The role of stress and dietary micronutrients in fish health

by

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A thesis submitted to the University of Plymouth

in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

(October 2008)

Department of Biological Sciences

Faculty of Science

in partnership with the Waltham Centre for Pet Nutrition

Word Count: 35,795

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ABSTRACT

The overall theme of this thesis has been the study of the effects of well known and potentially novel stressors on fish health and how some of these may be modulated by dietary supplements of micronutrients, using both *in vitro* and *in vivo* analysis. *In vitro* experiments with cultured fish cells (EPC-A1, GFSk-S1) evaluated the potential of niacin and selenium to reduce cytotoxicity and genomic instability (DNA damage) induced by ultraviolet radiation exposure, following assay validation with reference toxins. Whilst cytotoxicity was determined by the neutral red retention (NRR) assay, genomic stability was evaluated by either a standard or modified version of the single cell gel electrophoresis (SCGE) or 'Comet' assay. Niacin as nicotinamide (NAM) significantly reduced levels of UVB induced DNA damage (single strand breaks). Selenium supplements, as sodium selenite or seleno-L-methionine also showed a protective effect against H_2O_2, UVA and UVB induced cytotoxicity and oxidative DNA damage. An additional *in vitro* study was carried out to identify environmental nanoparticles as a potential novel source of stress for fish. Titanium dioxide (TiO_2) nanoparticle exposure to GFSk-S1 cells caused dose-dependent increases in cytotoxicity (NRR assay) and oxidative DNA damage (Comet assay). These effects were exacerbated by combined exposures of TiO_2 with UVA. Electron spin resonance (ESR) and spin trapping suggested that TiO_2 induced oxidative stress may be primarily due to the production of hydroxyl radicals (OH).

Two *in vivo* experiments were carried out in order to evaluate the effects of husbandry stress (netting and confinement stress) on antioxidant capacity, immune function and genomic stability in trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) . In both studies on trout and carp, certain health parameters were shown to be sensitive to husbandry stress, and may be useful biomarkers of stress in future studies. Total antioxidant capacity (TAC) was reduced in both carp and trout after stress. Respiratory burst capacity of blood leukocytes was also affected by stress but differently in carp than in trout. In carp, husbandry stress apparently stimulated the production of free radicals by leukocytes whereas in trout it was suppressed. In trout,

stress was also shown to increase oxidative DNA damage, as measured by the Comet assay. Health parameters that appeared not to be significantly affected by husbandry stress in this study include SOD activity, ALP activity, red blood cell fragility and complement activity (for carp only).

In the second experiment on carp, a 10 week feeding trial was also conducted prior to stress induction to evaluate the potential modulatory effects of selenium, zinc and vitamin E on any husbandry induced stress effects observed. Health parameters were also evaluated pre stress in order to elicit any effects of micronutrient supplementation on health parameters in unstressed fish. Selenium supplementation significantly increased glutathione peroxidase activity post stress, but not pre stress, indicating an increased requirement of selenium in stressed fish. No other differences were observed between dietary treatments for any parameter measured either pre stress or post stress, indicating that levels of selenium, zinc and vitamin E were probably sufficient in the control diet.

Further work is needed to enhance our understanding on the interaction between, and the role of, stress and dietary micronutrients in fish health using a suite of assays to monitor all aspects of fish health, both *in vitro* and *in vivo*.

All experimental work involving animals was carried out under the Home Office project licence #30/2135 and personal licence #30/7213

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LIST OF ABBREVIATIONS

2-SeCD	2-selenium-bridged β-cyclodextrin
8-OHdG	8-hydroxydeoxyguanosine
ABEL	analysis by emitted light
ACH50	alternative complement activity units
ADP	adenine dinucleotide phosphate
ALP	alkaline phosphatase
ALS	alkali-labile sites
BER	base excision repair
$BW.d^{-1}$	body weight per day
CO_2	carbon dioxide
CPD	cyclobutane pyrimidine dimers
DEPMPO	5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide
dH_2O	distilled H_2O
DMPO	5,5-dimethyl-1-pyrroline-*N*-oxide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DO	dissolved oxygen
ECACC	European Collection of Cell Cultures
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbant assay
EMS	ethyl methanesulphonate
EPC	*Epithelioma Papulosum Cyprini*
EPR	electron paramagnetic resonance
ESR	electron spin resonance
ESS	enzyme sensitive sites
FAAS	flame atomic absorption spectroscopy
FAO	United Nations Food and Agriculture Organisation
FBS	Fetal Bovine Serum
FBW	final body weight
FCR	feed conversion ratio
fMLP	formyl-methionyl-leucyl-phenylanaline
Fpg	formamidopyrimidine (fapy)-***DNA*** glycosylase
GFSk	Goldfish skin
GR	glutathione reductase
GSH	glutathione
GSSG	oxidised glutathione
GSHPx	glutathione peroxidase
GVB	gelatin veronal buffer
H_2O_2	hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
IBW	initial body weight
ICP-MS	inductively coupled plasma mass spectroscopy
LMP	low melting point
LWG	live weight gain
MEM	Minimal Essential Medium
MTT	-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
MPO	myloperoxidase
MS222	tricane methane sulphonate
NA	nicotinic acid
NAD	nicotinamide adenine dinucleotide

