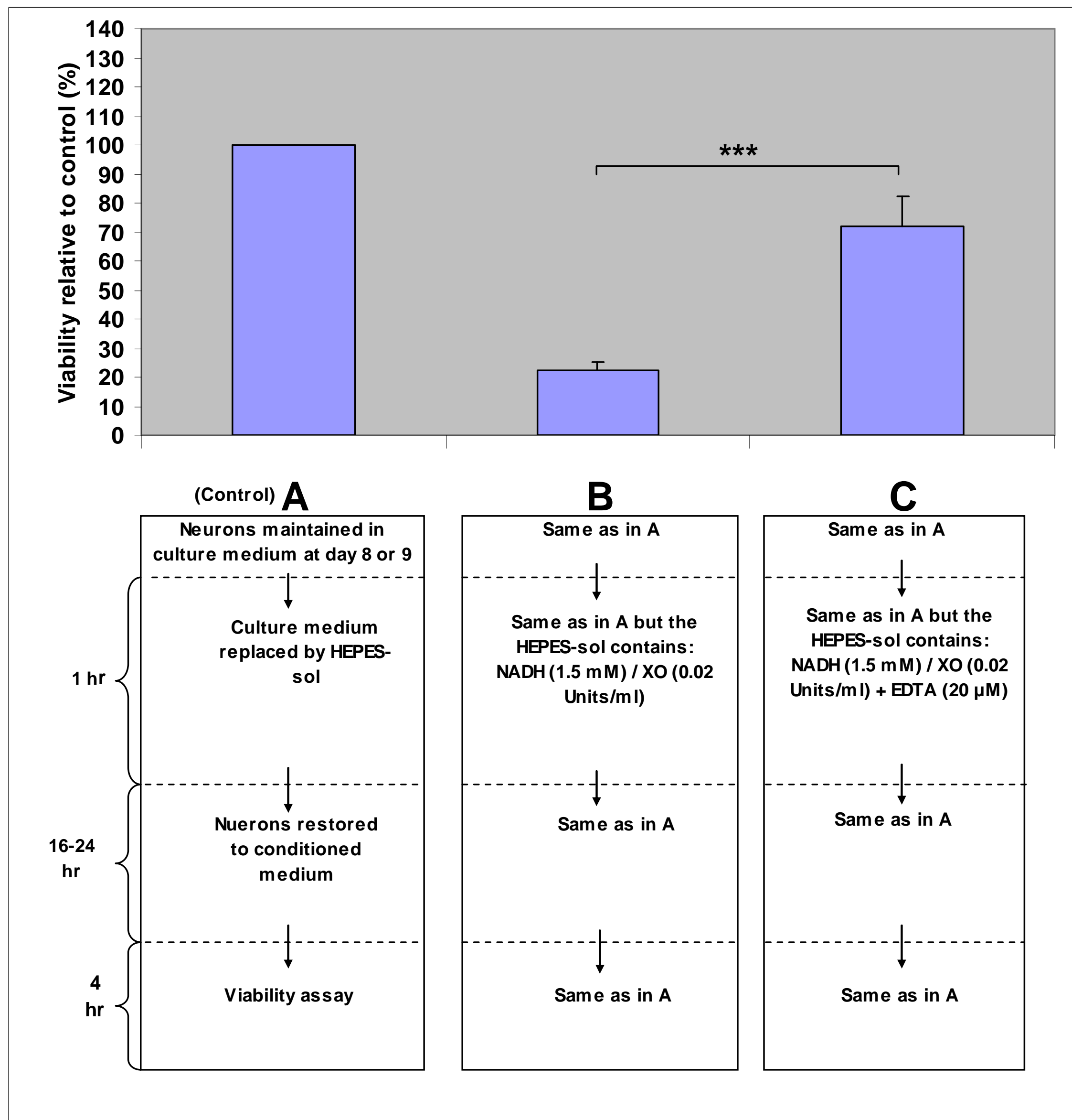

Figure 3-62: Effect of EDTA (20 µM) co-treatment on NADH / XO toxicity. NADH was used here at 1.5 mM just to show that even at higher toxicity level EDTA is still able to provide substantial protection against NADH / XO toxicity. **Notice:** in column C, HEPES-sol including XO was pre-incubated with EDTA for 3 hr (notice that this 3 hr pre-incubation was in a test tube without any contact with the CGNs cultures) and then NADH was added to the solution (to initiate the reaction), and then the mixture (including EDTA, NADH, and XO) was applied to the neurons as shown in the figure. The same was done to column B but without EDTA, and the same was done to column A but without either EDTA or the toxic insult. ***$p<0.001$. (n=5).

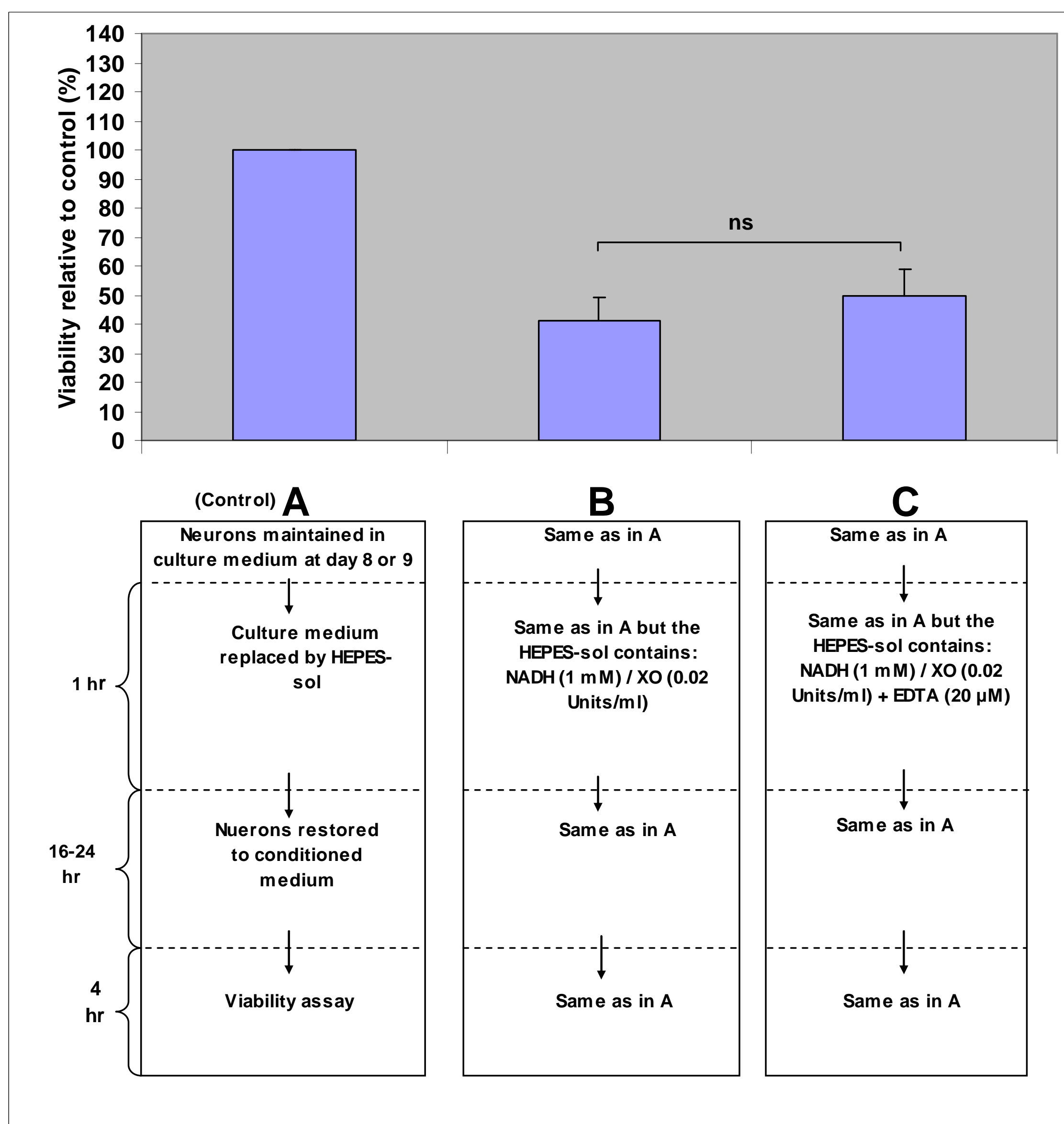

Figure 3-63: Effect of EDTA (20 µM) co-treatment when it was not pre-incubated with XO on the toxicity of NADH / XO combination. Notice: in column C, HEPES-sol (not including XO) was pre-incubated with EDTA for 3 hr (notice that this 3 hr pre-incubation was in a test tube without any contact with the CGNs cultures) and then both XO and NADH were added to the solution (to initiate the reaction), and then the mixture (including EDTA, NADH, and XO) was applied to the neurons as shown in the figure (notice that the volume of the EDTA-containing HEPES-sol before adding XO and NADH was more than 90% of its volume after adding XO and NADH). The same was done to column B but without EDTA, and the same was done to column A but without either EDTA or the toxic insult. ns: not significant. (n=5).

The effects of deferoxamine and EDTA co-treatments were also investigated against the toxicity of the X / XO combination. Deferoxamine at 300 µM (Fig. 3-64) or EDTA at 2, 20, or 200 µM (Fig. 3-65) was pre-incubated with XO for 3 hr in HEPES-sol in a test tube (again, this pre-incubation time was without any contact with CGNs). After the 3 hr, X was added to the solution in the test tube (to start the reaction) and the whole mixture (which contains X, XO, and the chelating agent) was applied to CGNs. Neither deferoxamine nor EDTA, when tried this way, was able to protect against X / XO

toxicity. Notice that neither deferoxamine (300 µM) nor EDTA (200 µM), when tried alone this way, was toxic to CGNs (data not shown).

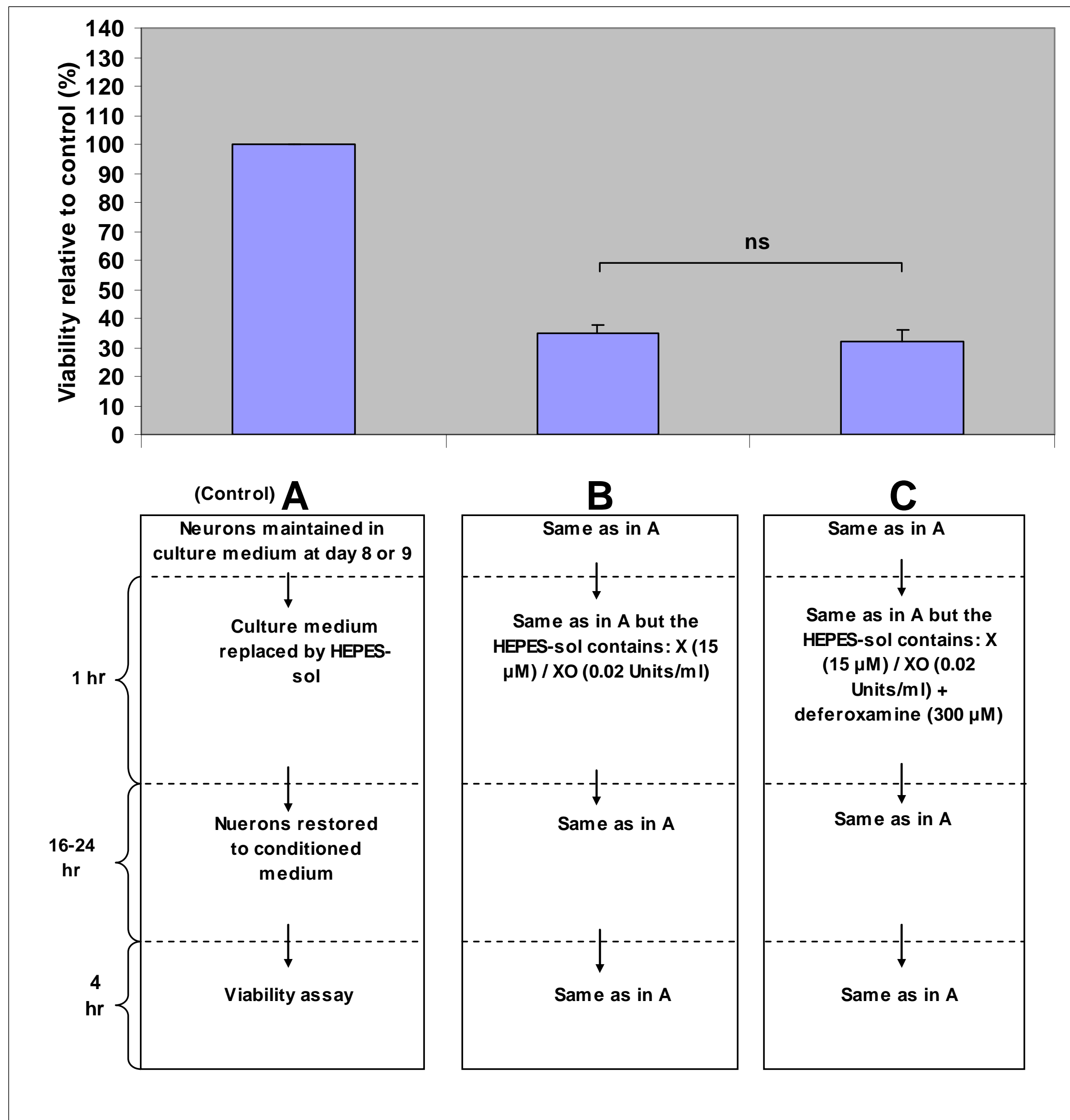

Figure 3-64: Effect of deferoxamine co-treatment on X / XO toxicity. Notice: in column C, HEPES-sol including XO was pre-incubated with deferoxamine for 3 hr (notice that this 3 hr pre-incubation was in a test tube without any contact with the CGNs cultures) and then X was added to the solution (to initiate the reaction), and then the mixture (including deferoxamine, X, and XO) was applied to the neurons as shown in the figure. The same was done to column B but without deferoxamine, and the same was done to column A but without either deferoxamine or the toxic insult. ns: not significant. (n=5).

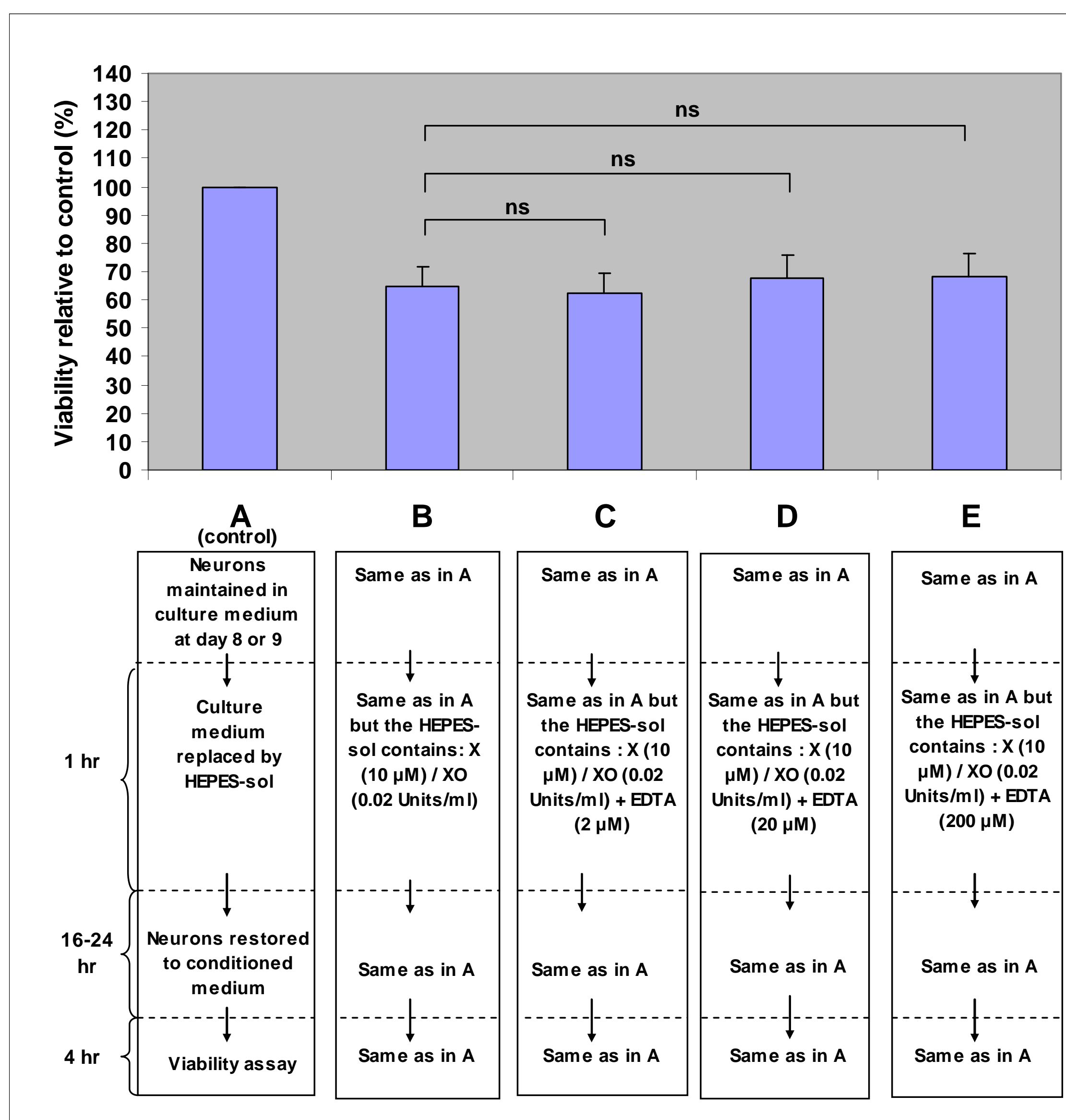

Figure 3-65: Effect of EDTA co-treatment on X / XO toxicity. X was used here at 10 instead of 15 µM just to show that even at lower toxicity level EDTA was not able to provide any protection against X / XO toxicity (also when X was used at 15 µM, EDTA (200 µM) could not protect (data not shown)) . **Notice:** in columns E, D, and C, HEPES-sol including XO was pre-incubated with EDTA at the shown concentrations for 3 hr (notice that this 3 hr pre-incubation was in a test tube without any contact with the CGNs cultures) and then X was added to the solution (to initiate the reaction), and then the mixture (including EDTA, X, and XO) was applied to the neurons as shown in the figure. The same was done to column B but without EDTA, and the same was done to column A but without either EDTA or the toxic insult. ns: not significant. (n=5).

3.3.2.6 Role of extracellular hydroxyl radical in NADH / XO and X / XO toxicities

Since SOD-1 (and Mn-SOD), catalase, and EDTA co-treatments protected against NADH / XO toxicity, there is a possibility that the toxicity was due to a Fenton reaction where hydrogen peroxide generated extracellularly was interacting with an extracellular contaminating metal (where this reaction can be mediated by superoxide) to produce the

very reactive and toxic hydroxyl radical. However, the treatment solutions used i.e. HEPES-sol and MEM-HEPES-sol contain at least two hydroxyl radical scavengers: HEPES (at 20 mM in HEPES-sol and 25 mM in MEM-HEPES-sol) and glucose (at 3 mM in HEPES-sol and 5 mM in MEM-HEPES-sol). This argues against the involvement of extracellular hydroxyl radical in the toxicity of either NADH / XO or X / XO combinations. To confirm or refute this, three additional hydroxyl radical scavengers were tried: mannitol, ethanol, and DMSO. None of these three compounds was toxic to CGNs when tried alone at 20 mM (data not shown). However, none of these three compounds was able to provide protection against NADH / XO (Fig. 3-66) or X / XO (Fig. 3-67) toxicity.