NADP	nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NAM	nicotinic acid amide or nicotinamide
NBT	nitroblue tetrazolium
NC	negative control
NEAA	Non Essential Amino Acids
NER	nucleotide excision repair
NMP	normal melting point
NO	nitric oxide
NRC	National Research Council
NRR	neutral red retention
$^1O_2$	singlet oxygen
$O_2^{\cdot-}$	superoxide radical anion
OH	hydroxyl radical
ONOO	peroxynitrite
PARP-1	poly (ADP-ribose) polymerase-1
PBS	phosphate buffered saline
PC	positive control
PMA	phorbol myristoyl acetate
POBN	α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone
RBC	red blood cells
RLU	relative light units
RNA	ribonucleic acid
ROS	reactive oxygen species
SCGE	single cell gel electrophoresis
SE	standard error
Se-M	selenomethionine
Se-scFv2F3	selenium-containing single-chain Fv catalytic antibody
SGR	specific growth rate
SIN-1	3-morpholino-sydnonimine HCl
SOD	superoxide dismutase
SSB	single strand breaks
TAC	total antioxidant capacity
TGC	thermal unit growth co-efficient
TiO_2	titanium dioxide
TR	thioredoxin reductase
UVA	ultraviolet A radiation
UVB	ultraviolet B radiation
UVC	ultraviolet C radiation
UVR	ultraviolet radiation
VEA	vitamin E analogue
WST-1	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium
XO	xanthine oxidase

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Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award. The study was funded by Waltham Centre for Pet Nutrition (WCPN). A number of scientific conferences were attended during this period of which contributions are listed below.

Signed .....

Date 19/05/09

Publication (included in appendix A).

Reeves, J. F., Davies, S. J., Dodd, N. J. F. and Jha, A. N. (2008) Hydroxyl radicals (OH) are associated with titanium dioxide (TiO_2) nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* **640 (1-2):** 113-122.

Posters

Rider, S. A., Reeves, J. F. Armstrong, K, Knight, J, Davies, S. J. and Jha, A. N. (2007) The effect of chronic husbandry stress on markers of genomic stability, antioxidant status and health in rainbow trout. In: Abstracts from the 7th International Comet Assay Workshop held at University of Ulster, Coleraine, Northern Ireland; 24th–27th June 2007. *Mutagenesis*, **22(6):** e1–e21.

Reeves, J. F., Davies, S. J. Alexander, L. and Jha, A. N. (2006). Effects of selenium supplementation on H_2O_2- and UVA-induced oxidative DNA damage in goldfish skin cells *in vitro.* Presented at the XII International Symposium on Fish Nutrition & Feeding (ISFNF) (Biarritz, France, 28th May – 1st June 2006).

Reeves, J. F., Davies, S. J. and Jha, A. N. Niacin modifies UVB-induced genetic damage in fish skin cells (2005). Presented at The Annual Meeting of the European Environmental Mutagen Society (EEMS) (Kos Island, Greece, 3-7th July 2005). Attendance funded by ECNIS.

Oral presentations

Reeves, J. F., Davies, S. J., Dodd, N. J. F. and Jha, A. N. (2007) Determination of oxidative stress induced by environmental agents and its modification by micronutrients in fish cells under in-vitro conditions. In: Abstracts from the 7th International Comet Assay Workshop held at University of Ulster, Coleraine, Northern Ireland; 24th–27th June 2007. *Mutagenesis*, **22:** (6), e1–e21.

Reeves, J. F., Davies, S. J. Alexander, L. and Jha, A. N. (2006). Effects of selenium supplementation on H_2O_2- and UVA-induced oxidative DNA damage in goldfish skin cells *in vitro*. Presented at the Annual Alltech Aquaculture Meeting (Dunboyne, Ireland, 7th November, 2006).

Other meetings attended

- Alltech Selenium Conference (Stamford, UK, 6th July 2007)
- World Aquaculture Society Conference (Florence, Italy, 9th – 13th May 2006)
- Fish Immunology Workshop (Wageningen Institute of Animal Sciences, Netherlands, 10-14th April 2005).

Courses

- General teaching Associates (GTA) course (University of Plymouth, 8-12th August 2005).
    - Certificate of Professional Development (UoP)
      Learning, Teaching and Assessment: Theory and Practice
- Home Office licence training – Modules 1-3 Fish (Marine Biological Association [MBA], Plymouth UK, 25/26th August 2005).

Acknowledgements

I would like to thank all the people who have helped me during the completion of this work. Dr. Simon Davies, my director of studies for all his help and guidance both as a supervisor and a friend. Dr. Awadhesh Jha, second supervisor for his help and guidance, especially with matters relating to genotoxicity. Also a special thank you to Lynne Cooper or her expertise related to cell culture methodology and Nick Dodd for his expertise in ESR.

I would also like to thank Sebastien Rider for collaborate work on the effects of husbandry stress on rainbow trout, both for his expert laboratory work and discussions of ideas. Furthermore, for his help in measuring selenium and zinc levels by ICP-MS and FAAS, respectively.

Dr. Abigail Stevenson, Dr. Lucille Alexander and Dr. Donna Snellgrove of the Waltham Centre for Pet Nutrition, for their sponsorship and for support and guidance throughout.

Most of all special thanks go to my dear friends and colleges in the fish nutrition group and ecotoxicology group who kept my spirits up during times when personal problems outweighed scientific ones – too many to mention, you know who you are.

Thanks also go to Professor Lucy Lee for kindly donating cultured goldfish cells, and Professor Andrew Collins for providing Fpg enzyme and for accommodating me in his lab in Norway for 3 days.

Thanks to Altech UK for suppling SelPlex® and Zinc BioPlex® for the carp trial.

Finally I would like to thanks my parents Andrew and Ruth for their emotional support throughout this difficult time.

Dedication

I would like to dedicate this thesis to my whole family, especially my mum and dad, Andrew and Ruth, my brother Dave and Sister Natalie.

CHAPTER 1: INTRODUCTION

1.1 Aquaculture

Aquaculture is growing more rapidly than all other animal food producing sectors. Worldwide, aquaculture has increased at an average compounded rate of 9.2% per year, compared with 1.4% for capture fisheries and 2.8% for terrestrial farmed meat production systems (FAO, 2002). Aquaculture production has become increasingly important as a source of dietary protein since capture fisheries have suffered tremendously from over-fishing and environmental degradation. It is estimated that 47% of the main stocks or species of fish are fully exploited and are therefore producing catches that have reached, or are very close to, their maximum sustainable limits.