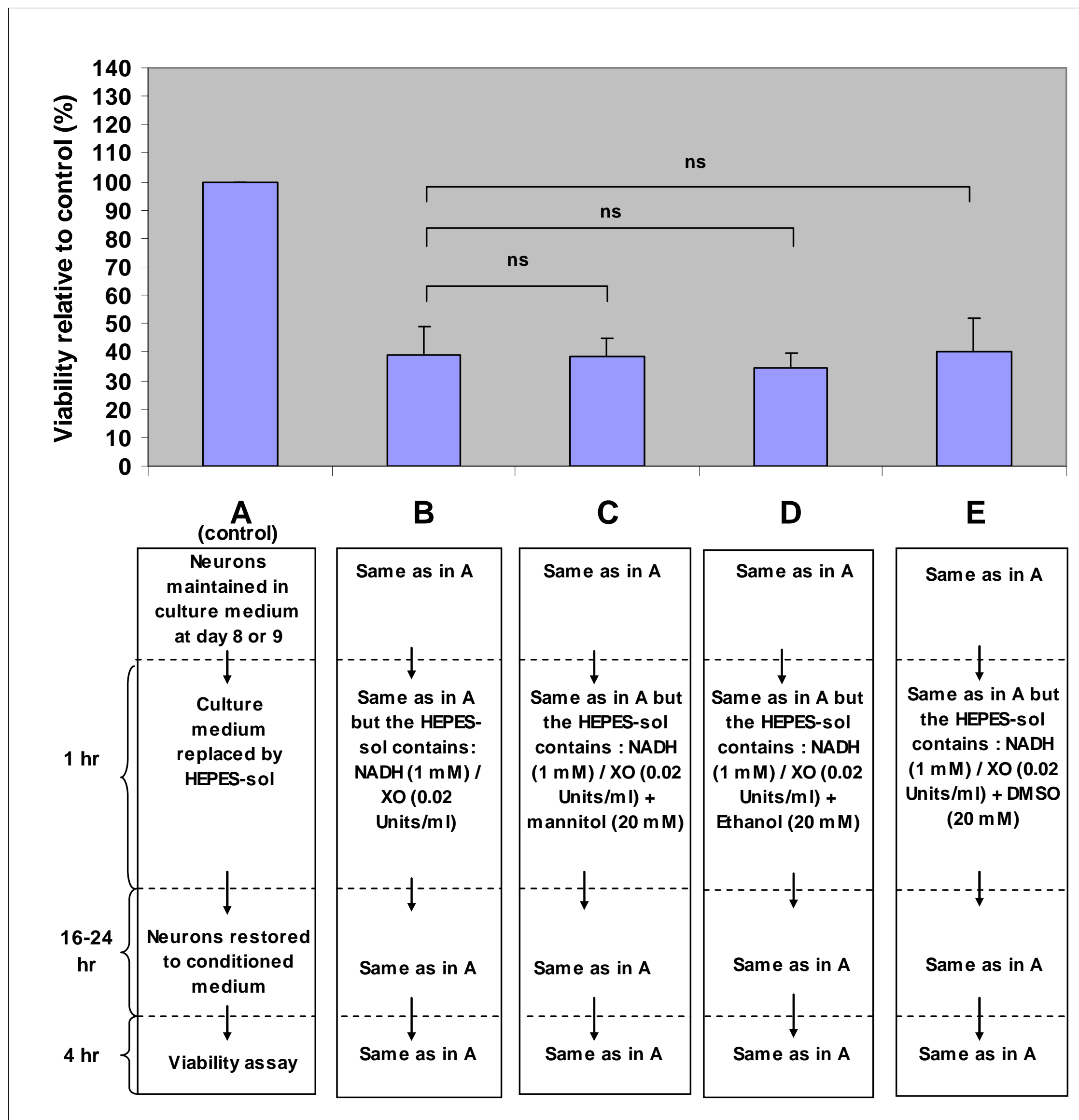

Figure 3-66: Effect of co-treatment with hydroxyl radical scavengers on NADH / XO toxicity. ns: not significant. (n=3).

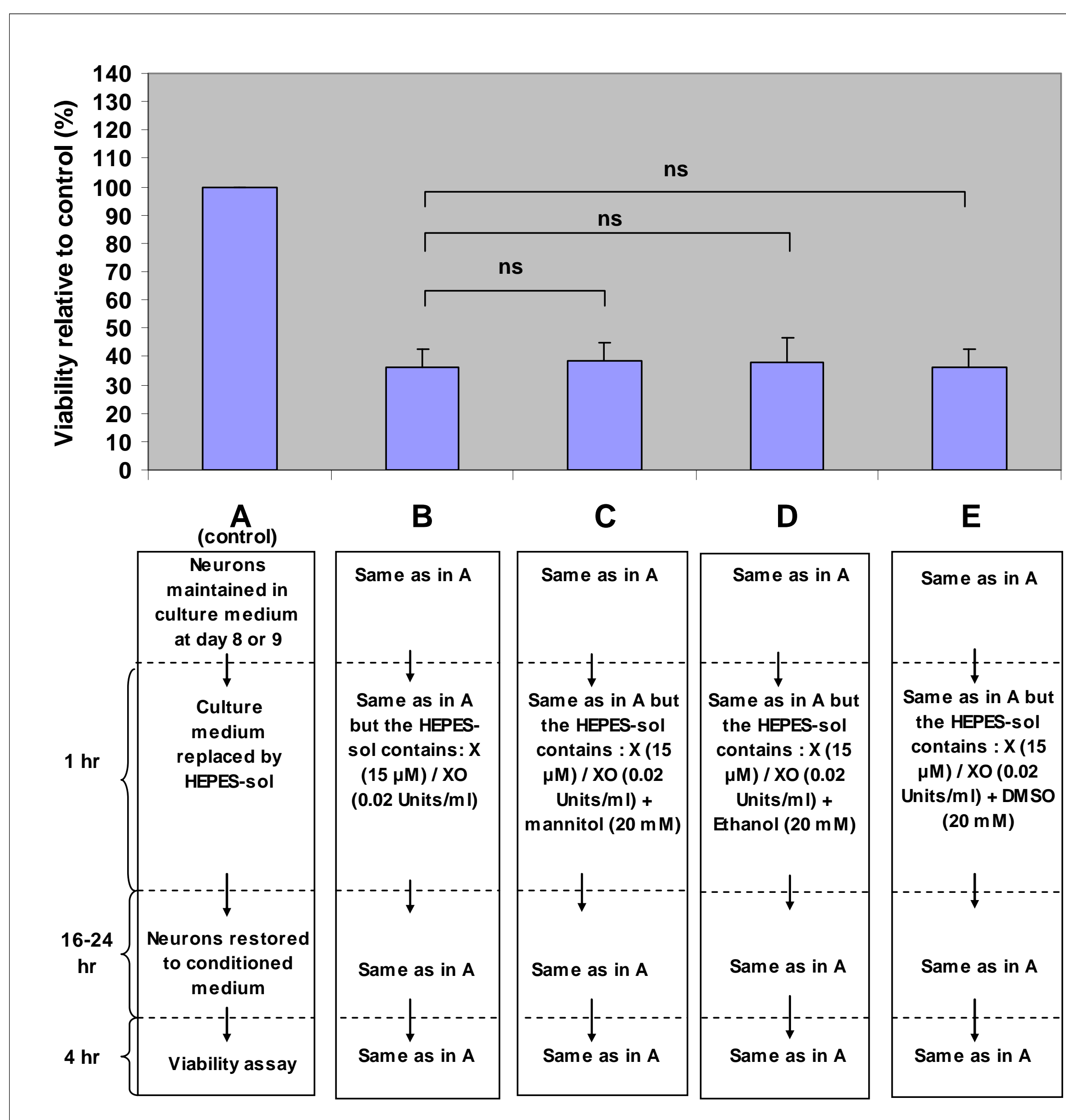

Figure 3-67: Effect of co-treatment with hydroxyl radical scavengers on X / XO toxicity. ns: not significant. (n=3).

3.3.3 Further investigation of X / XO toxicity

In addition to investigating the toxicity of the NADH / XO combination (which was rarely investigated in previous studies) and comparing it to the well investigated toxicity of the X / XO combination, another aim of this project was to address specific questions regarding those aspects of X / XO toxicity where there is uncertainties about them (see the section on Aim/Objectives in the Introduction). Some of these questions were addressed in the previous section (e.g. the possibility that XO is contaminated with iron/metal).

Another aspect of further investigation was the role of superoxide in X / XO toxicity. It was shown in the previous section that SOD-1 and Tiron failed to protect against X / XO toxicity despite the fact that the cell-free experiments showed that superoxide does indeed get generated from X / XO combination. The complete protection found with catalase indicates that hydrogen peroxide was a main toxic molecule in X / XO toxicity. Also, intracellular superoxide was shown previously to mediate the toxicity of extracellularly generated/applied hydrogen peroxide, though this was shown in tissue cultures other than CGNs [*Ito et al., 1992, Kyle et al., 1988, Hiraishi et al., 1994*]. For these reasons, there is a possibility that intracellular superoxide is involved in the X / XO toxicity in CGNs cultures used here, and that the failure of SOD-1 and Tiron to protect was due to both the failure of SOD-1 (and Tiron at the used concentration and study design) to enter the cells and also the failure of superoxide generated from X / XO combination to enter the cells.

Tiron, although it failed to protect against X / XO toxicity when it was applied as co-treatment at 50 µM (see earlier), was tried here as pre-treatment for 3 hr at 2 mM, aiming to give it a chance to get inside the neurons in high amount. When tried alone this way, Tiron was not toxic (data not shown). When tried this way, Tiron provided protection against X (15 µM) / XO toxicity (Fig. 3-68) or X (10 µM) / XO toxicity (Fig. 3-69). This suggests that, as suspected, intracellular superoxide might be involved in the toxicity of X / XO combination. However, it is not possible to rely heavily on the Tiron result alone, because there is a possibility that this compound was producing its protective effect through a mechanism different from scavenging intracellular superoxide e.g. chelating some intracellular metals [*Fridovich and Handler, 1962*]. Another way to test the involvement of intracellular superoxide in X / XO toxicity was through inhibiting intracellular SOD-1, where this inhibition should potentiate the X / XO toxicity if intracellular superoxide was mediating this toxicity. Diethyldithiocarbamate (DDC) was tried, which is a known and cell permeable inhibitor of SOD-1 (but of low specificity) [*Ito et al., 1992, Hiraishi et al., 1994, Blum and Fridovich, 1983, Benov and Fridovich, 1996*]. DDC pre-treated alone was not toxic (data not shown). The neurons were pre-treated with DDC at 50 µM for 1 hr before removing it and replacing it with X / XO combination, but DDC failed to show statistically significant potentiation of the toxicity (Fig. 3-70).

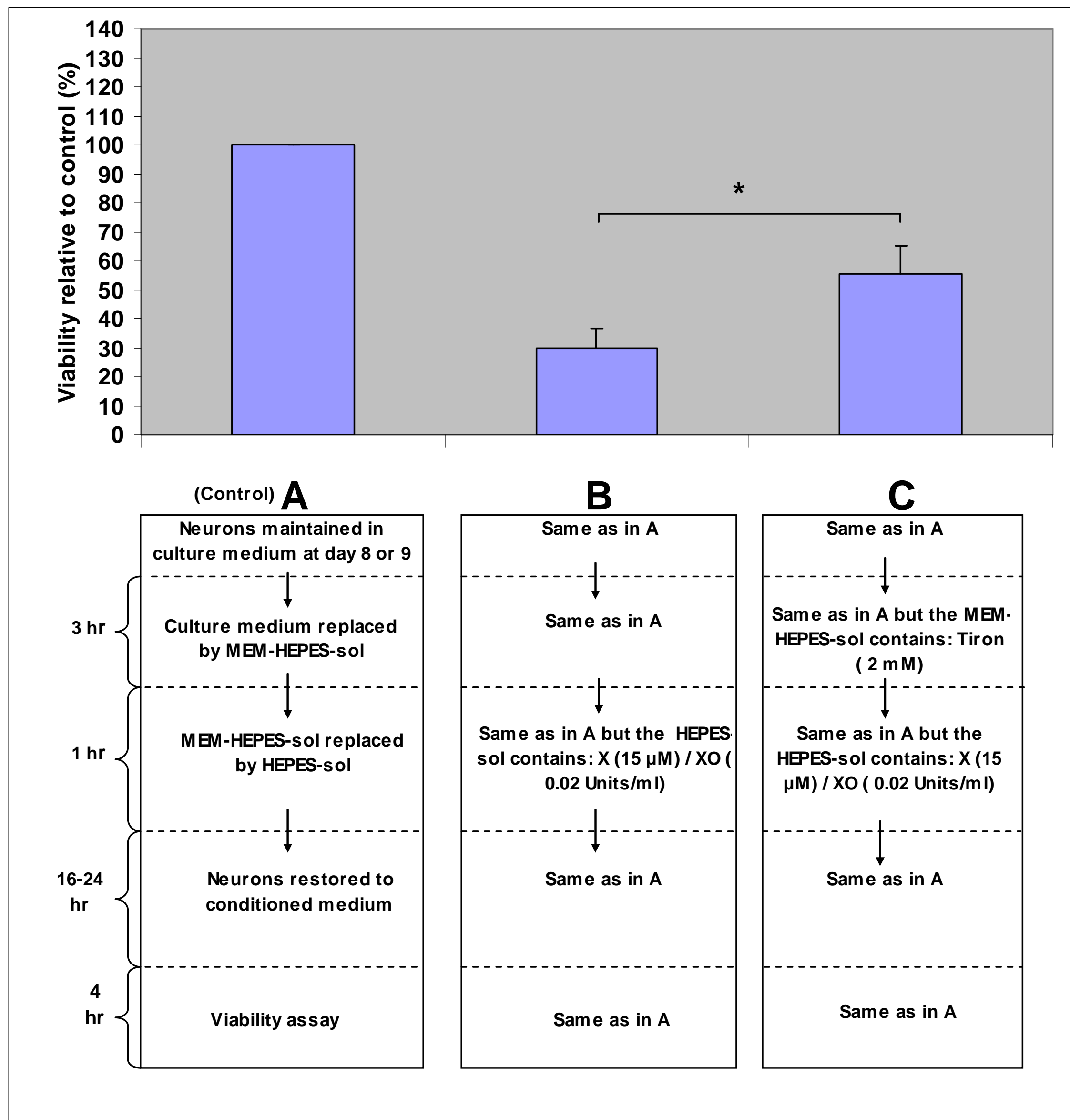

Figure 3-68: Effect of Tiron pre-treatment on the toxicity of X (15 µM) / XO (0.02 Units/ml) combination. Notice that Tiron was removed before applying the toxic insult. *$p<0.05$. (n=5).

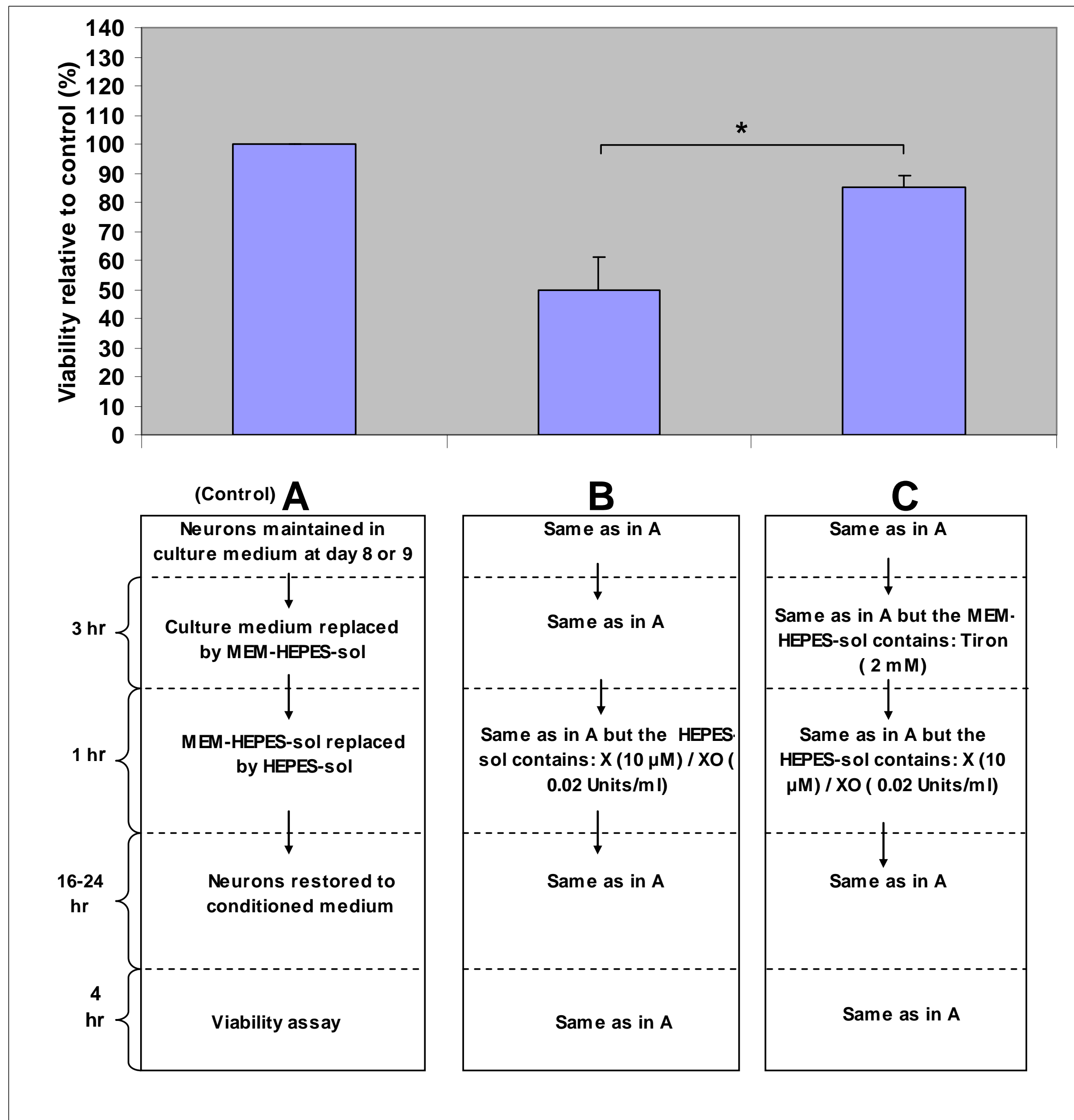

Figure 3-69: Effect of Tiron pre-treatment on the toxicity of X (10 µM) / XO (0.02 Units/ml) combination. Notice that Tiron was removed before applying the toxic insult. *$p<0.05$. (n=4).

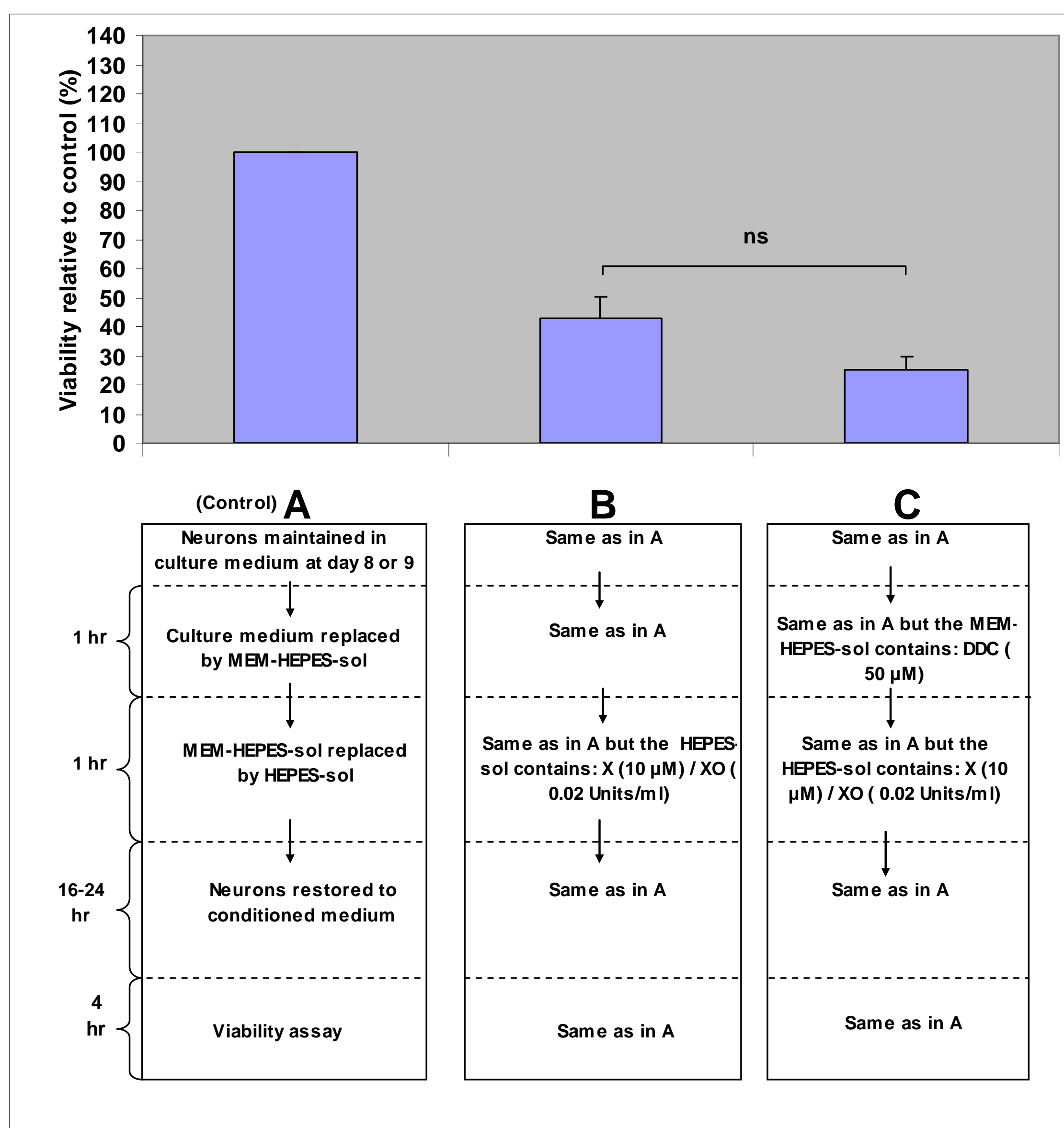

Figure 3-70: Effect of diethyldithiocarbamate (DDC) pre-treatment on X / XO toxicity. Notice that DDC was removed before applying the toxic insult. ns: not significant. (n=3).

The results with Tiron pre-treatment, deferoxamine pre-treatment, and catalase co-treatment (and also the results of many previous studies [*Satoh et al., 1998, Link and Riley, 1988, Mohsen et al., 1995, Duell et al., 1995, Hiraishi et al., 1987, Zigler et al., 1985*]) suggest that the toxicity of X / XO combination was due to hydrogen peroxide generated in the extracellular space and then entering the neurons and participating in a Fenton reaction with an intracellular iron (where this reaction was likely mediated by intracellular superoxide) to produce the very reactive and toxic hydroxyl radical (or a similar species). The failure of co-treatment with the hydroxyl radical scavengers tried in the previous section (mannitol, ethanol, and DMSO), although might argue against the involvement of extracellular hydroxyl radical in X / XO toxicity, does not necessarily

mean that intracellular hydroxyl radical was not involved. Therefore it was decided to use different hydroxyl radical scavengers (mannitol, ethanol, DMSO, and others) as pre-treatment (to give them a chance to accumulate inside the cells) before applying the X / XO combination. As a start, a compound called α-(4-Pyridyl N-oxide)-N-tert-butylnitrone (POBN) was tried, which has the ability to scavenge hydroxyl radical (but also many other free radicals) [*Mottley et al., 1986, Pérez and Cederbaum, 2001, Reinke et al., 1994*]. POBN is a member of a large group of compounds called spin traps, which are used essentially as detectors of free radicals, where a spin trap can react with a free radical (e.g. hydroxyl radical) to produce a new species (a more stable secondary radical) that can be detected by a method called electron paramagnetic resonance spectroscopy [*Reinke et al., 1994, Tarpey and Fridovich, 2001*]. In theory (which was also shown in some viability studies), since these detect free radicals by scavenging them, they might protect tissues from insults that involve generation of toxic free radicals. When POBN was applied alone as pre-treatment, it was not toxic to CGNs (data not shown). When the neurons were pre-treated with POBN at 20 mM for 1hr before removing it and replacing it by the X / XO combination, instead of protecting, it potentiated the toxicity (Fig. 3-71). Surprisingly, when POBN was applied as co-treatment rather than pre-treatment, it produced the opposite effect, showing significant protection against X / XO toxicity (Fig. 3-72) (see Discussion for possible explanations for these opposite effects).

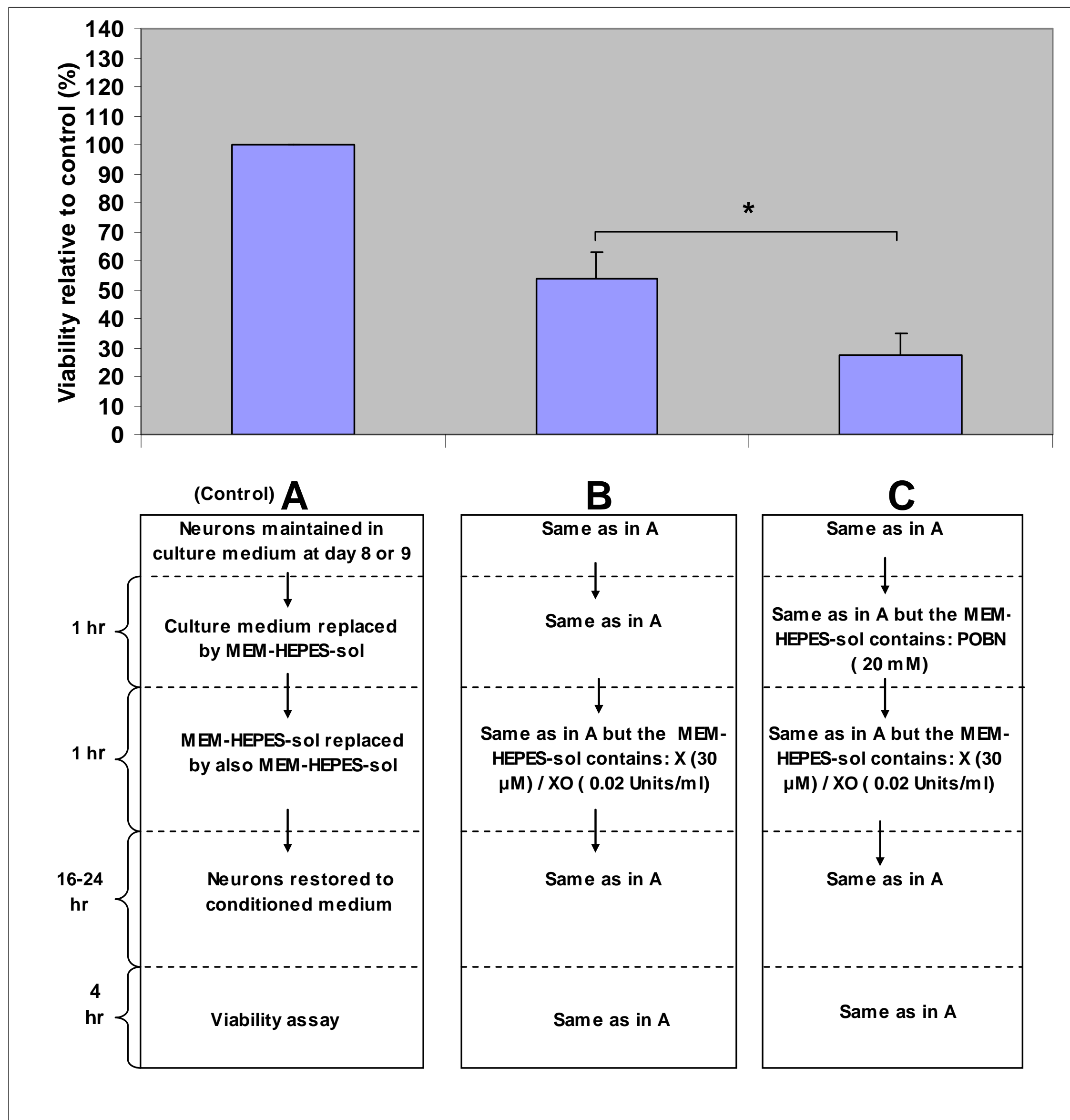

Figure 3-71: Effect of POBN pre-treatment on the toxicity of X / XO combination in MEM-HEPES-sol. Notice that POBN was removed before applying the toxic insult. *p<0.05. (n=5).

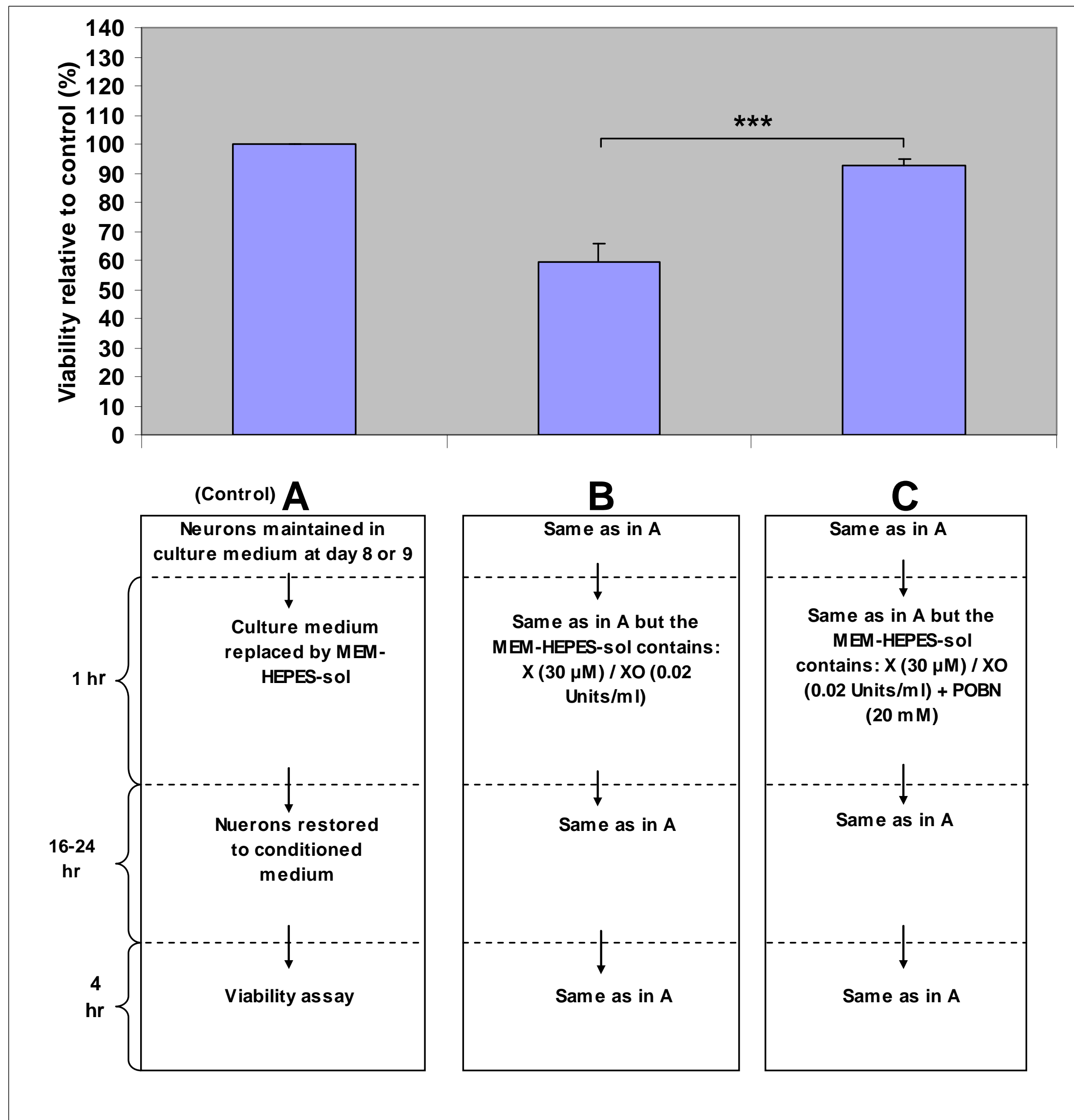

Figure 3-72: Effect of POBN co-treatment on the toxicity of X / XO combination in MEM-HEPES-sol. ***p<0.001. (n=5).

4 Discussion

4.1 Culturing and experimental conditions

Please notice that some detailed discussion of the justification of using neurons and CGNs in particular was mentioned in the Introduction (section **1.5**, pages 43 & 44).

4.1.1 Neuronal morphology

The culturing process produced viable CGNs. The cells appeared immediately after plating as round cells. In the first 24 hr of plating, the neurons started to grow processes, and also started to migrate and group with each other. On the other hand, many cells died in the first 24 hr of plating, even before adding the cytosine arabinoside. There is more than one possibility for this death. First, it is possible that these cells died as a result of physical damage in the isolation process i.e. the death was due to: chopping with the blade, trypsin treatment, trituration, etc. An attempt was undertaken here to assess the viability of the neurons immediately after isolation (and before plating) by using the Trypan blue exclusion test (see Introduction for the principle of this test), and it was found that most of the cells were viable (data were not shown). However, this test was not clear at all when tried. Neither the dead cells were clearly obtaining the dye, nor were the viable cells clearly excluding it. The presence of serum and/or some debris might have caused this obscurity in observing the uptake/exclusion of Trypan blue by cells. Therefore, it was not possible to prove or rule out this possibility as the reason for the neuronal death, though it might be at least partially responsible.

The second explanation for the death is that, since the fresh culture medium used in the culturing process contains glutamate, it might have been due to glutamate excitotoxicity after exposure to this medium. However, this is unlikely, since it was shown that glutamate does not cause toxicity to CGNs at this early stage [*Frandsen and Schousboe, 1990*] (also see later). The third explanation, which might contribute (at least partially), is that the majority of dead cells may did not necessarily die immediately after dissociation from brain, but rather they may have died gradually in the first 24 hr of plating, where the cells (neurons and non-neuronal cells) that died are those ones which

could not stand the culturing shock/stress induced by placing them in a new and strange environment drastically different from their normal in vivo environment.

After 8 days in culture, the neurons exhibited a normal phenotype, and showed the known characteristic morphology of cultured CGNs [*Peng et al., 1991, Parks et al., 1991*], where most of those cells that died in the first 24 hr of plating disappeared. The cytosine arabinoside added after 24 hr of plating should have ensured that most of the cells present after 8 days in culture are neurons. Also, the neuronal nature of these CGNs cultures, as well as the absence of a significant number of glial cells, was confirmed by immunocytochemistry in a previous project undertaken in this laboratory [*Smith, 2008*].

4.1.2 Morphological examination in viability experiments

It was a constant observation under the microscope that, after applying a toxic insult and then restoring neurons to the restoration medium, the neurons continue to deteriorate for up to 4-8 hr with no deterioration/recovery afterwards. Therefore, the restoration period (at least 16 hr) seemed to be enough for the damage to stabilize at a certain level after which the neurons were unlikely to recover/deteriorate.

Morphological examination is a very objective way of assessing the viability of neurons. However, taking images of the neurons can be less objective. The reason is that an insult-mediated damage to the neurons observed under the microscope in a well is, in many cases, not homogenous. Therefore, choosing the area in a well to take a photo can result in contrasting photos. For example, if a toxic insult caused moderate toxicity to neurons, it is possible to take the photos for the control group (from any area in a well) and the toxic insult group (from an area in a well that is not damaged) that make the toxic compound appears as if it was not toxic. That non-homogeneity in the damage observed under the microscope in neuronal cultures can occur was noticed previously [*Leahy et al., 1994*].

If so, why it is said above that morphological examination is a very objective technique? The reason is that, to take the above example, although a well in the insult group will contain some viable areas (not damaged), it is possible to move around the well to see all of its field, which will make it possible to see that other areas in the well are indeed

damaged. On the other hand, in the control group, no damage is seen whatever the area in a well is looked at. This applies to many experiments performed in this project.

There are some cases where taking photos can be as objective as observing the damage under the microscope. This happens when the toxic insult is so severe to the degree that the whole culture in wells is totally collapsed/damaged, and hence there will be severe damage observed whatever the area is chosen in a well to take a photo. This applies to some experiments performed in this project.

4.1.3 Alamar blue assay

The viability assay used, the Alamar blue reduction assay, although not free of drawbacks, is a reliable and very convenient measure of cell viability. It has been validated previously on its own and against other assays (e.g. Trypan blue exclusion method, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT-assay), or lactate dehydrogenase (LDH) release) in different types of cell cultures including CGNs, and using a variety of toxicity models including oxidative stress [*White et al., 1996, Nakayama et al., 1997, Back et al., 1999, O'Brien et al., 2000, Gonzalez and Tarloff, 2001, Hamid et al., 2004*]. Although it is generally thought that Alamar blue is exclusively reduced by mitochondrial enzymes, other enzymes are known to reduce it also (e.g. the cytosol is as efficient as the mitochondria in reducing it) [*O'Brien et al., 2000, Gonzalez and Tarloff, 2001, Hamid et al., 2004*]. Regardless of the site of its action, Alamar blue is recognized to be reduced mainly by living cells: its active ingredient, resazurin, was introduced more than 70 years ago to the food industry to detect and measure the presence of contaminating living microorganisms [*John, 1939, Nixon and Lamb, 1945, Straka and Stokes, 1957*]. The extent of its reduction is expected to reflect the viability status of the cultures.