Besides food production, aquaculture is also important in providing fish for the ornamental industry, especially in Asia where it is considered to have potential to contribute to the economy. A recent survey from the FAO estimates that worldwide annual sales of ornamental fish are close to US$ 200 million (FAO, 2002).

Given the importance of aquaculture it is necessary to explore avenues by which efficiency and production may be maximised. Maximum efficiency in aquaculture production depends on keeping fish healthy and this includes reducing any sources of stress they may unavoidably encounter.

1.2 Effects of stress on fish health

Generally speaking, stress can be defined as '*...the reactions of the body to deleterious forces (stress factors) that tend to disturb normal physiological equilibrium*' (Bly *et al.*, 1997). The rearing of fish unavoidably exposes them to both natural (environmental) and anthropogenic stress factors. Natural environmental stress factors that fish may encounter include changes in season, temperature, salinity, photoperiod, and UVR exposure, to name a few. Due to the high stocking densities seen in aquaculture facilities, social stress is also exaggerated (crowding and hierarchy). Anthropogenically induced stress factors include xenobiotic and metal contaminants, but stress may also be initiated through common husbandry techniques such as handling, grading, transportation and netting (Bly *et al.*, 1997; Iwama *et al.*, 1997). While in severe cases stress may result in massive mortality, sub-lethal stress can compromise various physiological and behavioural functions, leading to suppressed immunocompetence and disease resistance, decreased growth rates and an overall reduction in health, culminating in sub-optimal production (Barton & Iwama, 1991; Pickering, 1992; Bly *et al.*, 1997; Iwama *et al.*, 1997). Therefore, the management of stress, including its dietary modulation, is critical in running and maintaining a successful aquaculture system.

1.2.1 UVR exposure as a stress factor for fish

Ultraviolet radiation (UVR) occupies the 200-400 nm portion of the solar radiation spectrum. The predominant form of solar UVR reaching the Earth's surface is in the form of long wavelength ultraviolet A (UVA) (320-400 nm) and

only a minority (< 10%) is in the form of ultraviolet B (UVB) (280-320 nm) (Kadekaro, *et al.*, 2003). The short wavelength ultraviolet C (UVC) (200-280 nm) is highly energetic, but very little reaches the Earth's surface, as it is almost completely filtered out by the ozone layer.

In terms of unavoidable stressors that may influence fish health, exposure to ultraviolet radiation (UVR) is increasingly becoming recognised as important (Zagarese and Williamson, 2001). Finfish in outdoor aquaculture systems are particularly susceptible to the stresses of UVR exposure. Under natural conditions fish avoid exposure to potentially damaging solar radiation by moving to deeper waters or shade, but this is not necessarily a strategy that farmed fish can utilise. In most aquaculture systems, water conditions are often clear and shallow, and therefore are conducive to considerable penetration of UV radiation (Bullock, 1988). UVB, the most damaging wavelength reaching the Earth's surface, can penetrate to depths of water ranging from a few centimetres down to 20 meters, depending on water quality/chemistry (Smith *et al.*, 1992; Kirk, 1994; Huovinen *et al.*, 2000). With aquaculture systems at high altitudes there may be further increases in UVR penetration, due to the decreased atmospheric (ozone) density which limits the filtration of solar rays. Excessive overcrowding, and some gill diseases, associated with intensive aquaculture, aggravates the problem by forcing fish to swim at or near the water's surface, often with their head and backs out of the water (Woo and Bruno, 1999). Furthermore, commercially important species such as trout (e.g. cutthroat) have very little protective pigment in their skin compared to other fish species (Blazer *et al.*, 1997), and are therefore

overly sensitive to the effects of UVR. Finally, the early life stages of all cultivated fish species are more than likely to be at risk of UVR exposure. Eggs and larvae are typically translucent to UVR, lacking protection which may be afforded from scales and thick layers of pigmented skin in adults of some species (Blazer *et al.*, 1997; McFadzen *et al.*, 2000; Meador *et al.*, 2000).

UVR exposure causes a variety of adverse health effects in biological systems ranging from molecular and tissue damage to population level effects. At the molecular level, UVR is able to damage proteins, lipids and DNA either through direct absorption of incident photons or through oxidative stress (Kielblessa *et al.*, 1997; Duthie *et al.*, 1999; Ichihashi, *et al.*, 2003; Kaderko *et al.*, 2003). UVR-induced oxidative stress may result from the generation of excessive levels of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radical anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH) via the excitation of endogenous chromophores (photosensitisers) (Fig. 1.2). These ROS initiate macromolecular disruption either through direct oxidation reactions or through further activation of endogenous reactive intermediates.

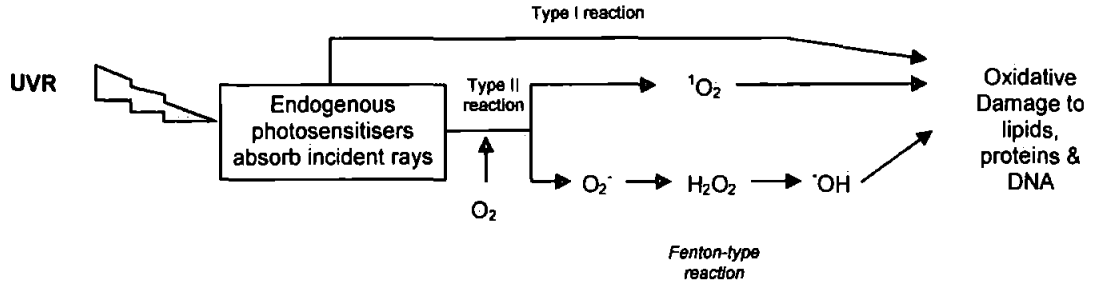


Figure 1.1 Mechanism of ROS generation by UVR

In humans acute bouts of UVR exposure may lead to erythema, a redness of the skin caused by capillary congestion, commonly known as sunburn. Chronic exposure may result in skin aging, cataracts, malignant and benign skin tumours as well as immune suppression (Cridland and Saunders, 1994).

For fish the most severe consequence of UVR exposure in fish is mortality, of which early life stages are to be particularly susceptible (Charron *et al.*, 2000; Browman *et al.*, 2003; Hakkinen *et al.*, 2004). However, it has also been demonstrated that UVR can cause a wide range of sublethal effects, similar to those experienced by terrestrial animals (humans) (Table 1.1). Although not immediately lethal, these pathologies may culminate in reduced growth and retarded development (Hunter *et al.*, 1979; Poston and Wolfe, 1985) and lead to substantial late mortalities (Hakkinen *et al.*, 2004).

One of the most sensitive biomarkers of UVR exposure in biological systems is damage to genetic material (DNA, RNA). Damage to DNA is of particular concern in biological systems as it can have cytotoxic or mutagenic consequences by interrupting DNA replication or altering gene regulation and subsequent protein expression. Mutations induced by DNA lesions in oncogenes and tumour-suppressor genes are believed to form the basis for photocarcinogenesis in animals (Black *et al.*, 1997). Damage to DNA is of further concern because of the delayed manifestation of the effects - inherited defects may not be expressed for several generations (O'Reilly and Mothersill, 1997).

There are several classes of UVR induced DNA damage, and these are broadly wavelength specific (Kielbassa *et al.*, 1997). UVB radiation is the most mutagenic waveband among types of solar radiation as DNA bases directly absorb incident photons within this wavelength range. The effects of UVB radiation on DNA are caused mostly by the formation of dimeric photoproducts between adjacent pyrimidine bases on the same strand (Cridland and Saunders, 1994; Kielbassa, *et al.*, 1997; Ichihashi *et al.*, 2003). The major class of these lesions are the cyclobutane pyrimidine dimers (CPD), with pyrimidine-pyrimidone (6-4) photoproducts being the second most prevalent adducts formed. Although DNA is not a chromophore for UVA, UVA (and UVB) radiation are found to produce another type of DNA lesion through the generation of ROS and oxidative stress (Cridland and Saunders, 1994; Kielbassa, *et al.*, 1997; Ichihashi *et al.*, 2003). For example, singlet molecular oxygen ($^1O_2$) and hydroxyl radicals (OH) produced by UVB and UVA radiation target the DNA base guanine, giving rise to 8-hydroxydeoxyguanosine (8-OHdG) in the strand DNA. Consequently, 8-OHdG is known to be a ubiquitous biomarker of oxidative stress. UVR also induces a much wider range of DNA damage, such a DNA-protein crosslinks, single strand breaks (SSB) and thymine glycol (Cridland and Saunders, 1994; Kielbassa, *et al.*, 1997; Ichihashi *et al.*, 2003).

For fish, numerous studies have demonstrated the induction of dimeric photoproducts in response to UVR exposure, both *in vitro* or *in vivo* (Ahmed and Setlow, 1993; Ahmed *et al.*, 1993; Uchida *et al.*, 1997; Meador *et al.*, 2000; Lesser *et al.*, 2001; Armstrong *et al.*, 2002; Browman *et al.*, 2003).

However, it appears that other types of DNA damage, such as oxidised bases (i.e. 8-OhdG), single strand breaks, and DNA protein crosslinks, have not been widely reported in fish in response to UVR exposure.

Table 1.1. Effects of UVR exposure on fish.

Observed effect	Species	Reference
Gross pathologies of the skin due to inflammation/necrosis (Erythema)	Anchovy, mackerel	Hunter *et al* (1979) Bullock (1988)
	Rainbow trout, koi	Ramos *et al.* (1994)
	Paddlefish	Blazer *et al.*, (1997)
	Cutthroat trout	McFadzen *et al.* (2000)
	Sole and turbot	
	Medaka	Armstrong *et al.* (2002)
Cataracts	Rainbow trout	Cullen *et al.* (1994) Laycock *et al.* (2000)
Physiological changes (respiratory, circulatory and neurological)	Sockeye salmon	Bell and Hoar (1950)
	Pike	Hakkinen *et al.* (2004)
Behavioural changes (e.g. swimming ability)	Sockeye salmon	Bell and Hoar (1950)
Immunosupression (from indirect and direct evidence)	Cutthroat trout	Blazer *et al.*, (1997)
	Sole and turbot	McFadzen *et al.* (2000)
	Roach	Jokinen *et al.* (2000)
	Carp	Salo *et al.* (2000) Markkula *et al.* (2005)
DNA damage	Platyfish	Meador *et al.* (2000)
	Atlantic cod	Lesser *et al.* (2001)
	Medaka	Armstrong *et al.* (2002)
	Atlantic cod	Browman *et al.* (2003)
Photosensitisation of pollutants (e.g. polyaromatic hydrocarbons [PAH])	*Review*	Barron and Ka'aihue (2001)

1.2.2 Husbandry induced stress in fish

Husbandry practices that may induce a stress response from fish include handling, weighing, grading netting and transportation. Forced crowding such as in high stocking densities or due to low water confinement may exacerbate these effects. During such practices fish evoke a so called 'stress response' in

an attempt to avoid being caught, handled or forced into confined spaces. It is a similar response that may be elicited in wild fish to cope with hostile environments and also avoid predation. However, in aquacultural conditions where there is no possible escape, the stress response may no longer be beneficial but in fact become damaging to health. During the stress response, fish switch from an anabolic to catabolic state. This primary stress response involves the release of many hormones, notably catecholamines (CAs) and glucocorticoids such as cortisol, changes of which are frequently used as an indicator for stress induction by husbandry techniques its severity (Ramsey *et al.*, 2006; Varsamos, *et al.*, 2006). The stress response is also associated with secondary effects. Modulations in blood glucose and hematocrit are often studied and used to determine the level of secondary stress effects (Barnton and Iwama 1991, Ruane *et al.*, 2002). Husbandry stress has been shown to modulate the immune system of fish (Yin *et al.*, 1995; Tort *et al.*, 1996). Components of the respiratory burst, a key response of the cellular immune response, are affected by acute physical stress, being either stimulated (Pulsford *et al.*, 1994) or suppressed (Vazzana *et al.*, 2002, Angelidis *et al.*, 1987).