In this project, when the difference in viability between two groups based on Alamar blue assay results is $\geq 20\%$, there is found a good (but not necessarily exact) correlation between the Alamar blue viability assay results and the morphological appearance of the neurons under the microscope. There was not even a single case where the neuronal appearance under the microscope was suggesting that Alamar blue was giving misleading false results. Actually, this good correlation between Alamar blue assay and the morphological examination was the reason for believing that the protection of

ethanol and DMSO against fresh culture medium toxicity was real and not due to an artefact in the Alamar blue viability assay, which led eventually to discovering the reason of fresh culture medium toxicity.
Moreover, it is unlikely that Alamar blue was interfering with any of the treatment compounds or even interfering with their delayed effects, since the end of the treatment period (when the treatment solutions that contain the test compounds are removed) and the addition of Alamar blue are separated by at least 16 hr of restoration period and also since, as mentioned above, the cell damage/morphology seemed under the microscope to stabilize within 8 hr of the restoration period (i.e. at least 8 hr before adding Alamar blue) and the cells do not deteriorate or recover after that.

As mentioned above, the good correlation between Alamar blue viability results and the morphological examination is only seen if the difference between two groups based on Alamar blue results is ≥ 20%. As this difference decreases more and more below 20 %, although the correlation between Alamar blue results and morphological examination can be noticed, it will start to be less and less clear. It is possible that Alamar blue has the ability to detect small changes in the viability of cells, where these small changes are not large enough to cause very clear changes in the morphology.

Also as mentioned above, the Alamar blue viability assay, like any other assay, is not free of drawbacks. For example, if an experiment contains two groups, a control group and a moderate insult group, then although the Alamar blue will be reduced more by the control group, if the dye is left in the cultures for too long a period, then the moderate insult group (and the control group) may reduce the remaining Alamar blue molecules that were not reduced initially, resulting in full reduction of the Alamar blue in the two groups, and hence equal viability values in the two groups. For this reason, an optimal incubation period and optimal concentration of Alamar blue have to be applied. Previous studies have applied an incubation time between 2 and 6 hr (4-6 hr if using CGNs cultures) [*White et al., 1996, Fatokun, 2006, Fatokun et al., 2007b*], where the Alamar blue viability results based on this incubation time accurately reflect the viability status of the cultures. In an experimental check performed in this project, there was no statistically significant difference between the viability result based on 4 and 6 hr Alamar blue incubation times, which suggests that both of the incubation times are appropriate (4 hr was used throughout this project) .

4.1.4 Effect of position in the plate on the viability of cultures

It was observed that neurons located in the edge wells look much stressed after 8 days in culture, which was likely due to a massive evaporation of liquids from these wells. This was evident from the observation that the medium volume was much reduced in those wells and was also looking very pinkish. Therefore, neurons in these wells were never used in this project.

Importantly, the results show that neurons located in the next-edge wells give consistently slightly (10-15%) higher viability values in the Alamar blue assay than neurons located in the inside wells. May be because these differences were small, it was not possible to decisively confirm them by the morphological examination under the microscope, though they were noticeable. If these differences were real, they are somehow unexpected. It is expected that, because the neurons in the edge wells were looking much stressed under the microscope, the viability of neurons should decrease rather than increase as the location of cultures gets closer to the edge of the plate. A speculative explanation is that, because the neurons in those experiments were under the influence of fresh culture medium toxicity (i.e. glutamate excitotoxicity, see later), the neurons in the next-edge wells (being under the stress of the edge effect, although much less than the stress of the neurons in the edge wells) were able to activate compensatory mechanisms during the 8 days in culture that made them more resistant than neurons in inside wells when they were challenged at day 8 or 9 by fresh culture medium (i.e. glutamate) excitotoxicity.

Regardless of whether these relatively small differences in the viability between next-edge wells and inside wells were real or were artefacts in the Alamar blue assay, it was clear that such differences could lead to significant misleading conclusions. For example, if the real difference in viability between two treatment groups, one with a toxic insult and the other with this toxic insult plus a protective agent, was around 5% in favour of the latter group (which might not be statistically significant), placing the former group in inside wells and the latter group in next-edge wells will give a difference, in the Alamar blue assay, of around 15-20% in favour of latter group, which can be statistically significant, but misleading. For this reason, next-edge wells were never used after this finding.

4.1.5 Fresh culture medium toxicity

It turned out that the immediate damage seen in the neurons (including those in the control group) when they are manipulated at the experiments/intervention day (i.e. day 8 or 9) was due to glutamate excitotoxicity (through activating NMDA receptors). The source of glutamate was the fresh serum (which we were unaware of its content of glutamate) which is present at 10% V/V in the fresh culture medium that is added to the cultures at that day. The discovery in this project of the involvement of NMDA receptors in the fresh culture medium toxicity was serendipitous. This occurred when ethanol and DMSO, which were tried for another purpose (they were tried as hydroxyl radical scavengers), prevented this toxicity, and it was found in the literature that they can block NMDA receptors in neurons and can prevent glutamate toxicity [*Lin et al., 2003, Dildy and Leslie, 1989, Lovinger et al., 1989, Cebere and Liljequist, 2003, Danysz et al., 1992, Wegelius and Korpi, 1995, Lu and Mattson, 2001*]. When MK-801 [*Fatokun et al., 2008b & c*], a specific NMDA receptor blocker was tried here, it blocked the toxicity. After this finding, an early report was found [*Schramm et al., 1990*] that clearly demonstrated the role of NMDA receptors in fresh serum toxicity (likely through the activation of these receptors by glutamate already present in fresh serum) in CGNs cultures. Therefore, we kind of reached the same conclusion independently, which gives it more support. The very low concentration at which MK-801 was found to be protective here (20 nM) is in accord with this report of Schramm and co-workers (1990) which found a similar potency with MK-801.

A series of experiments was conducted to substantiate this conclusion. The first was using another blocker of NMDA receptors, kynurenic acid [*Perkins and Stone, 1982, Fatokun et al., 2008b*]. Since the mechanism of blocking NMDA receptors by MK-801 (blocks the ion channel in the receptor) is somewhat different from that of kynurenic acid (blocks the glycine binding site on the receptor) [*Fatokun et al., 2008b*], it was useful to try the latter to see if fresh culture medium toxicity can be prevented by two different ways of blocking NMDA receptors, which was the case. The relatively high concentration of kynurenic acid needed to block the toxicity (1 mM) is in accord with a previous study that showed that this activity is relatively weak in cell cultures [*Hilmas et al., 2001*]. Kynurenic acid possesses other known activities e.g. blocking some nicotinic receptors and blocking non-NMDA glutamate receptors [*Hilmas et al., 2001, Fatokun et al., 2008b*]. However, it is unlikely that non-NMDA glutamate receptors were involved in

fresh culture medium toxicity, since the specific NMDA receptor blocker MK-801 provided almost complete protection, and also since previous studies found that blocking non-NMDA glutamate receptors does not prevent glutamate toxicity in CGNs [*Eimerl and Schramm, 1991,Fatokun et al., 2008c, Resink et al., 1994*].

Further evidence for the role of glutamate and NMDA receptors in fresh culture medium toxicity came from an experiment in which fresh culture medium was behaving like glutamate in producing the so-called preconditioning effect. Marini and Paul (1992) have observed that acute pre-treatment (minutes/hours) with a subtoxic concentration of glutamate in CGNs can protect against a subsequent lethal exposure to glutamate itself (acute preconditioning effect). The same was found here with fresh culture medium, where acute pre-treatment with a reduced amount of fresh culture medium protected against a subsequent exposure to a lethal full amount of fresh culture medium itself. The protective mechanism of pre-treatment with subtoxic glutamate in CGNs was shown to be mediated by a subtle (subtoxic) activation of NMDA receptors [*Marini and Paul, 1992*]. The experiment performed here with pre-treatment with reduced amount of fresh culture medium shows that as the volume of fresh culture medium applied as pre-treatment is reduced, the protection increased, suggesting that with reducing the volume in the pre-treatment (assuming that glutamate concentration is reduced in parallel) the effect of fresh culture medium (i.e. glutamate) moves away from a toxic effect towards a preconditioning protective effect against a subsequent exposure to full and lethal amount of fresh culture medium (i.e. glutamate) itself.

The experiments with ethanol, DMSO, MK-801, kynurenic acid, and pre-treatment with reduced amount of fresh culture medium showed that protection in these experiments against fresh culture medium toxicity was sustained even after these protective interventions were terminated and followed by addition of fresh culture medium (which contains glutamate) for 16-24 hr before the viability assay. Although it is possible that the mechanism of the sustaining of protection of ethanol, DMSO, MK-801, and kynurenic acid is similar in these experiments (since they work by blocking NMDA receptors), this may not necessarily be the same mechanism responsible for the sustaining of protection of pre-treatment with reduced amount of fresh culture medium (where this protection was likely mediated by subtle activation of NMDA receptors).

When a glutamate degrading enzyme, glutamic-pyruvic transaminase [*Matthews et al., 2000*], was used here it provided almost complete protection against fresh culture medium toxicity. Since glutamate is present in fresh serum-containing culture medium (like the one used here) at concentrations more than 50 μM [*Aronica et al., 1993, Schramm et al., 1990*], this effect of glutamic-pyruvic transminase confirms that the glutamate molecule itself (and not a similar NMDA receptor agonist) was responsible for fresh culture medium toxicity. This adds a direct proof to the study of Schramm and co-workers (1990) where they suggested glutamate to be responsible for fresh serum toxicity based on the presence of glutamate in fresh serum and based also on their finding of protection by blocking NMDA receptors. Therefore, the combined results of these two independent investigations makes it very likely that the toxicity of glutamate already present in fresh culture medium was exerted through activating NMDA receptors. This does not rule out that the toxicity of glutamate was potentiated by other factors in the fresh culture medium. It was shown previously that glutamate toxicity can be potentiated by serum, where serum albumin was likely the component responsible for this potentiation [*Schramm et al., 1990, Eimerl and Schramm, 1991*].

When glutamate was applied here to CGNs in a glutamate-free and a serum-free solution at concentrations of 300 or 30 μM for only one hour, it caused significant toxicity. This shows that CGNs are indeed susceptible to glutamate excitotoxicity, and also shows that serum is not required for this toxicity (although it can potentiate it). It is worth mentioning that the solution used here as a vehicle to externally add glutamate (MEM-HEPES-sol) does not contain added glycine. However, it is very likely that glycine was still required for glutamate toxicity, since it is known to be required as a co-substrate in NMDA receptor-mediated glutamate excitotoxicity in CGNs [*Beaman-Hall et al., 1998, Fatokun et al., 2008b & c*]. Since the concentration of glycine needed for such an action can be quite low (in nM concentrations) this amount could easily have been provided by the cells themselves, as suggested previously [*Beaman-Hall et al., 1998, Parks et al., 1991*]. Also, since kynurenic acid provided substantial protection (likely through blocking the glycine binding site), this suggests that glycine was present and was activating its kynurenic acid-inhibitable binding site. Another point worth mentioning is that, although MEM-HEPES-sol contains magnesium at nearly 0.8 mM which is known to be a physiological blocker of NMDA receptors, it was shown previously that under depolarizing conditions, as applied here (25 mM potassium

chloride), NMDA receptors can be activated even in the presence of magnesium [*Marini and Paul, 1992, Eimerl and Schramm, 1991*].

By discovering that glutamate already present in the fresh culture medium was responsible (through activating NMDA receptors) for the immediate damage induced by manipulating the neurons at the intervention day, many previously unexplained observations in this project seemed to be explainable at once. One observation was that the damage induced to the neurons at the intervention day was severe in this project but not severe in two previous projects undertaken in this laboratory [*Fatokun, 2006, Smith, 2008*]. The cell density observed under the microscope immediately after plating tended always to be much higher in this project compared to the previous two projects, which was likely due to a small difference in the culturing procedure undertaken at the plating day (i.e. day zero). It seems now that the higher cell density in this project was responsible for the severe damage observed by adding fresh culture medium (i.e. glutamate) at the intervention day, since glutamate excitotoxicity was shown previously to be highly dependent on the neurons density in CGNs cultures [*Ciotti et al., 1996*].

Another observation was that the toxicity of the added fresh culture medium tended always to be more severe if this medium was looking more pinkish (i.e. more alkaline). Although close adjustment of this fresh medium pH resulted in a considerable improvement in cell viability, the damage by adding this fresh medium was still severe. Also, when a plate containing the cultures was placed outside the incubator for more than an hour, although this made the culture medium that the neurons were maintained in very pinkish (i.e. very alkaline) and although this also likely reduced the temperature of the cultures to the room temperature, this did not damage the neurons (at least no immediate damage was observed). It seemed at that time that although increasing the pH per se (at day 8 or 9) of the culture medium that the neurons are maintained in does not kill the neurons, increasing the pH of the fresh culture medium that is added at day 8 or 9 potentiates its toxicity. The increase in damage by increasing fresh culture medium pH can now be explained by the fact that NMDA receptor-mediated glutamate toxicity in CGNs is greatly potentiated by the increase in pH [*Eimerl and Schramm, 1991*], which is because increasing the pH relieves a proton-mediated block of NMDA receptors [*Traynelis and Cull-Candy, 1990*]. However, because NMDA receptor-mediated glutamate toxicity does indeed operate at pH 7.4 [*Eimerl and Schramm, 1991*], this also explains the damage observed here by adding fresh culture medium (i.e. glutamate) even with close

adjustment of pH (though this adjustment improved the viability). Also, the fact that NMDA receptors require the ligand (i.e. glutamate) to cause toxicity may also explain the observation that the increase in the pH per se (at day 8 or 9) of the culture medium that the neurons are maintained in (which does not contain significant amount of glutamate) does not kill the neurons.

A third observation that was not explained previously was that the culture medium does not kill the neurons when they are maintained in it before the intervention day. Although there were many neurons dying in the first 24 hr of plating, fresh culture medium was not suspected to cause this death for two reasons. Firstly, this culture medium is universally used to culture many types of cells including neurons, and there was no reason to suspect that this medium might be toxic. Secondly, if it was responsible for the death observed at the plating day (i.e. day zero), why did it not kill the viable neurons when they were maintained in it from day 1 until the intervention day? Actually this was the reason that in the beginning a toxic effect of the added fresh culture medium was not suspected as the reason for the damage observed at the intervention day, where alternative explanations were explored at that time. However, this can now be explained by the following scenario of events. In the first few days, glutamate can not cause damage to neonatal CGNs as was shown previously [*Frandsen and Schousboe, 1990, Resink et al., 1994*], and this is likely because the glutamate receptors subunits that can mediate the toxicity are not matured/functioning at this early stage [*Frandsen and Schousboe, 1990, Resink et al., 1994, Schramm et al., 1990, Leist et al., 1997*]. Since the brain quickly clears any glutamate that is present extracellularly, the glutamate present in the fresh culture medium is quickly removed by CGNs as was shown previously where glutamate concentration drops in fresh serum-containing culture medium in CGNs cultures from more than 50 µM to only 5 µM in the first 24 hr, and then drops further in the next two days to around 2 µM and is maintained at this low level in the subsequent days [*Aronica et al., 1993*]. By the time that glutamate receptors subunits that can mediate toxicity are matured (likely after 5-6 days of plating [*Resink et al., 1994*]) there will be no significant amount of glutamate left in the culture medium to cause toxicity, but when fresh culture medium (which contains more than 50 µM glutamate) is added to the neurons at the intervention day (i.e. day 8 or 9), glutamate binds to the matured NMDA receptors to induce severe toxicity to the CGNs.

Schramm and co-workers (1990) have discussed the pathological relevance of the presence of glutamate in serum (i.e. circulation), and the potentiation of its toxicity by some serum components. They proposed that in some pathological conditions e.g. hemorrhagic stroke or brain trauma, circulating glutamate might contribute to cell damage. This is a valid proposal and this might be a unique mechanism of in vivo toxicity, since the exposure of neurons to circulating glutamate in such pathological conditions might initiate toxicity without the pre-request of the depolarization/damage-induced glutamate release from the neurons. The same authors also speculated that such a circulating glutamate might cause chronic toxic effects by leaking through the blood brain barrier (BBB) in elderly people where this barrier is expected to be fragile.

Given the central excitatory role played by glutamate in the brain, the wide diversity of its receptor sub-classes, its presence in the circulation, and its ability to cause neuronal damage in many situations, it is not surprising that every time a new role is postulated/discovered for the glutamate system in neuronal pathological conditions (studied in vivo or in vitro) that were not shown/suspected initially to be related to this system. Therefore, in studying neuronal pathologic conditions thought not to be related to glutamate system, it should be considered that the master, highly diverged, and widely distributed glutamate system might be interfering with the condition under investigation, and that checking for this involvement may resolve some unexplained observations.

4.2 Oxidative stress experiments performed before solving the problem of fresh culture medium toxicity

The interpretation of the results of the experiments discussed in this section is complicated by two facts. These experiments were performed before solving the problem of fresh culture medium toxicity, which means that the cultures (including those in the control groups) were under the influence of glutamate excitotoxicity. Also, some of these experiments were performed before finding that the neurons in next-edge wells give consistently higher viability readings than the neurons in inside wells, so some of the treatment groups in these experiments were containing next-edge wells (which may result in misleading conclusions as discussed before). However, it was appropriate to discuss some of those experiments (performed in that period) that were

consistent and provided some valuable information. Due to the uncertainties mentioned above, there will be only a brief discussion of the results in this short section, without detailed discussion on why a compound produced an unexpected effect or failed to produce an expected effect.

The idea was to test the susceptibility of CGNs to different types of oxidative stress insults, which can lay the foundation for detailed investigation of XO toxicity. Hydrogen peroxide externally applied showed toxicity to CGNs in a dose dependent manner, which is in agreement with previous studies that externally applied this toxic insult to CGNs [*Fatokun et al., 2007b, Götz et al., 1999*]. Due to the relatively modest reactivity of hydrogen peroxide, it usually exerts its toxic effects through its conversion to more reactive species, usually hydroxyl radical. This conversion to hydroxyl radical, called Fenton reaction, requires a metal, usually iron or copper (see Introduction). When deferoxamine, an iron chelator with high affinity for the oxidized form of iron (i.e. Fe^{3+}) [*Keberle, 1964*], was tried, it provided protection, raising the possibility that hydrogen peroxide toxicity was due to Fenton reaction. To confirm this mechanism of toxicity, a hydroxyl radical scavenger, mannitol, was tried, but failed to show any protection. Although the failure of mannitol to protect can be explained by the lack of a role of Fenton reaction and hydroxyl radical in the toxicity, there are many alternative explanations (see later in section **4.3**).

Another toxic insult, the X / XO combination, was tried and it showed significant and consistent toxicity to CGNs when applied at X (100 μM) / XO (0.02 Units/ml). The toxicity of this combination is expected since it is known to produce toxicity in different types of cell cultures [*Fatokun et al., 2007a, Satoh et al., 1998, Link and Riley, 1988, Mohsen et al., 1995, Duell et al., 1995, Simon et al., 1981, Hiraishi et al., 1987, Zigler etal., 1985, Valencia and Morán, 2004*]. Since this combination is known to directly produce hydrogen peroxide and superoxide (hydrogen peroxide should also be produced by the spontaneous dismutation of superoxide that is directly produced), catalase and SOD-1 were tried against the toxicity of this combination. Catalase produced complete protection, but SOD-1 failed to produce protection.. Most previous studies in cultures found that catalase protects whereas SOD does not protect against X / XO toxicity [*Fatokun et al., 2007a, Satoh et al., 1998, Link and Riley, 1988, Mohsen et al., 1995, Duell et al., 1995, Simon et al., 1981, Hiraishi et al., 1987, Zigler etal., 1985*].

In the same experiment, in addition to testing the effects of catalase and SOD-1, two XO inhibitors were tried against X / XO toxicity: allopurinol (a blocker of the X binding site, the Mo site) and DPI (a blocker of the NADH binding site, the FAD site) (see Introduction). Both of allopurinol and DPI failed to provide statistically significant protection, which was unexpected since both (especially allopurinol) are known to block X oxidation by XO (but see next section **4.3** for detailed discussion of experiments performed after solving the problem of fresh culture medium toxicity where these two compounds were tried against X / XO toxicity).

Since catalase provided complete protection against X / XO toxicity, this suggests that hydrogen peroxide is a main toxic molecule, which makes this toxicity model somehow similar to the toxicity model of externally applying hydrogen peroxide. Since deferoxamine provided protection against externally applied hydrogen peroxide, it was also tried against X / XO toxicity. Although deferoxamine at the concentration (and incubation time) that was protective against externally applied hydrogen peroxide did not show protection against X (100 μM) / XO toxicity, it was protective when the toxic insult was reduced to X (30 μM) / XO, which suggests that it did not protect in the first experiment because the toxic insult was too severe for it to provide significant protection. Also the protection with deferoxamine against X / XO toxicity was increased when its pre-treatment time was increased, and since this chelator is expected to be slow in entering the cells [*Porter et al., 1988*], this suggests that its site of action is intracellular (see section **4.3** for more discussion on deferoxamine).

A third type of oxidative stress insults, a nitric oxide donor, SNAP, was also applied to CGNs cultures to see if they are also susceptible to this type of toxicity. Nitric oxide is a free radical that can cause damaging effects (probably indirectly) under some conditions, which was demonstrated previously in CGNs cultures [*Leist et al., 1997*]. SNAP treated here for 24 hr showed a dose dependent toxicity. However, it may be that this effect of SNAP was not due to providing nitric oxide. The reason is that the SNAP powder was dissolved and the aliquots of the solution kept in a freezer until the experiment day. If SNAP in solution instantly generates nitric oxide (as expected), this raises the possibility that the nitric oxide, which is a short lived free radical, generated would have been long degraded before the experiment day. A better way would have been to dissolve SNAP powder and then add it to the cultures instantly.

There is more than one explanation for the observed toxicity with SNAP. One possibility is that it was still due to, at least partially, nitric oxide spontaneously released by SNAP at the time of treatment. This is because, based on the published data on SNAP stability in solutions under different conditions (where SNAP $t_{1/2}$ can be up to hours) [*Megson et al., 1997, Ioannidis et al., 1996, Singh et al., 1996, Mathews and Kerr, 1993, Arnelle and Stamler 1995*], there is still a possibility that the SNAP stock solution prepared under our conditions (the powder was dissolved in distilled water to a concentration of 10 mM at room temperature and ambient oxygen, aliquoted, protected from light, and then immediately kept in freezer until the experiments day) was still containing a significant amount of intact SNAP when the frozen aliquots were thawed and SNAP was applied to CGNs. A second possibility, also assuming the presence of a significant amount of intact SNAP at the time of addition to cultures, is that the toxicity was not due to nitric oxide spontaneously released by SNAP, but rather was due to other actions of SNAP itself that may or may not involve production of nitric oxide (but not spontanously released from it). This is consistent with some previous studies which showed that some biological actions of SNAP, although might be due to production of nitric oxide, are not due to nitric oxide that is spontanously released from it [*Kowaluk and Fung, 1990, Singh et al., 1996, Mathews and Kerr, 1993, Arnelle and Stamler, 1995*]. A third possibility, assuming that SNAP was long degraded before the experiments time, is that the toxicity was not due to nitric oxide released spontaneously from SNAP, and was not due to other actions of intact SNAP itself, but rather was due to toxic effects of some degradation products of SNAP (generated before the time of addition to cultures) other than nitric oxide.

Overall, these experiments performed before solving the problem of fresh culture medium toxicity gave some helpful information. They showed that CGNs are susceptible to perhaps more than one type of oxidative stress injury. They also showed that the toxicity models of both externally applying hydrogen peroxide and externally applying the X / XO combination are similar in that both are completely (but not necessarily exclusively) mediated by hydrogen peroxide, where an iron ion was likely mediating hydrogen peroxide toxicity. These experiments leave unanswered the effect of SOD, allopurinol, or DPI on X / XO toxicity, but a detailed investigation of the effect of these compounds was carried out in the experiments performed after solving the problem of fresh culture medium toxicity, which will be discussed in the next section (**4.3**).

4.3 Oxidative stress experiments performed after solving the problem of fresh culture medium toxicity

After solving the problem of fresh culture medium toxicity, it was possible to conduct reliable experiments. In this stage of the project, it was possible to answer many of the questions stated earlier in the section on the Aim/Objectives in the Introduction. This section is divided into two main sections, the first is a comparison between X / XO and NADH / XO toxicities, and the second is a further investigation of X / XO toxicity.

4.3.1 Comparison between X / XO and NADH / XO toxicity models

Although a combination of XOR and X (or other substrates that bind to the molybdenum site) is a widely used model in cell culture studies to generate ROS and to study their effects, the toxicity/effect of the combination of XOR and NADH (which binds the FAD site) in cell cultures has not been investigated in detail previously. Some possible reasons for this lack of interest to investigate this combination were mentioned in the Introduction, and it was also mentioned in the Introduction that the in vivo effect/toxicity of the oxidation of NADH by XOR might have been underestimated previously.

The idea was to compare X / XO and NADH / XO toxicity models in their potency, their response to blocking the different sites of XO, and the type of ROS and metals responsible for toxicity. The results show that NADH / XO and X / XO combinations are toxic to cultures of CGNs. However, the concentration of NADH needed to cause the toxicity was much higher than that of the other substrate, X, which is in agreement with previous cell-free experiments that showed that NADH is a much weaker substrate than X for the bovine milk XO used here [*Gilbert, 1963, Liochev et al., 1989, Nakamura, 1991*]. However, some other forms of the enzyme (e.g. bovine milk XDH, human milk XO, human milk XDH, human liver XO, rat liver XDH, and rat liver XO) have more/much more efficiency in oxidizing NADH than the bovine milk XO used here [*Maia et al., 2007, Sanders et al., 1997, Zhang et al., 1998*], and even some of them oxidize NADH with similar oxidation kinetics to the oxidation of X by the bovine milk XO. Therefore, much less concentration of NADH might have been enough to cause toxicity

if some of these other forms were used instead. Bovine milk XO was used in this project because of the availability, and also because it is the most studied form of the enzyme.

The NADH / XO combination was applied for only one hour, where lower concentrations of NADH might have been enough to induce toxicity if applied for longer durations. However, it was not possible to apply the NADH / XO for longer durations, because a previous study in this laboratory showed that XO applied alone for 6 hr caused significant toxicity to CGNs (probably through oxidizing xanthine produced by the neurons, since allopurinol attenuated this toxicity) [*Fatokun et al., 2007a*].

In both of NADH / XO and X / XO toxicities (especially the latter), the damage tended always to be more severe and more consistent in HEPES-sol compared to MEM-HEPES-sol. There are many differences between these two treatment solutions which makes it difficult to know the reason(s) of this interesting difference in the susceptibility to toxicity without a systematic investigation. Also, this is complicated by that the opposite was observed when NADH was applied alone, where it was toxic at 2 mM (but not 1 mM) in MEM-HEPES-sol but not in HEPES-sol. This toxicity of NADH alone in only one of the treatment solutions is also difficult to explain without a systematic investigation. These observations, however, were not considered to affect the main conclusions drawn from this project. Notice that it is unlikely that any of the observed effects of NADH applied alone or in combination with XO is due to an artefact due to a direct interaction (e.g. reduction) between NADH and the Alamar blue dye used in the viability assay, since as mentioned before the test compounds (including NADH) were not present together with Alamar blue. The test compounds were removed and replaced by conditioned medium for at least 16 hr before applying Alamar blue.

4.3.1.1 Effects of inhibiting the different sites on XO

In agreement with previous cell-free experiments, it was found that blocking the site of X binding (the Mo site) with allopurinol failed to prevent the damage induced by the NADH / XO combination, although it prevented the damage induced by the X / XO combination. This result is consistent with the previous proposal that the failure of allopurinol in preventing tissue damage in some previous studies where XOR-mediated damage was suspected [*Allen et al., 1990, Benders et al., 2006, Mosler et al., 2005, Coetzee et al., 1996*] might be theoretically explained by the inability of allopurinol to prevent

NADH oxidation by XOR, and hence its inability to prevent the tissue damage [*Berry and Hare, 2004, Harrison, 2002, Sanders et al., 1997, Zhang et al., 1998*].

Blocking the site of NADH binding (the FAD site) with DPI at 100 nM attenuated the damage induced by the NADH / XO combination applied in either HEPES-sol or MEM-HEPES-sol, which was expected since DPI is known to block this site. This (along with the failure of allopurinol to protect) suggests that the toxicity of the NADH / XO combination was mediated/initiated by direct enzymatic oxidation of NADH by XO and was not due to merely (or exclusively) non-specific interaction between the enzyme and the substrate. DPI also blocked the toxicity of the X / XO combination applied in HEPES-sol., but failed to show statistically significant protection against this combination when applied in MEM-HEPES-sol. In any case, the results show that DPI can prevent X / XO toxicity in HEPES-sol, which is in agreement with previous cell-free experiments that showed that the FAD site is the site of ROS generation regardless of whether the reducing substrate binds to the Mo site (i.e. X or HX) or to the FAD site (i.e. NADH) [*Komai et al., 1969, Sanders et al., 1997, Olson et al., 1974, Nakamura, 1991, Berry and Hare, 2004, Harrison, 2002*].

4.3.1.2 Identification of ROS and metals responsible for the toxicity

The effects of SOD-1 and catalase on the toxicity of the X / XO and the NADH / XO combinations were investigated in this study. In the case of X / XO toxicity, although catalase provided almost complete protection, SOD-1 failed to produce any effect, implicating that although hydrogen peroxide is required for this type of toxicity, extracellularly generated superoxide is not (may be apart from its spontaneous dismutation to hydrogen peroxide). Also when the superoxide scavenger, Tiron [*Greenstock and Miller, 1975, Hassan et al., 1980*], was tried as a co-treatment, it failed to provide protection against X / XO toxicity. The lack of protection by co-treatment with SOD-1 against X / XO toxicity is in agreement with many previous culture studies which have used different types of cell/organ cultures including CGNs [*Fatokun et al., 2007a, Satoh et al., 1998, Link and Riley, 1988, Mohsen et al., 1995, Duell et al., 1995, Simon et al., 1981, Hiraishi et al., 1987, Zigler et al., 1985*], but with at least one exception which found protection with 6 hr co-treatment with SOD-1 against X / XO toxicity in cultures of CGNs [*Valencia and Morán, 2004*]. The protection found by the study of Valencia and Morán (2004) suggests that SOD-1 co-treatment under some conditions can protect

against X / XO toxicity, although no clear difference in the experimental conditions was suspected to account for the difference in the observed effect between this and the many other studies that did not find protection with SOD co-treatment.

One may think that the observation (found here and in many previous studies) that SOD does not increase or decrease X / XO toxicity is an odd result. To put it in other words, SOD should either potentiate the toxicity if it is a hydrogen peroxide-dependent (and not superoxide-dependent) (since SOD will convert superoxide to hydrogen peroxide) or attenuate the toxicity if it is a superoxide-dependent, so the result that SOD has neither of these two effects indeed needs an explanation. That SOD does not attenuate the toxicity can be explained by the lack of a role of superoxide produced extracellularly in the toxicity (may be apart from its spontaneous dismutation to hydrogen peroxide). On the other hand, a possible explanation for the inability of SOD to potentiate the toxicity (assuming that it is a hydrogen peroxide-dependent and not superoxide-dependent) is that most of ROS produced directly by X / XO (around 80%) are known to be in the form of hydrogen peroxide, while the remaining 20% will be in the form of superoxide (although this may change depending on the experimental conditions) [*Fridovich, 1970*]. Therefore, adding SOD, which will convert two molecules of superoxide into one molecule of hydrogen peroxide, will result in only a small increase in hydrogen peroxide production (around 10%), and so there will be no significant increase in the X / XO toxicity. An additional explanation for the inability of SOD to potentiate the toxicity (again assuming that it is a hydrogen peroxide-dependent and not superoxide-dependent) is that even in the absence of SOD, all superoxide produced by X / XO would quickly and spontaneously dismutate to hydrogen peroxide in the extracellular compartment (if given the time, which might be the case because superoxide may not be able to enter the cells, and also because superoxide production ceases long time before the end of the 1 hr treatment, see later). This means that the same result (i.e. dismutation of superoxide to hydrogen peroxide extracellularly) was going to be produced regardless of whether SOD is present or not.

The fact that superoxide can quickly and spontaneously dismutate to hydrogen peroxide does not of course mean that SOD activity is not important as an in vivo defence mechanism against oxidative stress, because it is the difference in the efficiency between the enzymatic and the non-enzymatic (spontaneous) dismutation of superoxide that matters [*Fridovich, 1983*]. Although a portion of the superoxide produced in vivo

will immediately and spontaneously dismutate to hydrogen peroxide, a remaining portion may stay active and travel relatively long distance before it eventually spontaneously dismutates, so unless the very efficient SOD is present, a superoxide-dependent effect/toxicity may occur.

There is a possibility that the way in which the X / XO mixture was added to the cells in this study may have masked a toxic role of superoxide generated from this combination. This is because whenever X and XO were added together to the treatment solution, this mixture-containing solution was warmed in the water bath for few minutes before adding to cultures. The cell-free experiments in Figures 3-50 and 3-51 clearly showed that superoxide generation by X / XO combination ceases by less than 10 minutes after starting the reaction, where after this 10 minutes most of the short-lived superoxide would have already been spontaneously dismutated to hydrogen peroxide. Therefore, by the time of adding the mixture to cells (5-10 minutes after starting the reaction), there might not be a significant amount of superoxide that would otherwise produce a specific toxicity (may be through entering the cells). A better way of conducting the viability experiments was to start the X / XO reaction in the vicinity of cells, which would give the generated superoxide time to be in contact with cells where it may produce a specific toxic effect through entering the cells or directly interacting with them. Not only this, but also catalase (to deactivate extracellular hydrogen peroxide) should be present when the reaction is started in the vicinity of cells to make sure that an observed toxicity of X / XO combination is due to superoxide entering the cells (or directly interacting with them) and not merely due to its extracellular spontaneous dismutation to hydrogen peroxide. If there is still toxicity observed with this experimental design, a role of superoxide can then be confirmed by using SOD.

A pilot study was undertaken for the experimental design detailed above (n = 1). In this one trial, the experiment was performed as follows: the reaction of X (100 μM) / XO (0.02 Units/ml) was started in the vicinity of neurons in the presence or absence of catalase (10 Units/ml) in HEPES-sol. Even with this design, catalase provided complete protection (data were not shown). It was mentioned in the Results section that in HEPES-sol the X / XO combination always produces large and consistent toxicity at X concentration of only 15 μM, and that it produces almost complete toxicity at X concentration of 30 μM. Therefore, using X here at 100 μM was expected to cause almost complete toxicity (which was the case), and importantly was also expected to

produce large amount of superoxide in the vicinity of neurons. Notice that in the viability experiments shown in section **3.3.2.4** of the Results section, catalase was tried at 3 Units/ml, but it was tried in this one trial at 10 Units/ml, and this was done to make sure that it is able to deactivate most of hydrogen peroxide generated from X / XO combination, especially since X was used at 100 μM where large amounts of hydrogen peroxide were expected to be produced. Although it was shown in the cell-free experiments in the Results section that catalase is contaminated with some SOD activity, the contamination was observed at catalase (1000 Units/ml) and was less at catalase (300 Units/ml), with no observed contamination at catalase (3 Units/ml), so it is unlikely that there was a significant contamination with SOD activity at the catalase level (10 Units/ml) used here. Therefore, the absence of any observed toxicity of superoxide generated in the vicinity of neurons (in the presence of catalase) suggests that (as suggested by the completed experiments and by many previous studies) superoxide generated extracellularly from X / XO combination has no role in the toxicity of this combination (may be apart from its extracellular dismutation to hydrogen peroxide). However, this was only a single trial experiment (n = 1), so it was not possible to confirm this observation.

In the case of the NADH / XO combination, the experiments were conducted in the same way as those with the X / XO combination i.e. NADH and XO were added to the treatment solution which was then warmed in the water bath for few minutes before adding to cultures. However, the reaction between NADH and the bovine milk XO is expected to be slow (since NADH, is relatively a very week substrate for this isoform of XO). Therefore, it is possible that most of the oxidation of NADH by XO was occurring during the one hour application to cells (not during the few minutes of warming as in the X / XO system), although this can not be confirmed.

Although NADH / XO toxicity was similar to X / XO toxicity in the complete protection afforded by catalase, the former differed in that SOD-1 provided substantial protection. It is likely that, for many reasons, this protection by the co-treatment with SOD-1 was due to its elimination (dismutation) of superoxide and not due to any of the known non-specific actions of SOD-1 that may not involve elimination of superoxide. Firstly, SOD-1 was protective at concentrations as low as 3 Units/ml (which is one of the lowest concentrations tried in previous toxicity studies), whereas the non-specific actions of SOD-1 are expected to occur at high concentrations [*Liochev and Fridovich,*

2007]. Secondly, Mn-SOD (3 Units/ml), which is known to be free of at least some of the non-specific actions of SOD-1 [*Sankarapandi and Zweier, 1999, Liochev and Fridovich, 2000*], was as protective as SOD-1. Thirdly, SOD-1 was protective in both bicarbonate/CO_2-containing solution (MEM-HEPES-sol) and a solutions without added bicarbonate/CO_2 (HEPES-sol), which argues against a role for a peroxidase activity of SOD-1 in the observed protection, since this non-specific activity was shown to be dependent on bicarbonate/CO_2 [*Goldstone et al., 2006, Sankarapandi and Zweier, 1999, Liochev and Fridovich, 2004*]. Fourthly, substantial protection against NADH / XO toxicity was observed by co-treatment with the superoxide scavenger, Tiron (though Tiron is also known to be (among other actions) an effective chelator of some metals including iron and molybdenum [*Fridovich and Handler, 1962*], an activity that can not be ruled out as the reason for its protection).

It is likely that the protection by SOD-1 (and Mn-SOD) (especially since that it was used as co-treatment rather than pre-treatment, that it was protective at a low concentration (3 Units/ml), and was applied for only 1 hr) was due to an extracellular action of this enzyme. Some previous reports suggested that SOD-1 is unable to quickly/easily penetrate the cell membrane even at high concentrations. In neuronal cultures, acute co-treatment with SOD-1 (or even pre-treatment with SOD-1 for 8-24 hr) did not prevent glutamate-receptor mediated excitotoxicity, despite the fact that in those studies, intracellular production of superoxide was found to mediate the toxicity [*Patel et al., 1996, Lafon-Cazal et al., 1993a & b*]. Also, using a very specific detection method of intracellular superoxide, it was shown that SOD-1, although applied as a 1 hr pre-treatment (which gave prolonged contact with cells) and at a much higher concentration than 3 Units/ml (up to 100 units/ml), could not scavenge intracellular superoxide in cultures of bovine aortic endothelial cells [*Zhao et al., 2005*]. However, uncertainty remains; since other reports showed clearly that SOD-1 can enter cells under some conditions. SOD-1 (500 Units/ml) co-treated for 1 hr was able to produce a protective effect by entering cultured hepatocytes by endocytosis [*Kyle et al., 1988*], where this endocytosis was also observed by another study that followed it using a different approach [*Dini et al., 1995*]. However, the protection by SOD-1 against a similar insult using the same cell culture type (rat hepatocytes), as well as the entrance of SOD-1 to those cells, was not observed in another study [*Ito et al., 1992*], which suggests that small differences in the experimental conditions may have large consequences. In neurons, there were also some studies where SOD-1 was producing effects suggestive of it being

entering the neurons e.g. SOD-1 produced a protective effect in cultures of spinal cord neurons against glutamate excitotoxicity, but SOD-1 in that study was not applied as co-treatment but rather as pre-treatment for 2 hr and at a high dose [*Michikawa et al., 1994*].