Oxidative stress may also be expected to occur in husbandry stressed fish as energy is reallocated to intensify activities such as locomotion and respiration (Bonga, 1997). During hyper-metabolic states associated with stress, levels of ROS are elevated due to electron leakage from the electron transport chain during oxidative respiration in mitochondria. This has been observed in exercised rats (Bejma & Ji, 1999; Leeuwenburgh & Heinecke, 2001).

Furthermore, a study in cattle concluded that transportation stress causes oxidative stress as measured by reduced TAC and increased lipid peroxidation (Chirase *et al.*, 2004). As with mammals, increased exercise in fish has been found to cause oxidative stress and associated DNA damage (Aniagu *et al.*, 2006).

1.2.3 Nanoparticle exposure in fish

Nanotechnology concerns the development and use of substances which have a particle size <100 nm, the aim being to significantly increase the surface area to mass ratio, thereby greatly enhancing chemical/catalytic reactivity (amongst other properties), compared to normal-sized particles of the same substance. Although this provides many benefits for industry, there is increasing concern that substances previously considered biologically inert may indeed become toxic in a nanoparticulate state due to their increased reactivity and possible easier penetration of cells. In this context, normal-sized (>100 nm) TiO_2 has been considered to be biologically inert to animals and humans (Chen & Fayerwhether, 1988; Bernard *et al.*, 1990) and it is widely used as an ingredient in white paint, food colourant, sunscreen and cosmetic products (Gurr *et al.*, 2005). TiO_2 nanoparticles, on the other hand, are incorporated into cellular membranes and cytoplasm of mammalian cells in culture (Sakai *et al.*, 1994; Wamer *et al.*, 1997), although studies on the toxic effects of TiO_2 nanoparticles provide conflicting evidence. Zhang and Sun (2004) found human colon carcinoma cells were still 90% viable even after a 24 hour exposure to 1000 $\mu g\ ml^{-1}$ TiO_2 nanoparticles. Nakagawa *et al.* (1997)

reported no effect of ultra-fine (25 nm) TiO_2 on a series of genotoxicity parameters measured in mouse lymphoma and Chinese hamster cells, including the Comet assay, microbial and cell mutation assays and chromosomal aberrations. TiO_2 nanoparticles also had no cytotoxic (cell growth) or genotoxic (micronuclei induction) effect on cultured rat liver epithelial cells (Linnainmaa *et al.*, 1997). Conversely, exposure of Syrian hamster embryo cells to 1 $\mu g\ cm^{-2}$ TiO_2 (<20 nm) for 12–72 hours caused a significant dose-dependent increase in the induction of micronuclei and apoptosis (Rahman *et al.*, 2002). Additionally, anatase (10 and 20 nm) TiO_2 particles, in the absence of photo-activation, induced oxidative DNA damage, lipid peroxidation, micronuclei formation, and increased hydrogen peroxide and nitric oxide production in a human bronchial epithelial cell line (Gurr *et al.* 2005). Wang *et al.* (2007) also found that TiO_2 nanoparticles are cytotoxic (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide [MTT] assay), genotoxic (Comet and micronucleus assays) and mutagenic (hypoxanthineguanine phosphoribosyltransferase gene mutation assay) towards cultured human lymphoblastoid cells. Long *et al.* (2006) found that TiO_2 causes oxidative stress in brain microglia cells under *in vitro* conditions. *In vivo* toxicity studies have also demonstrated that inhalation of TiO_2 nanoparticles causes pulmonary inflammation in rats and mice (Bermudez *et al.*, 2004). This was characterised by increased numbers of macrophages and neutrophils and increased concentrations of soluble markers in bronchoalveolar lavage fluid. Many studies have documented the phototoxic and photogenotoxic effects of TiO_2 (both normal and nano-sized) (Nakagawa *et al.*, 1997; Wamer *et al.*, 1997; Uchino *et al*, 2002; Zhang and Sun, 2007;

Dunford *et al.*, 2007) and consequently its properties as a photo-catalytic compound have been applied to waste water disinfection (Lui *et al.*, 2006) and photodynamic therapy of certain cancers (Ackroyd *et al.*, 2001). Whilst, there is ample evidence of the formation of reactive oxygen species (ROS) when TiO_2 is exposed to UV light (Cai *et al.*, 1992; Konaka *et al.*, 2001; Uchino *et al*, 2002; Chen *et al.*, 2004), there is disagreement as to the exact nature of the species produced and their involvement in cell death. Possible ROS that could be formed are hydroxyl radicals (OH), superoxide radical anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$).

Despite growing concern over the potential risk of manufactured nanoparticles to the environment (Owen & Depledge, 2005; Moore, 2006), there is a paucity of information on their potential toxic effects on natural biota. In particular, except for a 48 hour acute toxicity test on the fresh water flea, *Daphnia magna* (Lovern & Klaper, 2006), there is no information available in the literature on potential detrimental effects of TiO_2 nanoparticles on aquatic organisms, including fish. However, exposure to another type of manufactured nanoparticle, fullerenes, was shown to shown to cause lipid peroxidation in the brain of Largemouth Bass (Oberdorster, 2004).