Although it is more likely that SOD-1 (and Mn-SOD) was working here extracellularly rather than intracellularly to produce protection against NADH / XO toxicity, this does not answer the question of whether SOD was blocking an extracellular toxic action of superoxide or was deactivating it extracellularly before it could cross the cell membrane and exert an intracellular toxic action? This question is difficult to answer from the available results. Although superoxide (which is an anionic radical) is known to be generally very poor in crossing biological membranes [*Takahashi and Asada, 1983, Liochev and Fridovich, 2005*], there are some exceptions in which it was shown to be able to do so [*Liochev and Fridovich, 2005*]. Actually, even in CGNs, activating the glutamate NMDA-receptors was shown to lead to an intracellular production of superoxide that was able to exit the neurons and be detected in the extracellular compartment [*Lafon-Cazal et al., 1993b, Atlante et al., 1997*]. However, it is unclear if activating NMDA-receptors in those studies had led to the opening of some channels or pores (or to some defects in membrane integrity) that allowed intracellular superoxide to exit the neurons through these channels/pores that would be otherwise impermeable to superoxide. Therefore, it is unclear if superoxide generated here from the NADH / XO combination was able to enter the cells. The possibility that superoxide was not entering the neurons, and was instead producing its toxic action in the extracellular compartment, might be supported by the observed protection by co-treatment with EDTA. This general chelator of cations and metals [*Hutcheson et al., 2004*] is regarded as a biological membrane-impermeable compound [*Gazaryan et al., 2007, Frederickson et al., 2002, Azuma et al., 2001, Abeijon and Hirschberg, 1990*] (an exceptional previous study showed that EDTA was able to enter cells by endocytosis, but it was used at a very high concentration (6 mM) [*West and Brownstein, 1988*], whereas EDTA was protective here at concentrations as low as 2 μM). Also the mode of EDTA protection here suggests that is was blocking an extracellular metal-dependent toxic action (see later). Although this effect of EDTA does not necessarily mean that SOD (and Tiron) was blocking an extracellular toxic action of superoxide, it suggests so. This is because in many cases, superoxide exerts its toxicity through reacting with metals, and since both of SOD and EDTA (which were likely working extracellularly) were protective, an extracellular toxic interaction between superoxide and a metal might have been responsible for the toxicity.

If SOD was exerting its protection through blocking an extracellular toxic action of superoxide, this can be through blocking one of many candidate toxic actions of superoxide. Superoxide can mediate a metal-catalyzed toxicity (as mentioned above) e.g. through mediating the conversion of hydrogen peroxide to the very reactive and toxic hydroxyl radical (or a similar species) [*Fong et al., 1976, Halliwell, 1978, McCord and Day, 1978*]. This conversion in biological systems results from the reaction between hydrogen peroxide and a reduced metal ion, usually iron or copper (Fenton reaction). Since the extracellular metal ion that might be present in the treatment solutions as a contaminant is likely to be in the oxidized form, superoxide will be required for its reduction, making it able to react with hydrogen peroxide to produce hydroxyl radical. Even if the metal is present in the reduced state, superoxide will be required to reduce it back when it is oxidized by hydrogen peroxide, and hence to continue the reaction. The presence of NADH in the system makes this possibility more likely. This is because it was shown previously that, in the presence of reduced iron and NADH, externally added hydrogen peroxide generates much more hydroxyl radicals than it generates in the presence of only reduced iron [*Rowley and Halliwell, 1982*]. Interestingly in this study by Rowley and Halliwell (1982), hydroxyl radical generation was blocked by SOD, implying that superoxide was both produced and required, may be to reduce back the Fe^{3+} that was converted from Fe^{2+} upon oxidation by added hydrogen peroxide. In the case of the NADH / XO combination applied here, superoxide, in addition to its possible generation by such a reaction, is actually directly generated by the oxidation of NADH by XO, and hydrogen peroxide is also directly produced by this oxidation and will also be produced by the spontaneous dismutation of superoxide. If a contaminant metal ion is also present in the system, the requirement for extracellular production of a large amount of hydroxyl radical from the NADH / XO combination seems to be fulfilled.

To test this explanation, two metal chelators were tried, deferoxamine and EDTA. Although co-treatment with deferoxamine did not show any protection against NADH / XO toxicity, EDTA co-treatment was able to protect, as mentioned above, at concentrations as low as 2 μM. On the other hand, neither deferoxamine (300 μM) nor EDTA (2, 20, or 200 μM) protected against X / XO toxicity. Therefore, the protection by the cell-impermeable EDTA seemed to be specific for NADH / XO toxicity and enforces the suspicion that the protection offered by SOD (and Tiron) against this toxicity was due to blocking a superoxide-dependent extracellular toxic interaction

between hydrogen peroxide and a metal to produce hydroxyl radical (or a similar species). The very low concentrations at which EDTA was protective argues against the chelation of Ca^{2+} (present at 2 mM) or magnesium (Mg^{2+}) (present at 0.8 mM) in the treatment solution as the reason (or the sole reason) for the protection by EDTA. Also, EDTA was shown to efficiently chelate iron ions even in the presence of a large excess of Ca^{2+} [*Hutcheson et al., 2004*].

The failure of deferoxamine co-treatment to protect against NADH / XO toxicity, even though it will chelate contaminating iron present free in the treatment solution (since it was left with XO for 3 hr in this treatment solution (without contact with cells) before adding NADH and then applying to cells, and also since the volume of the treatment solution containing deferoxamine and XO before adding NADH was 95% of its volume after adding NADH), indicates that the NADH / XO toxicity is unlikely to involve an iron-mediated generation of hydroxyl radicals in the extracellular treatment solution. However there is a possibility that an iron contaminant was associated/bound with XO and was difficult to remove (to the degree that even 3 hr of contact between deferoxamine and XO before applying to cells was not enough for chelating this iron), which was able to mediate the toxicity. Iron is known to contaminate XO by binding loosely to it, where this iron contaminant has been shown to resist significant chelation by deferoxamine while remaining susceptible to other chelators (this contaminating iron influenced ROS generation by XO) [*Britigan et al., 1990*].

On the other hand, EDTA co-treated the same way as deferoxamine was protective, as mentioned above, at concentrations as low as 2 μM. Interestingly, this protective effect of EDTA seemed to be dependent on pre-incubating it with XO before starting the treatment. This is evident from that when the treatment solution (which contains EDTA, XO, and NADH) was applied to cells but without prior contact between EDTA and XO, EDTA no longer protected, despite the fact that EDTA was left alone in the treatment solution for 3 hr (without contact with cells) before adding XO and NADH and then applying to cells, and also despite the fact that the volume of the treatment solution containing EDTA before adding XO and NADH was more than 90% of its volume after adding XO and NADH. This suggests two things, firstly, that EDTA was protective by interacting directly with XO, likely chelating a contaminating metal ion associated with the enzyme, but not present free in the treatment solution and not associated with NADH. Secondly, the interaction between EDTA and XO must be slow (since prior

contact for some time (3 hr was tried) between XO and EDTA before starting the treatment was required for the protection).

The unidentified metal contaminant associated with XO could be iron, despite the fact that deferoxamine co-treatment failed to protect, and this is because of the above mentioned possibility that this iron was associated (loosely bound) with XO and was difficult to remove. Another metal ion which might have been responsible for the toxicity is molybdenum, since it has been shown to participate with superoxide and NADH in a potentially toxic reaction [*Darr and Fridovich, 1984*]. Molybdenum ion may have dissociated from XO as a result of freezing and thawing of the enzyme, making it available to participate with NADH and superoxide in a toxic reaction. There may be flexibility in the dissociation of molybdenum ion (which is an integral part of XO) from the enzyme, since 40% of the bovine milk XO molecules are known to be molybdenum-free [*Harrison, 2002*]. Contaminating copper ion associated with XO is also a candidate, especially since EDTA always inhibits copper-mediated hydroxyl radical generation [*Que et al., 1980, Aruoma et al., 1991, Makrigiorgos et al., 1995, Samuni et al., 1983, Shinar et al., 1983, Cui et al., 1994, Lloyd and Phillips, 1999*], while it can (depending on the experimental conditions) inhibit or stimulate iron-mediated hydroxyl radical generation [*Graf et al., 1984, Hutcheson et al., 2004, Halliwell and Gutteridge, 1981, Grootveld and Halliwell, 1986, Engelmann et al., 2003, Gutteridge, 1987*]. Also, it can not be ruled out that a metal contaminant-independent pharmacological action of EDTA was involved, possibly involving a slowly developing, direct inhibition of XO. However, the possibility of a metal contaminant-independent direct inhibition of XO is difficult to explain in the light of the failure of EDTA to protect against X / XO toxicity, even if it is assumed that EDTA was blocking the FAD site, since blocking this site (which is always the site of ROS generation) should block the toxicity of X / XO combination, as mentioned before. On the other hand, a metal contaminant-dependent protection by EDTA can be explained even with the failure of EDTA to protect against X / XO toxicity, since in the NADH / XO system the presence of NADH might have well led to an EDTA-inhibitable metal-mediated toxicity as mentioned before, and as will be discussed further later on.

Notice that the protective effect of EDTA was likely exerted in the extracellular compartment, for two reasons. Firstly, EDTA as mentioned before is considered a cell-impermeable chelator, so it is unlikely that it was entering the cells, especially at the

very low concentration tried, and especially that it was applied as co-treatment and was not given any time to be in contact with cells before applying the toxic insult. Secondly, it is unlikely that EDTA was producing its protective effect intracellularly, because of the observation mentioned before that XO has to be pre-incubated with EDTA in the treatment solution (before any contact with cells) for the latter to be protective.

Besides the problem of indentifying the metal responsible for mediating the production of hydroxyl radical, there is also another two problems in proposing hydroxyl radical (free in the solution) as the extracellular toxic product in NADH / XO system. First, is that hydroxyl radical is a very short-lived species, where it would degrade/disappear not far from its site of generation in the extracellular compartment. Second, is the presence of at least two hydroxyl radical scavengers in the treatment solutions, namely: HEPES (at 20 mM in HEPES-sol, and 25 mM in MEM-HEPES-sol) and glucose (at 3 mM in HEPES-sol, and 5 mM in MEM-HEPES-sol) [*Grady et al., 1988, Hicks and Gebicki, 1986, Halliwell et al., 1987, Shiraishi et al., 1993, Liochev and Fridovich, 1991, Sagone et al., 1983, Luo et al., 2001*]. Even NADH will be a target for hydroxyl radical. Also, when three hydroxyl radical scavengers (mannitol, ethanol, and DMSO) were tried as co-treatment, they failed to show any protection against NADH / XO toxicity (as they failed to protect against X / XO toxicity). However, it can be argued that the extracellularly generated hydroxyl radical in the NADH / XO system was scavenged by these scavengers, but the result of that was the production of secondary radicals (e.g. HEPES-radical and glucose-radical) where some of these secondary radicals are known to be toxic/reactive themselves [*Liochev and Fridovich, 1991, Luo et al., 2001*]. Therefore, EDTA and SOD, through blocking hydroxyl radical production, may have been protective by preventing the production of hydroxyl radical-derived secondary radicals.

If the NADH / XO toxicity was not due to an extracellular production of hydroxyl radical, an alternative possibility is that this toxicity was due to a superoxide-dependent hydrogen peroxide-accumulating free radical chain reaction which has been described in some cell-free systems that contain NADH, superoxide, and a metal (or a similar factor), where SOD (although through converting superoxide into hydrogen peroxide) paradoxically decreases (through blocking the superoxide-dependent hydrogen peroxide-accumulating chain reaction from the start) the overall production/accumulation of hydrogen peroxide. The literature abounds with reports of potentially toxic reactions that involve participation of superoxide and NADH [*Liochev*

et al., 1989, Liochev and Fridovich, 1989, 1990, 1991, Darr and Fridovich 1984, Fridovich, 1989, Chan and Bielski, 1974, Imlay and Linn, 1988, Rowley and Halliwell, 1982]. Excess of added NADH (which is a weak substrate for the used form of XO) will not be oxidized directly by the enzyme, especially in the early phase of the reaction, and could participate with superoxide (produced by the direct oxidation of NADH by XO) to produce toxicity. However, superoxide does not interact quickly with NADH at physiological pH except in the presence of a suitable mediating agent such as a metal. In such a system, the events may proceed through a series of free radical chain reactions where superoxide and the metal participate in the oxidation of a molecule of NADH, leading to a long chain process [*Liochev et al., 1989, Liochev and Fridovich, 1989, 1990, 1991, Darr and Fridovich 1984, Fridovich, 1989, Imlay and Linn, 1988*], such as the following [*Darr and Fridovich 1984, Fridovich, 1989*]:-

- superoxide + metal → metal-superoxide complex (1)

- metal-superoxide complex + NADH → NAD radical + metal + hydrogen peroxide (2)

- NAD radical + oxygen → NAD^+ + superoxide (3)

- superoxide + superoxide → hydrogen peroxide (4)

The metal regenerated in (2) and superoxide in (3) can recycle via reaction (1), propagating a chain reaction. Hydrogen peroxide may accumulate in the system because it is usually the stable molecule to which superoxide will eventually be converted (equations (2) and (4)) [*Darr and Fridovich 1984, Fridovich, 1989, Liochev and Fridovich, 1990, Misra and Fridovich, 1972, Marklund and Marklund, 1974, Heikkila and Cohen, 1973*]. The net effect of adding SOD (which will block the hydrogen peroxide-accumulating chain reaction from the start) to such reactions would then be (although through converting superoxide into hydrogen peroxide) a decrease in the overall production/accumulation of hydrogen peroxide in the system. In this situation hydrogen peroxide, not hydroxyl radical, is a major product of the interaction between superoxide, metal, and NADH (molybdenum ion is a good candidate metal for such a sequence of reactions [*Darr and Fridovich 1984*]). Also in this situation, EDTA protective effect would be due to blocking such an extracellular reaction (and not due to blocking extracellular production of hydroxyl radical). Also, may be due to the absence of such a NADH-dependent reaction in X / XO system, EDTA co-treatment failed to protect in that system, although the same metal contaminating XO was likely present in the X / XO system (since this is the same commercial preparation of XO).

More support for the possibility that the NADH / XO toxicity was due to a superoxide-dependent extracellular accumulation of hydrogen peroxide came from the observation that, in cell-free experiments, SOD-1 largely inhibited hydrogen peroxide production/accumulation in the system. This effect of SOD-1 seemed to be specific for the NADH / XO system, since it did not influence hydrogen peroxide production/accumulation in the X / XO system in these cell-free experiments. However, these assays are catalase-based, and since superoxide is known to interact significantly with catalase (inhibits it) [*Kono and Fridovich, 1982*], it is not possible to rule out other confounding mechanisms (other than the proposed mechanism which is the blocking of superoxide-dependent accumulation of hydrogen peroxide) as the reason for the observed effect of SOD-1 in this assay. The lack of effect of SOD-1 on hydrogen peroxide production/accumulation in the X / XO system in this assay does not necessarily mean that the NADH / XO system was free of the above mentioned confounding mechanisms that might involve interaction between superoxide and catalase. This is because in the X / XO system, by the time of adding catalase to the mixture (1 hr after starting the X / XO reaction), all superoxide produced would have already been spontaneously dismutated to hydrogen peroxide (since, as mentioned earlier, the superoxide generation from X / XO combination ceases by less than 10 minutes after starting the reaction), so there will be no superoxide present to react with catalase. On the other hand, in the NADH / XO system, it possible that superoxide was still being produced in the system by the time of adding catalase (i.e. 1 hr after starting the NADH / XO reaction). In any case, if the effect of adding SOD-1 to NADH / XO system was due to blocking superoxide-dependent accumulation of hydrogen peroxide in the system and not due to any other confounding mechanism, this supports the free radial chain reaction explanation for the apparently paradoxical protection by SOD against the toxicity of the NADH / XO combination. Almost exactly the same degree of inhibition of hydrogen peroxide production/accumulation in the NADH / XO system by SOD-1 was also observed in this assay when the reaction was carried out in a HEPES-free solution (Dulbecco's phosphate-buffered saline (DPBS)) instead of the HEPES-sol. This rules out that HEPES, which is know to interfere with many free radical reactions [*Grady et al., 1988, Hicks and Gebicki, 1986, Halliwell et al., 1987, Shiraishi et al., 1993, Liochev and Fridovich, 1991, Hodges and Ingold, 2000, Habib and Tabata, 2004, Kirsch et al., 1998*], was responsible for the observed effect of SOD-1 in this cell-free assay.

Notice that the failure of SOD-1 at even 300 Units/ml to influence hydrogen peroxide production/accumulation in the X / XO toxicity in these cell-free experiments (as well as its failure in the viability experiments at this high concentration to attenuate X / XO toxicity) argues against the possibility of the contamination of SOD-1 (especially at 3 Units/ml) with catalase activity.

In accord with the possibility that the NADH / XO toxicity was due to a superoxide-dependent extracellular accumulation of hydrogen peroxide is the observation that pre-treating (but not co-treating) the neurons with deferoxamine was protective. This effect of deferoxamine suggests that hydrogen peroxide is a main toxic molecule generated extracellularly in the NADH / XO system, and that it was exerting its toxicity through crossing the cell membrane and then probably reacting with an intracellular deferoxamine-sensitive iron ion to produce intracellular toxic hydroxyl radical. Also deferoxamine might have been protective through directly scavenging intracellular hydroxyl radical and/or other radicals e.g. lipid radicals [*Hoe et al., 1982, Hartley et al., 1990*]. Although deferoxamine has the ability to block peroxynitrite-mediated effects [*Bartesaghi et al., 2004*], pre-treatment with an inhibitor of nitric oxide synthase (L-NAME, 1 mM) [*Patel et al., 1996, Gunasekar et al., 1995*], aiming to block nitric oxide-mediated peroxynitrite production, failed to protect against NADH / XO toxicity, which argues against the blockade of peroxynitrite-mediated effects as the mode of protection by the deferoxamine pre-treatment. Regardless of the mechanism of NADH / XO toxicity that was inhibitable by co-treatment with SOD, Tiron, catalase, and EDTA (and by pre-treatment with deferoxamine), this toxicity was likely initiated by superoxide produced by the direct enzymatic oxidation of NADH by XO because both of the enzyme and the substrate were required and also because, as mentioned before, DPI (which blocks the site of NADH oxidation) was protective. Fig. 4-1 shows the sequence of the more likely reactions leading eventually to the toxicity of the NADH / XO combination as suggested by the available results.

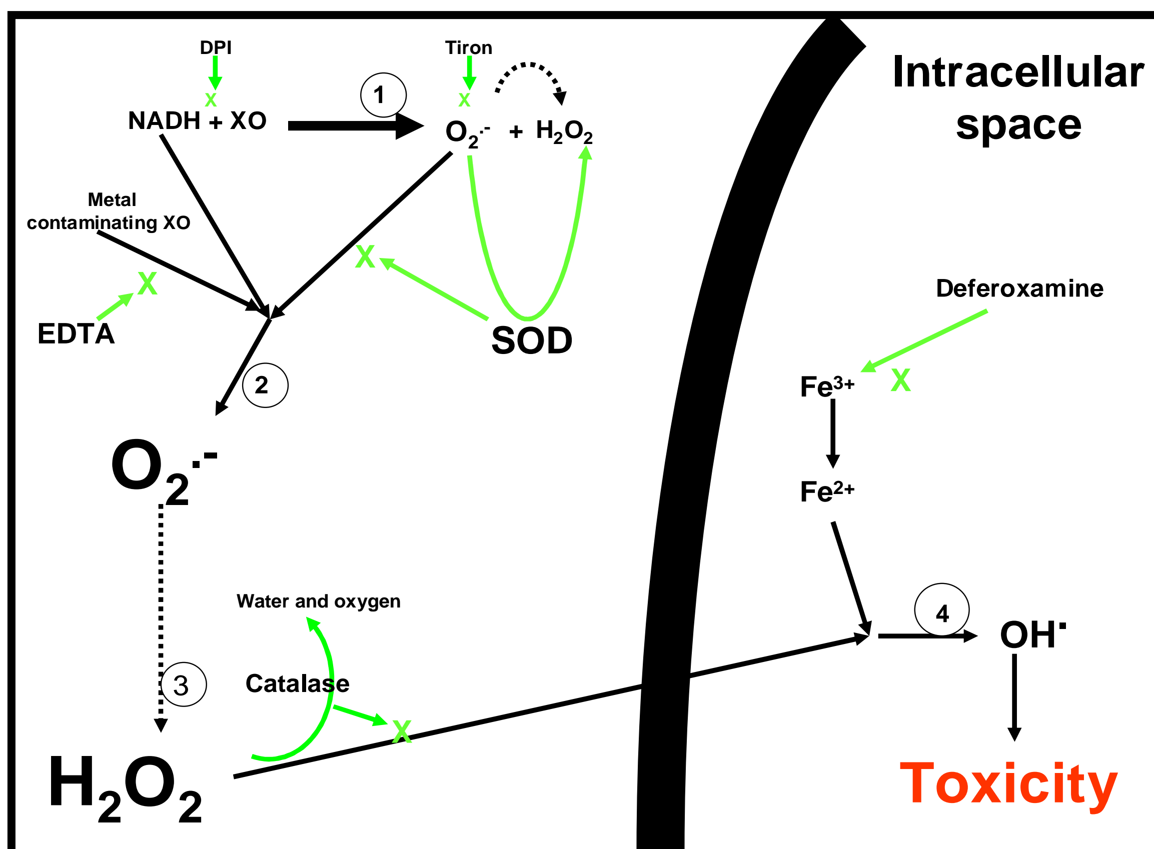

Figure 4-1: Diagram showing the sequence of the more likely events leading eventually to cell damage in the NADH / XO system, in the light of the available results. Initially, XO directly oxidizes NADH extracellularly (step 1), which can by blocked by DPI. This generates (directly) superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). Superoxide, unless participating in a faster reaction, can quickly and spontaneously dismutate to hydrogen peroxide (curved dashed black arrow). However, the results suggest that superoxide participates in a faster reaction, which is likely to be a free radical chain reaction involving (in addition to superoxide) both a metal contaminating XO (which can be silenced by EDTA) and NADH (which is likely available from the large excess of added NADH that is not directly oxidized by XO). This free radical chain reaction re-generates superoxide (in a much higher amount than the superoxide that initiates the chain reaction) (Step 2). The majority of superoxide radicals do not enter the cells, and hydrogen peroxide, the stable form to which all superoxide does eventually convert, accumulates in the system (Step 3). SOD, through its very efficient deactivation of superoxide can block (although through converting superoxide into hydrogen peroxide) the chain reaction from the start, and hence can prevent the accumulation of hydrogen peroxide in the system (which is supported by the cell-free experiments). Also, Tiron, through scavenging superoxide, can prevent it from initiating the chain reaction. Catalase, through deactivating hydrogen peroxide once produced in the system, can prevent its production/accumulation, and hence can prevent it from entering the cells. The final step in the toxicity is that hydrogen peroxide readily crosses the cell membrane, where it participates with intracellular iron ion (Fe^{2+}) to generate the very reactive and toxic hydroxyl radical ($OH^{\cdot}$) (Step 4), which can be blocked by pre-treatment (but not co-treatment) with deferoxamine.

Interestingly, as in the case with NADH / XO toxicity, pre-treatment (and not co-treatment) with deferoxamine was protective against X / XO toxicity. This suggests that hydrogen peroxide was a main toxic molecule produced extracellularly in the X / XO system (as in the NADH / XO system) that was crossing the cell membrane and exerting intracellular iron-mediated toxicity. However, hydrogen peroxide production in the X / XO system was likely occurring through a mechanism (mainly direct production from

the combination) different from the mechanism of its production in the NADH / XO system (likely through a superoxide-, NADH-, and metal-dependent free radical chain reaction), since SOD, Tiron, and EDTA co-treatments protected against the toxicity of the latter but not the former system. In addition to the protection found by pre-treatment with deferoxamine, X / XO toxicity was similar to NADH / XO toxicity in the failure of the pre-treatment with L-NAME to provide protection, which also argues against the blocking of intracellular peroxynitrite-mediated effects as the reason for the protection of deferoxamine pre-treatment against this combination (see later for further investigation of X / XO toxicity).

4.3.1.3 Feasibility of in vivo toxicity of NADH oxidation by XOR

The intracellular concentrations of free NADH are reported to be in the micromolar range [*Yu and Heikal, 2009*]. However, there seems to be uncertainty regarding the concentration of intracellular free NADH, which might have been due to the difficulty in measuring this concentration [*Canelas et al., 2008*]. The uncertainty also extends to the ratio of free to bound intracellular NADH. For example, Vishwasrao and co-workers (2005) suggested that this ratio might be higher than previously estimated, and that as much as 40% of NADH might be present free intracellularly. It is feasible that the oxidation of NADH by some forms of XOR (other than the one used here) that are known to be very efficient in oxidizing this substrate might produce in vivo toxicity, as suggested previously [*Berry and Hare, 2004, Harrison, 2002, Sanders et al., 1997, Zhang et al., 1998*].

In adding the NADH / XOR combination to cells in vitro (like in this study), the oxidation of NADH by XOR will likely take place in the extracellular compartment, whereas lower concentrations of NADH might cause toxicity if oxidized by intracellular XOR (because lower levels of ROS are expected to cause toxicity if produced intracellularly in the vicinity of critical targets rather than extracellularly). Also, in cell cultures, many cells die in the first hours of plating (as in this study), where these might be those cells that can not resist the culturing stress. Hence, in vivo, those cells that are less resistant to stress/toxicity (which will die immediately if plated in vitro) might be damaged by lower concentrations of an insult than if applied in vitro. Also, it is possible that many compensatory protective mechanisms that might be induced in vitro in response to the culturing stress (that allow many cells to survive) do not operate in vivo,

where cells can be killed by the oxidation (by XOR) of lower NADH concentrations than if applied in vitro. However, it can be argued for an opposite possibility, where in vivo tissues might be better equipped with protective mechanisms (e.g. they have richer antioxidant environment) to cope with insults than cells in vitro [*Halliwell, 2003*], and hence higher concentrations of toxic insults might be required to kill cells in vivo than if applied in vitro.

An important fact that might limit the significance of the oxidation of NADH by XOR in vivo is the presence of NAD^+ in much higher concentrations than NADH, where the free cytoplasmic $NADH/NAD^+$ ratio was reported to be very low (< 0.01) [*Canelas et al., 2008, Sanders et al., 1997, Park et al., 1998*]. NAD^+, as mentioned before, can potently inhibit NADH oxidation by the predominant intracellular isoform of XOR i.e. XDH (but notice that NAD^+ is weak in inhibiting XO compared to XDH). However, NAD^+ can not completely inhibit NADH oxidation by XDH, since a previous cell-free study has shown that XDH is still able to oxidize NADH to produce ROS even in the presence of high concentrations of NAD^+ [*Harris and Massey, 1997*].

It is possible that the toxicity of NADH oxidation by XOR might occur/increase in some pathological situations where NADH levels are known to increase on the expense of the decrease of NAD^+ levels. For example, under severe ischemic conditions, the cytoplasmic $NADH/NAD^+$ ratio in the heart was shown to increase approximately 30-fold [*Park et al., 1998*]. In such a situation, a significant toxicity might be produced from the oxidation of NADH by the predominant intracellular isoform i.e. XDH. Also, even if NAD^+ concentration is so high to the degree that it can potently inhibit ROS generation by XDH, it is known that under some pathological situations the XO level increases either by the conversion from XDH or by the increase in the expression/activity of the total enzyme i.e. XOR [*Wiezorek, 1994, Phan et al., 1989, Osarogiagbon et al., 2000, Thom, 1992, Schröder et al., 2006, Ischiropoulos et al., 1996, Park et al., 1998, Berry and Hare, 2004, Harrison, 2002*]. In such a situation, XO might significantly oxidize NADH (where NAD^+ is relatively weak in inhibiting this oxidation) which might lead to significant toxicity.

Although there might not be a significant amount of NADH present extracellularly in vivo, NADH is used in clinical trials as a therapeutic drug to treat some chronic illnesses e.g. Parkinson’s disease, Alzheimer’s disease, and chronic fatigue syndrome

[*Birkmayer et al., 1993, Forsyth et al., 1999, Rex et al., 2004*], where administering it will increase greatly its availability in the circulation. Since XOR is present predominantly in the XO form in the circulation, a toxic effect might arise under some circumstances from the oxidation of the administered NADH by XO (e.g. in some acute pathological situations where XO levels are reported to increase by hundreds-fold [*Harrison, 2002*]), especially since there might not be significant amount of NAD^+ present in the circulation to inhibit this oxidation (anyway, NAD^+ is a weak inhibitor of the XO form).

Although this investigation of NADH / XO toxicity suggests that it was dependent on a XO-contaminating metal ion, this does not mean that this type of toxicity is irrelevant to the in vivo situations. It is known that, at least in some pathological conditions, traces of reactive metals are present in vivo either free or bound/chelated to certain molecules/proteins, where this binding may not prevent the reactivity of these metals (actually it may enhance their reactivity/toxicity in some situations) [*Graf et al., 1984, Hutcheson et al., 2004, Engelmann et al., 2003, Gutteridge, 1987, Sayre et al., 1999, Ong and Halliwell, 2004, Thompson, 2001, Darley-Usmar and Halliwell, 1996, Halliwell and Gutteridge, 1992, Halliwell, 2006*]. Moreover, XO was shown to contain a high affinity binding site for iron, where contaminating iron (as the one proposed in this study) can bind to it, and it was shown that, as mentioned before, it is difficult to eradicate this contaminant iron if present (such a contaminant iron will be different from the iron atoms that are integral parts of XO). This has led some authors to discuss the possibility that this binding site on XO might be occupied by an exogenous iron ion in vivo, which can catalyse toxic reactions [*Vile and Winterbourn, 1986, Britigan et al., 1990*].

The results suggest that the toxicity of NADH / XO combination was initiated by superoxide generated from the direct enzymatic oxidation of NADH by XO (see before). However, the results also suggest that it is possible for an in vivo toxic interaction to occur between NADH and XOR even in the absence of direct oxidation of NADH by XOR. That is, in a situation where XOR produces superoxide through oxidizing X (or HX), a toxic effect can result from the participation of this produced superoxide, NADH, and a metal in a free radical chain reaction (or other toxic reactions) even in the absence of direct oxidation of NADH by XOR. In such a situation, low concentrations of NADH might be enough to cause the toxicity (since the weak oxidation of NADH by some forms of XO will not be a factor in the availability of superoxide, and also since the inhibition of NADH oxidation by XOR (especially XDH)

by NAD^+ will not be a factor in the availability of superoxide). Therefore, in vivo, NADH can react (in the presence of a suitable free or chelated metal e.g. iron, copper, etc.) with superoxide (generated by NADH, X, or HX oxidation by XOR) to cause a toxic effect.

In summary, the toxicity of NADH oxidation by XOR seems to be feasible in vivo. The results in this project can not prove or disprove this feasibility. However, if the toxicity of NADH oxidation by XOR does occur in vivo (which is likely to be the case), then the results in this project provide some suggestions on what might be the nature of the toxic ROS/metals that mediate this in vivo toxicity. Indeed, proving this in vivo toxicity would require in vivo studies. However, it will be difficult to prove or rule out this toxicity, since the NADH binding site on XOR (the FAD site), unlike the X binding site (the Mo site), still has no specific in vivo blockers. Although DPI can block this FAD site in vivo, this inhibitor is not specific and can inhibit many other enzymes [*Harrison, 2002, Berry and Hare, 2004*]. Therefore, there is a need for developing in vivo specific blockers of this site, especially since, as suggested by this cell-containing and previous cell-free studies, blocking the Mo site is unlikely to block a toxicity of NADH oxidation by XOR, and also since blocking the FAD site can inhibit ROS generated by either X or NADH oxidation by XOR. Also, in investigating this toxicity in vivo, it should be considered that toxicity might result from an indirect interaction between NADH and XOR.

4.3.2 Further investigation of the X / XO toxicity model

In addition to investigating the toxicity of the NADH / XO combination (which was rarely investigated in previous studies) and comparing it to the well investigated toxicity of the X / XO combination, another aim of this project was to address specific questions regarding those aspects of X / XO toxicity where there are uncertainties about them (see the section on Aim/Objectives in the Introduction). Some of these questions were addressed (at least partially) in the previous section (e.g. the possibility that XO is contaminated with iron/metal).

4.3.2.1 Role of intracellular superoxide in X / XO toxicity

The results in this and many previous studies suggest that, as discussed, superoxide generated from the X / XO combination in the extracellular compartment has no role in the toxicity of this combination (may be apart from its extracellular spontaneous dismutation to hydrogen peroxide). However, some previous reports showed that intracellular superoxide production mediates the toxicity of hydrogen peroxide that is either produced extracellularly from the X / XO combination or applied directly, though this was demonstrated in cell culture types other than CGNs [*Ito et al., 1992, Kyle et al., 1988, Hiraishi et al., 1994*]. To test this possibility in CGNs, the superoxide scavenger, Tiron (which failed to protect against X / XO toxicity when applied as a co-treatment at 50 μM, as mentioned before), was tried here as a pre-treatment for 3 hr at 2 mM, aiming to give it a chance to get inside the neurons in high amount. With this experimental design, Tiron was able to protect against X / XO toxicity, which suggests the involvement of intracellular superoxide, especially since the Tiron-containing treatment solution was removed before applying the toxic insult. However, Tiron is also known to be an effective chelator of some metals including iron and molybdenum [*Fridovich and Handler, 1962*], an activity that can not be ruled out as the reason for its intracellular protective effect.

An attempt was also undertaken to inhibit intracellular SOD-1 by diethyldithiocarbamate (DDC, a potent and cell-permeable inhibitor of intracellular SOD-1 [*Ito et al., 1992, Hiraishi et al., 1994, Blum and Fridovich, 1983, Benov and Fridovich, 1996*], but of low specificity), where this inhibition was expected to potentiate the toxicity of the X / XO combination if intracellular superoxide is involved. Although DDC is not very specific in inhibiting intracellular SOD-1, it is likely that its potentiation of hydrogen peroxide-dependent toxicity in at least some of previous studies was indeed due to inhibiting intracellular SOD-1, for many reasons. Firstly, Hiraishi and co-workers (1994) showed that the potentiation of hydrogen peroxide toxicity closely paralleled its ability to inhibit intracellular SOD-1. Secondly, DDC was not found to inhibit some other intracellular antioxidant enzymes e.g. catalase, glutathione peroxidase, or glutathione reductase [*Ito et al., 1992, Hiraishi et al., 1994, Blum and Fridovich, 1983*]. Thirdly, Hiraishi and co-workers (1994) showed that DDC did not potentiate some other types of toxicity that are not known to be dependent on hydrogen peroxide/superoxide, and only potentiated hydrogen peroxide-dependent toxicity.

Fourthly, Benov and Fridovich (1996) showed that the potentiation of the toxicity of an oxidative stress model by DDC was reversed by a cell-permeable SOD mimetic, suggesting that DDC potentiated the toxicity through inhibiting intracellular SOD-1.

There was no signficant potentiation of X / XO toxicity by DDC in this study. There was no time left in this project to try higher concentrations or longer incubation times of DDC (a previous study showed that increasing the pre-treatment time of DDC from 1 to 2 hr resulted in a very significant decrease in the activity of intracellular SOD-1 [*Ito et al., 1992*]). Although the failure of DDC to potentiate X / XO toxicity might suggest that intracellular superoxide was not involved, this will be difficult to explain in the light of both the protection found by Tiron pre-treatment and the results of previous studies. DDC is known to interact with XO (it can be oxidized initially by the enzyme, but the product will inhibit the enzyme) [*Fried, 1976, Kober et al., 2003*], and although this is unlikely to influence the activity of our added XO (since DDC and XO were not present together), DDC might affect the activity of intracellular XOR. However, it is not clear if this can explain the failure of DDC to potentiate X / XO toxicity. Fig. 4-2 shows the sequence of the more likely events leading eventually to cell damage in the X / XO toxicity model based on the available results.

The possibility raised by this study (and supported by the previous studies quoted earlier) that intracellular superoxide is involved in X / XO toxicity, suggests that the failure of SOD co-treatment (and Tiron co-treatment) to protect was due to both the failure of SOD to cross the cell membrane (and hence its failure to block the toxicity mediated by intracellular superoxide) and also the failure of superoxide generated extracellularly from X / XO combination to cross the cell membrane (and hence its failure to increase the pool of intracellular superoxide that was mediating the toxicity). Therefore, in vivo, in XOR-related disorders, the oxidation of substrates by XOR, whether takes place intracellularly or extracellularly, might produce a toxicity that can be mediated by intracellular superoxide. This validates targeting superoxide in investigating/treating disorders where XOR is suspected to a play a role.