The input of engineered nanoparticles in the aquatic environment is likely to be due to industrial (i.e. initial and downstream manufacturers) or non-industrial sources. Non-industrial sources include consumer products (sunscreens and cosmetics) from both direct (e.g. bathing) and indirect (sewer) sources, leaching from landfill or soil-applied sewage sludge and atmospheric sources

from waste combustion (Owen & Depledge, 2005). Given that nanotechnology industries plan large scale production, it is inevitable that these products and their by-products will accumulate in the aquatic environment (Daughton, 2004; Moore, 2006), and their potential genotoxic effects could have short and long term consequences for the biota (Jha, 2004). Furthermore, although only a small percentage of solar ultraviolet radiations (UVR) reaches the Earth's surface, they may penetrate to depths of more than >20 m in the water column (Huovinen *et al.*, 2000; Tadetti & Sempere, 2006) and as discussed earlier such radiation has been suggested to have deleterious effects on aquatic organisms, including fish.

1.3 Importance of micronutrients in fish health

It is widely accepted that, in addition to antigens and genetic and environmental factors, health is hugely influenced by food components – macro- and micronutrients. The lack of these components or their unbalanced intake can bring about many deleterious health effects. For example, in humans, diet has been implicated in 6 out of the 10 of the leading causes of death of Americans (Davies and Milner, 2004). In terms of formulated diets for cultured fish, gross malnutrition is generally no longer a problem, however, we are now faced with the more subtle effects that micronutrients, and their interactions with other dietary components, have on the various aspects of fish health (Lall, 2000; Halver, 2001). The challenge is to not only to establish optimal levels for normal growth, metabolism and disease resistance, but also

how requirements may change under variable environmental conditions and stress.

1.3.1 Niacin

Niacin (vitamin B_3) is the generic descriptor for nicotinamide (nicotinic acid amide; NAM) and nicotinic acid (pyridine-3-carboxylic acid; NA) both of which have identical vitamin activities. NAM and NA are the substrates for the synthesis of nicotinamide adenine dinucleotide (NAD) and the phosphorylated derivative NADP, co-enzymes found in all living cells which have a number of functions (Fig. x). The main function of these co-enzymes is to act as electron carriers during redox reactions in normal cellular metabolism. For example, during oxidative phosphorylation and generation of energy as ATP. Apart from it's role in metabolism, NAD is also a cofactor in the synthesis of nucleotides and DNA and is also the sole substrate for the enzyme poly (ADP-ribose) polymerase-1 (PARP-1), which has various functions including DNA repair (see review by Hageman and Stierum, 2001). PARP-1 aids in base excision repair (BER) by binding sites with single strand breaks through its N-terminal zinc fingers and will recruit DNA ligase III and DNA polymerase beta and a kinase to the nick.

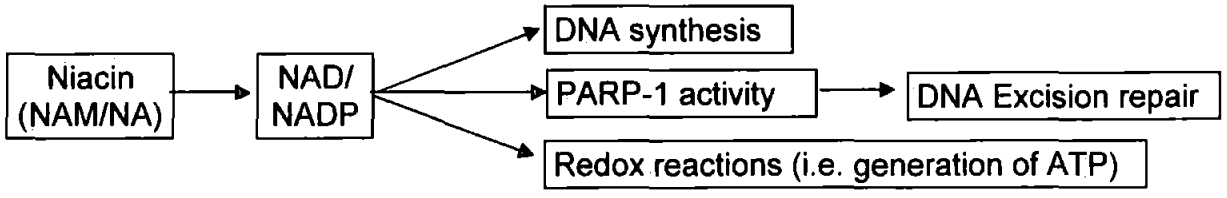


Figure 1.2. Functional roles for niacin and the co-enzymes NAD and NADP

The role of PARP-1 in genomic stability has been confirmed in mice and cells defective in PARP-1; these had elevated spontaneous genetic rearrangements and abnormal responses to DNA damaging agents (de Murcia *et al.*, 1997; Wang *et al.*, 1997; LeRhun *et al.*, 1998; Spronck & Kirkland, 2002). Furthermore, mammalian cells and cell lines having low or depleted intracellular NAD have been found to be more sensitive to cytotoxic effects of DNA damaging agents (Durkacz *et al.*, 1980; Boulton and Durkacz, 1997). This sensitivity is thought to be due to reduced availability of NAD resulting in impaired PARP-1 function and inhibition of base excision repair and DNA strand rejoining.

In addition to being a precursor for the production of NAD, niacin itself has also been reported to possess oxygen radical scavenging activity (Hageman and Stierum, 2001). In rats, niacin deficiency lowers PARP-1 and NAD concentration in the liver, but also increases susceptibility of DNA to oxidising agents (Zhang *et al.*, 1993; Rawling *et al.*, 1994; Boyonoski, *et al.*, 1999). The latter may be due to a reduced cellular antioxidant capacity since NAM has been reported to possess antioxidant activity comparable to that of ascorbic acid (vitamin C) (Kamat and Devasagayam, 1996).

In human trials, niacin supplementation (100 mg NA/day) has been reported to reduce levels of basal DNA damage and increase resistance against *ex vivo* hydrogen peroxide-induced strand breaks (Weitberg, 1989). Additionally, daily supplementation of 100 mg NAM in combination with zinc and carotenoids has been shown to increase resistance against oxidative DNA damage and enhance DNA repair in peripheral blood lymphocytes of human volunteers

(Sheng *et al.*, 1998). It is not known whether the reduction in oxidative DNA damage was the result of enhanced PARP-1 function and enhanced DNA repair, or due to the antioxidant activity of niacin.

Niacin may also have a role to play in the prevention of carcinogenesis, including UVR-induced skin cancer formation. The incidence of UV-induced skin tumours in mice was found to be reduced by topical application of NAM and by supplementation with 0.5% and 1.0% niacin in their diet. In mice orally supplemented with niacin, NAD levels in the skin were increased. Niacin was also found to reduce photoimmunesupression. It was concluded that niacin supplementation may contribute to the prevention of UV-induced skin cancer, through maintenance of skin NAD levels and possibly through enhancement of PARP-1 function (Gensler, 1997; Gensler, *et al.*, 1999).