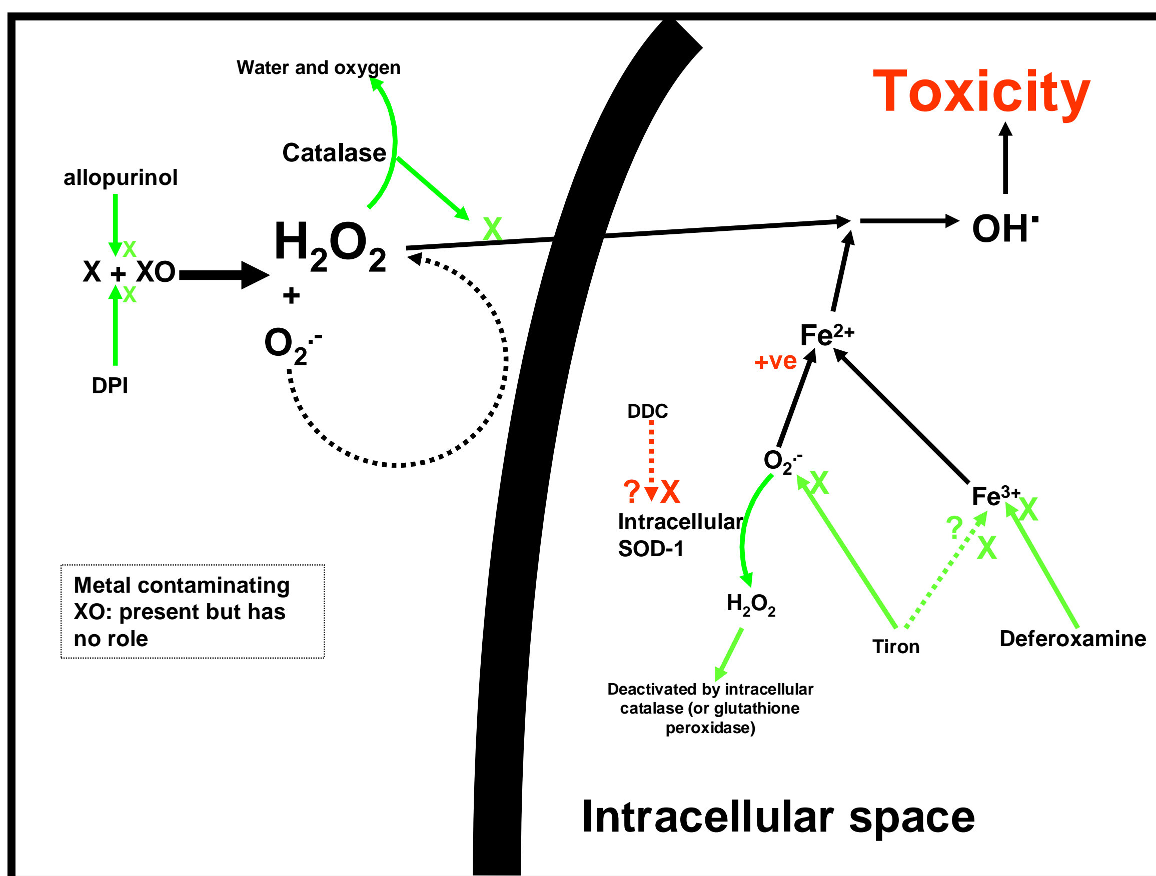

Figure 4-2: Diagram showing the sequence of the more likely events leading eventually to cell death in the X / XO system, in the light of the available results. Initially, XO directly oxidizes X extracellularly, which can be blocked by blocking the Mo site on XO with allopurinol, and also probably by blocking the FAD site with DPI. This oxidation directly produces ROS i.e. hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) as co-products in this process. The majority of ROS produced directly will be in the form of hydrogen peroxide (80%), while the remaining will be in the form of superoxide (20%). Most of the directly produced superoxide radicals do not enter the cells, and they spontaneously dismutate to hydrogen peroxide in the extracellular compartment. Hydrogen peroxide produced directly from XO and also from the spontaneous dismutation of superoxide readily crosses the cell membrane to cause intracellular toxicity, where externally added catalase can deactivate hydrogen peroxide before it can enter the cells. Notice that although the metal contaminating XO that was playing a role in NADH / XO toxicity was likely present in this system (since this is the same commercial preparation of XO), this metal has no role in X / XO toxicity. In the intracellular space, hydrogen peroxide participates with intracellular reduced iron ion (Fe^{2+}) to produce the very reactive and toxic hydroxyl radical (Fenton reaction), which can be blocked by pre-treatment (but not co-treatment) with deferoxamine. Intracellular superoxide mediates this intracellular toxicity of hydrogen peroxide through increasing the availability of the reduced form of iron as shown (→+ve) (or by other mechanisms), which can be blocked by pre-treatment (but not co-treatment) with the superoxide scavenger, Tiron (though Tiron is also known to be an effective chelator of some metals including iron, which might be an alternative explanation of its protection, as shown). Intracellular SOD-1 limits this toxic action of superoxide through dismutating it to hydrogen peroxide, where this small amount of hydrogen peroxide is deactivated by the intracellular hydrogen peroxide-deactivating enzymes. Therefore, inhibiting intracellular SOD-1 may result in the potentiation of the toxicity, but although this potentiation of toxicity by inhibiting intracellular SOD-1 was demonstrated in previous studies by using DDC, this was not found here (since DDC effect failed to reach statistical significance in the viability assay).

4.3.2.2 Role of intracellular hydroxyl radical in X / XO toxicity

The results with Tiron pre-treatment, deferoxamine pre-treatment, and catalase co-treatment (and also the results of many previous studies [*Satoh et al., 1998, Link and Riley, 1988, Mohsen et al., 1995, Duell et al., 1995, Hiraishi et al., 1987, Zigler et al., 1985*]) suggest that the toxicity of X / XO combination was due to hydrogen peroxide generated in the extracellular space and then entering the neurons and participating in a Fenton reaction with an intracellular iron (where this reaction was likely mediated by intracellular superoxide) to produce the very reactive and toxic hydroxyl radical (or a similar species). The failure of co-treatment with the hydroxyl radical scavengers (mannitol, ethanol, and DMSO) mentioned earlier, although might argue against the involvement of extracellular hydroxyl radical in X / XO toxicity, does not necessarily mean that intracellular hydroxyl radical was not involved. Therefore, the idea was to investigate the involvement of intracellular hydroxyl radical through using different scavengers of it as pre-treatment (to give them a chance to accumulate inside the cells) before applying X / XO combination.

Initially, a compound called POBN, which has the ability to scavenge hydroxyl radical (but also many other free radicals) was tried [*Mottley et al., 1986, Pérez and Cederbaum, 2001, Reinke et al., 1994*]. POBN is a member of a large group of compounds called spin traps, which are used essentially as detectors of free radicals, where a spin trap reacts with a free radical (e.g. hydroxyl radical) to produce a new species (a more stable secondary radical) that can be detected by a method called electron paramagnetic resonance spectroscopy [*Reinke et al., 1994, Tarpey and Fridovich, 2001, Halliwell and Whiteman, 2004*]. In theory (which was also shown in some viability studies), since these detect free radicals by scavenging them, they might protect tissues from insults that involve generation of toxic free radicals. However, when the neurons were pre-treated here with POBN at 20 mM for 1hr, instead of providing protection, it significantly potentiated X / XO toxicity. Surprisingly, when POBN was applied as co-treatment rather than pre-treatment, it produced the opposite effect, showing significant protection against X / XO toxicity.

The opposite effects exerted by co-treatment and pre-treatment with POBN can not be easily explained. Therefore, the following discussion is mostly speculative. The potentiation of the toxicity by the POBN pre-treatment might have been due to the

accumulation of POBN inside the cells (since the POBN-containing treatment solution was removed before applying the toxic insult). Although intracellular POBN was not toxic itself (since pre-treatment with POBN alone was not toxic), it was able to potentiate the toxicity of the X / XO combination applied afterwards. This intracellular toxic effect of POBN might have or have not been due to free radicals (e.g. hydroxyl radicals) spin-trapping. If it was due to spin-trapping a free radical, it is possible that a species produced from spin-trapping the free radical by POBN (likely to be a secondary radical) was more toxic than the free radical being spin-trapped itself. This can happen e.g. some secondary radicals produced from scavenging hydroxyl radical were shown/proposed to be more toxic than (or at least as toxic as) hydroxyl radical itself, may be because they have longer half lives and/or more lipid solubility [*Luo et al., 2001, Liochev and Fridovich, 1991*]. For such a possibility, some authors have warned against overlooking the effects of secondary radicals produced in studies that use scavengers of hydroxyl radical (or other radicals) as a therapeutic means [*Liochev and Fridovich, 1991*]. Another fact that suggests that trying to directly scavenge free radicals may not be a good way to treat oxidative stress-related disorders is that, as mentioned in the Introduction, a free radical (especially hydroxyl radical) is generally non selective in its reactions, so to scavenge it, a scavenger needs to be applied in a very high concentration in order to outcompete the many vulnerable biological targets (i.e. scavengers) of the free radical. A better way is to prevent the generation of free radicals (for example by using metal chelators).

If the potentiation of toxicity by intracellular POBN was not due to free radical spin-trapping, an alternative possibility is that it might have been due to its known ability to reduce Fe^{3+} to Fe^{2+} [*Reinke et al., 1994*]. This possibility means that POBN in the intracellular compartment was catalysing the same or a similar reaction to that POBN was supposed to scavenge its toxic product i.e. Fenton reaction.

The opposite effect found by POBN co-treatment (i.e. protection) is also difficult to explain, even if it is assumed that it was exerted extracellularly. Since POBN applied as co-treatment (but not pre-treatment) was present together with the X / XO combination, one explanation for its protection is that it was inhibiting XO. However, this explanation is ruled out by a previous observation that POBN even at 100 mM does not significantly influence XO activity [*Britigan et al., 1991*]. Also, it is unlikely that the protection with POBN co-treatment was due to spin-trapping superoxide radicals generated

extracellularly from X / XO combination because POBN was shown to be slow in reacting with superoxide [*Britigan et al., 1991*], and also because it was shown in this and many previous studies that extracellular superoxide is not involved in X / XO toxicity. Moreover, it is unlikely that the protection by POBN co-treatment was due to scavenging hydrogen peroxide generated in the system because POBN does not interact significantly with hydrogen peroxide [*Britigan et al., 1991*]. Moreover, the hydroxyl radical scavengers (mannitol, DMSO, and ethanol) tried here as co-treatments had no effect on the toxicity, as mentioned before, arguing against spin-trapping extracellular hydroxyl radicals as the reason for protection by POBN co-treatment. Alternatively, one might assume that POBN (applied as co-treatment) was exerting its protective effect intracellularly, but this is even more difficult to explain, since POBN pre-treatment potentiated the toxicity.

If additional spin-traps (or hydroxyl radical scavengers) were used (especially as pre-treatment), this would have given both an explanation for the observed effects of POBN and more verification of the proposed role for intracellular hydroxyl radical in X / XO toxicity (see later for suggestions for future studies).

In summary, although it is likely that intracellular hydroxyl radical (or a similar species) is involved in X / XO toxicity, the preliminary studies did not support or argue against trying to directly scavenge hydroxyl radicals as a possible means to prevent this toxicity. However, it seems from the preliminary studies (and from the nature of hydroxyl radical) that it is better to prevent the generations of hydroxyl radicals than to try to directly scavenger it. This might also be true for some other radicals. This might explain the failure of some direct free radicals scavengers in clinical trials, especially since in clinical trials the scavengers are usually administered after the onset of the attack (e.g. stroke) where the targeted radical might have long been produced and probably caused its toxic reactions before the drug was able to reach to the damage site. However, this might be the only feasible way to treat free radicals-induced damage in the clinical situations, since preventing the generation of free radicals might not be feasible. However, it is possible that both trying to prevent the generation of certain free radicals and even trying to directly scavenge them might produce beneficial effects in human diseases where the oxidative stress damage is chronic e.g. Parkinson’s disease.

4.4 Future work

Generalization is difficult in cell culture studies, so some of the observations found here might be different under different experimental conditions. Therefore, to obtain more generalization, more verification of the experimental conditions is suggested. In particular, since the results suggest that a metal contamination of XO was involved in NADH / XO toxicity, future studies should investigate more the likely contamination of XO by a metal, which can be achieved through pursuing two lines of investigations. The first is using many preparations of the bovine milk XO (purchased from different sources), where if EDTA protects against the toxicity of all these preparations, it will be unlikely that all were contaminated with metals, and this would suggest other alternative mechanisms for EDTA protection. The second is screening various metal chelators (in addition to EDTA) that have different selectivity for metals against NADH / XO toxicity. Pursuing these two lines of investigations will hopefully confirm or rule out a role for metal contamination of XO in this toxicity.

Also, to have more insights into the NADH / XOR toxicity, it would be very helpful if some other forms of XOR (that are known to be much more efficient in oxidizing NADH than the bovine milk XO used here) were used and compared with each other.

Another suggestion is to exclude HEPES buffer from the treatment solutions (it was present in both HEPES-sol and MEM-HEPES-sol). HEPES has the ability to interfere with many free radical reactions, as mentioned before, so it is probable that some of the reactions here might have been mediated, inhibited, or diverted by the presence of HEPES. The only experiment where the effect of HEPES was tested was the cell-free experiment that investigated the effect of SOD-1 on hydrogen peroxide production/accumulation in the NADH / XO system, where the same result was obtained in the presence and absence of HEPES (see earlier). In future viability studies, it is better to try different buffer solutions (and different media), since no one seems to be ideal.

The results of this and many previous studies suggest that intracellularly (but not extracellularly) produced superoxide is involved in X / XO toxicity. For future work, there are two suggestions. The first is to confirm the role of intracellular superoxide in X / XO toxicity in CGNs by trying cell-permeable SOD mimetics (e.g. MnTBAP [*Patel*

et al., 1996]), and also by trying a better way of assessing the role of intracellular SOD-1 than using the SOD-1 inhibitor, DDC, tried here (e.g. knocking out the SOD-1 gene). The second suggestion is to investigate the probability that intracellular superoxide also mediates the toxicity of NADH / XO combination as it is the case with X / XO combination (since in both systems, extracellularly produced/accumulated hydrogen peroxide is a main toxic molecule, where intracellular superoxide was shown to mediate such a toxicity).

Since the results of this and previous studies suggest the involvement of intracellular hydroxyl radical (or a similar species) in the X / XO toxicity, there are two suggestions for future studies. First, since the preliminary experiments in this study did not show that the X / XO toxicity can be prevented by directly scavenging hydroxyl radical, this can be investigated by using many spin-traps and hydroxyl radical scavengers, especially as pre-treatment. Second, this investigation can be extended to the NADH / XO toxicity, since intracellular hydroxyl radical is likely to be involved in the toxicity of that system as well.

Investigating the effects of uric acid (which is produced from the oxidation of X by XO) on X / XO toxicity might produce interesting results, which could also have significant in vivo implications. Uric acid is known to produce both protective and detrimental effects [*Feig et al., 2008, Dimitroula et al., 2008*]. One way to do that is through comparing the toxicity of the X / XO combination with the toxicity of the acetaldehyde / XO combination, since although the substrate oxidation in the two systems takes place at the Mo site [*Simon et al., 1981*] which leads to ROS production at the FAD site, the oxidation of acetaldehyde by XO will not produce uric acid. Substrates other than acetaldehyde that can bind to the Mo site but their oxidation does not yield uric acid can also be tried.

Finally, broader avenues of research that can be followed include investigating the interplay of XOR toxicity with other toxic pathways. There are two interesting examples. First, it was mentioned in the Introduction that intracellular XOR, through a non clear mechanism, augmented the toxicity of the endogenous toxic metabolite, 3-Hydroxykynurenine (3-HK), when the latter was applied to neuronal cultures. Therefore, it will be interesting to characterize the mechanism of this toxic augmentation. Also, since 3-HK is just one of many products of a large metabolic

pathway, the kynurenine pathway, it will be interesting to investigate the interplay of XOR with this pathway, especially since this pathway is known to be intimately connected to oxidative stress [*Stone and Darlington, 2002*]. Second, since an augmentative interplay in neurons between glutamate receptor-dependent excitotoxicity and some oxidative stress components was demonstrated previously, as mentioned in the Introduction, and also since blocking the glutamate NMDA-receptors was shown to inhibit X / XO toxicity [*Satoh et al., 1998*], it will be interesting to further characterize the interplay between X / XOR (and NADH / XOR) toxicity and the glutamate system in CGNs.

5 Conclusions

A: Conclusions regarding culturing/experimental conditions

Some culturing/experimental optimizations were found to both improve the status of the cultures and increase the reliability of the viability experiments in CGNs cultures. These include: **1)** either a serum-free medium or a conditioned medium (i.e. glutamate-free serum-containing medium), but not fresh serum-containing medium (which will contain glutamate), should be used as the vehicle to add test compounds. This is because glutamate already present in the fresh serum-containing medium can cause severe toxicity to CGNs; **2)** for the same reason, conditioned medium can be used as the medium to which the neurons are restored at the end of the treatment period. In this regard, conditioned medium is preferred to serum-free medium, since although the latter has the advantage of being free of glutamate it might cause damage to the neurons if they are left in it withdrawn from serum (which might be necessary for the viability of cells) for a prolonged restoration period (like the 16-24 hr applied here); **3)** the edge wells in a 96-well plate should not be included in viability experiments in CGNs cultures, since these will likely be affected by the edge effect; **4)** if Alamar blue assay is being used to assess the viability of CGNs, in addition to the edge wells, also the next-edge wells should not be included in the experiments, and only inside wells should be used. This is because it was found here that the neurons in next-edge wells give consistently slightly higher viability readings in the Alamar blue assay than the neurons in the inside wells, where although these differences are relatively small, they might lead to misleading conclusions. In this regard, edge wells and next-edge wells should not be left blank, but a cell-free medium can be added to them; **5)** if a treatment medium uses bicarbonate/CO_2 as a buffering system, a harmful rise in the pH can easily occur (due to the release of CO_2), and to overcome this problem, such a medium can be placed in a vented-cap flask (i.e. permeable to gases) and placed in a CO_2-incubator, and retuned to this incubator immediately after each usage.

B: Conclusions regarding investigating XO toxicity

1- The combination of NADH and the bovine milk XO induces damage to CGNs. It is feasible that the oxidation of NADH by some forms of XOR (other than the one used here) that are known to be very efficient in oxidizing NADH might produce in vivo toxicity. However, it will be difficult to prove or rule out this toxicity, since the NADH binding site on XOR (the FAD site), unlike the X binding site (the Mo site), still has no specific in vivo blockers. Although DPI can block the FAD site in vivo, this inhibitor is not specific and can inhibit many other enzymes [*Harrison, 2002, Berry and Hare, 2004*]. Also, blocking the Mo site with allopurinol did not prevent NADH / XO toxicity in this study, which is in agreement with previous cell-free studies, which might have therapeutic implications. Therefore, there is a need for developing in vivo specific blockers of the FAD site, especially since, as suggested by this cell-containing and previous cell-free studies, blocking the FAD site can inhibit ROS generation regardless of whether the reducing substrate binds to FAD site (i.e. NADH) or to the Mo site (i.e. X or HX).

2- A possibility raised by this study is that a metal (like the one proposed to contaminate XO used in this study) might contribute to XOR toxicity in vivo, where such a metal might either potentiate a toxicity induced by XOR directly oxidizing the substrate or mediate an indirect interaction between XOR and the substrate. For example, in vivo, in cases where superoxide is generated by a direct oxidation of X (or HX) by XOR, a toxic effect can result from the participation of this produced superoxide, NADH, and a metal in a free radical chain reaction (or other toxic reactions) even in the absence of a direct oxidation of NADH by XOR.

3- Superoxide often mediates XOR toxicity, and the failure of SOD to prevent X / XO toxicity in cell cultures does not necessarily rule out a role for superoxide. This is because in many cases in cell culture studies, SOD might not be able to enter the cells, where intracellularly generated superoxide (it does not need to be generated from intracellular XOR) can mediate the toxicity of hydrogen peroxide generated extracellularly from the X / XO combination. This means that in vivo, superoxide can mediate the toxicity of XOR when oxidizing substrates either extracellularly or intracellularly.

4- The results add support to many previous studies which suggested that intracellular hydroxyl radical (or a similar species) is involved in XOR toxicity. However, the preliminary experiments did not support or argue against directly scavenging hydroxyl radical as a possible means to prevent this toxicity. However, it seems from the preliminary experiments (and from the nature of hydroxyl radical) that it is better to prevent its generation than to try to directly scavenge it.

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