Overall, it appears that the size of the intracellular NAD pool, and a sufficient supply of dietary niacin are considered important for PARP-1 function and for genomic stability in mammals, both in terms of DNA repair and prevention of oxidative DNA damage, as well as in the suppression of photocarcinogenesis.

As far as it can be seen there is no available literature on the role of niacin in genomic stability in fish. However, niacin is recognised an important vitamin that must be supplemented in formulated feeds (Lovell, 1998). This is because although niacin can be synthesised from the amino acid tryptophan, the utilisation of dietary tryptophan as a precursor of niacin is not entirely effective, especially in salmonids. It is also known that, in trout for example, niacin

deficiency can lead to poor growth, anaemia and gill swellings, associated with flexing of the operculae (Lovell, 1998). Additionally in the rainbow trout, an adequate supply of niacin has been shown to be necessary not only for optimal growth and feed utilization, but also in the prevention of fin damage due to UVR ('sunburn') (Poston and Wolfe, 1985). Insufficient niacin supply resulted in severe erosion of both the pectoral fins and the caudal fin when fish were subsequently exposed on two consecutive days to UV radiation. This was attributed to the lack of cellular NAD, which as mentioned above, has antioxidant properties and is able to remove reactive oxygen species that are generated by UVR.

1.3.2 Selenium

Selenium is an essential trace element for animal nutrition. In humans, an adequate supply of dietary selenium is important for the protection of cells and tissues from oxidative injury, maintenance of genomic stability and cancer prevention (including photocarcinogenesis), and for proper functioning of the immune system (See reviews by McKenzie, et al., 1998; McKenzie, 2000; Rayman, 2000; El-Bayoumy, 2001; Ferencik and Ebringer, 2003; Sies and Stahl, 2004; Lipinski, 2005).

As selenocysteine, the '21st' amino acid, selenium is an integral part of selenoproteins, which have important enzymatic and structural roles. All these enzymes are selenium dependent, generally with selenocysteine at the active site. Probably the most widely recognised family of enzymes are the

glutathione peroxidases (GSHPx 1-4), cytosolic and membrane-bound antioxidant enzymes, which remove hydrogen peroxide, and lipid and phospholipids hydroperoxides, respectively. This limits oxidative damage to important macromolecules such as lipids, lipoproteins and DNA. About 35 selenoproteins have been identified, though many have roles that have not been fully elucidated. Table 1.2. gives details known selenoproteins that carry out nutritional functions of selenium.

Biological utilization and distribution throughout the tissues depends on the form of selenium the organism receives. In terms of dietary selenium supplements, a common source is selenates and selenites. Of organic sources, selenocysteine can be found mainly in foods of animal origin (as part of selenoproteins). On the other hand selenomethionine (which cannot replace methionine in proteins) is to be found mainly in plants. Therefore, selenomethionine is more effective in supplying selenium to the organism than selenocysteine because it is not incorporated into proteins from which it would first have to be released. On the other hand selenomethionine has no catalytic activity since it is not part of any selenoenzymes. It has to be metabolised to an organic precursor, selenite, that can be converted to selenophosphate that then serves as a precursor of selenocysteine, the active form of selenium, which can be built into selenoproteins (Fig.1.1) (Allen *et al.*, 1999).

Se-M → Selenite (Na_2SeO_3) → Selenophosphate → Se-Cysteine → Selenoprotein

(*Selenophosphate synthetase*)

Figure 1.3. Use of selenomethionine (Se-M) for the synthesis of selenoproteins.

Table 1.2. Known selenoproteins that carry out nutritional functions of selenium.

Selenoprotein	Function
Glutathione peroxidases (GSHPx1-4)	Antioxidant enzymes: remove hydrogen peroxide, and lipid and phospholipid hydroperoxides (thereby maintaining membrane integrity, modulating eicosanoid synthesis, modifying inflammation and likelihood of propagation of further oxidative damage to biomolecules such as lipid, lipoproteins and DNA)
(Sperm) mitochondrial capsule selenoprotein	From GSHpx4: shields developing sperm cells from oxidative damage and later polymerises into structural protein requires for stability/motility of mature sperm
Iodothyronine deiodinases (3 isoenzymes)	Production and regulation of level of active thyroid hormone, T3, from thyroxine, T4
Thioredoxin reductase (3 isoenzymes)	Reduction of nucleotides in DNA synthesis; regeneration of antioxidant enzymes; maintenance of intracellular redox state, critical for cell viability and proliferation; regulation of gene expression by redox control of binding of transcription factors to DNA
Selenophosphate synthetase, SPS2	Required for biosynthesis of selenophosphate, the precursor of selenocysteine, and therefore for selenoprotein synthesis
Selenoprotein P	Found in plasma and associated with endothelial cells. Appears to protect endothelial cells against damage from peroxynitrite.
Selenoprotein W	Needed for muscle function
Prostate epithelial protein	Found in epithelial cells of ventral prostate. Seems to have redox function (resembles GSHPx 4), perhaps protecting secretory cells from against development of carcinoma
DNA-bound spermatid selenoprotein	GSHPx-like activity. Found in stomach and nuclei of spermatozoa. May protect developing sperm
18 kDa selenoprotein	Important selenoprotein, found in kidney and large number of other tissues. Preserved in selenium deficiency

In view of these reactions, selenates and selenites appear to be preferable sources of selenium, because they can be directly converted to selenophosphate. However, the use of these inorganic selenium compounds is hampered by the fact that they are more difficult to reabsorb from the digestive tract than organic selenium compounds, and they are also a great deal more toxic (Ferencik and Ebringer, 2003). Another reason is that there is evidence that selenites stimulate macrophages to produce pro-inflammatory cytokines, while selenomethionine does not have this effect (Jonhson *et al.*, 2000).

Selenium nutrition has been studied in some species of fish (mostly salmonids and catfish), but nowhere near as comprehensively as in mammals (humans). Table 1.3 summarises selected nutrition studies that have taken place with fish. One thing that is immediately clear is that, although it has been established that selenium is important for fish for optimal growth, feed conversion, GSHPx activity and immune function, there is a paucity of information regarding the role of selenium in maintenance of genomic stability. Also there is a lack of studies on ornamental fish. In terms of the dietary selenium source, fish nutrition trials have showed that organic sources (selenomethionine, selenocysteine and seleno-yeast) are more bioavailable and better utilised than inorganic forms (i.e. sodium selenite) (Lorentzen *et al.*, 1994; Jovanovic *et al.*, 1997; Wang *et al.*, 1997; Wang and Lovell, 1997). This is in agreement with the mammalian literature, as discussed above.

Table 1.3. Nutrional roles for selenium as evaluated in fish studies

Nutritional role	Fish species	References
Optimal growth and feed conversion	Channel catfish	Gatlin and Wilson (1984)
		Gatlin *et al.* (1986)
		Wang *et al.* (1997)
		Wang & Lovell (1997)
	Atlantic salmon	Bell *et al.* (1987)
	Rainbow trout	Hilton *et al.* (1980)
		Bell *et al.* (1986)
Optimal GSHPx (and other antioxidant enzymes) activity and retardation of oxidative stress indices	Tilapia	Diminov *et al.* (1998)
	Carp	Jovanovic et al. (1997)
	Channel catfish	Gatlin & Wilson (1984)
		Gatlin *et al.* (1986)
		Wise *et al.* (1986)
		Wang & Lovell (1997)
	Atlantic salmon	Bell *et al.* (1987)
	Chinook salmon	Thorarinsson *et al.* (1994)
	Coho salmon	Felton *et al.* (1996)
	Rainbow trout	Hilton *et al.* (1980)
		Bell et al. (1986)
Optimal Immune function	Channel catfish	Wise *et al.* (1986)
		Wang *et al.* (1997)
	Striped bass	Jaramillo & Gatlin (2004)
Protection against toxic effects of heavy metals (e.g. Cu, Cd, Hg)	Rainbow trout, Atlantic salmon	Hilton (1989)
	Rainbow trout hepatic cells *in vitro*	Al-Sabti (1995)
	Atlantic salmon	Lorentzen *et al.* (1998)

1.3.3 Zinc

Zinc is an essential element for all animals. More than 300 zinc-containing enzymes have been characterised for which zinc is directly involved in catalysis and interacting with the substrate molecules undergoing transformation (Hambidge, 2000). Zinc in ubiquitous in sub-cellular metabolism and is an essential component for the active site or sites for at least one enzyme in every enzyme classification (McCall *et al.,* 2000) These enzymes include carbonic anhydrase, carboxypeptidase A and B, dipeptidase, pyruvate carboxylase, superoxide dismutase, alkaline phosphatase, DNA polymerase, RNA polymerase and many others.

Zinc ions are also structurally important in a large number of proteins involved in gene transcription, DNA synthesis and repair, genomic plasticity and apoptosis (Rhodes & Klug, 1993; Berg & Shi, 1996). Zinc is also important for the transcription and induction of metallothioneins, a group of low molecular weight proteins that aid in the detoxification of toxic metals and the homeostatic control of essential metals such as copper and zinc itself (Maret, 2000). They also help maintain the intracellular redox potential by counteracting the depletion of glutathione during periods of oxidative stress. Furthermore an adequate supply of zinc is necessary for proper functioning of the immune system (Ferencik and Ebringer, 2003).

Research into the nutritional roles of zinc in fish is fairly limited. However, zinc evidently does play important roles in growth, reproduction, development,

vision and immune function in a variety of fish species (reviewed by Watanabe *et al.*, 1997). Consequently for fish, of the essential metals, zinc is considered second in quantitative importance only to iron (Watanabe *et al.*, 1997).

1.2.4 Vitamin E

Vitamin E is the generic descriptor for all molecules that possess the biological activity of α-tocopherol. Vitamin E is regarded as the primary lipid-soluble biological antioxidant which is required to protect polyunsaturated fatty acids (PUFAs) in cells against oxidative degeneration by terminating free radical initiated chain reactions, thus preventing oxidative damage to cell membranes, proteins and DNA (Burton and Traber, 1990). In humans, Important functions such as protection against oxidative damage, immune response, and the propensity of platelets to adhere to the vessel wall are related to vitamin E intakes (Webber *et al.*, 1997). Indeed, many observational studies have reported vitamin E to reduce the risk of cardiovascular disease.

In fish vitamin E deficiency results in decreased growth, muscular dystrophy, edema of the heart, muscle and other tissues due to increased capillary permeability, anaemia and impaired erythropoiesis, depigmentation and ceroid pigmentation in the liver (NRC, 1999). Vitamin E deficient fish have also been reported to have significantly reduced immune and non-specific responses to infection (Wise *et al.*, 1993). Furthermore, supplementing vitamin E to the diet in the form of α-tocopherol enhanced specific immune response, nonspecific

resistance factors and disease resistance of healthy and aflatoxin B1-induced immune-compromised Indian major carp (Sahoo and Mukherjee, 2002).

1.4 General aims

Overall, this study aims to investigate the effects of environmentally induced stress on (ornamental) fish health and also to establish how supplementation of various micronutrients may modulate some of these effects. A number of in *vitro* experiments were carried to achieve the following aims:

1. Develop and validate sensitive and reliable assays for the evaluation of cytotoxicity and genotoxicity in cultured fish cells.

2. Evaluate the potential protective effects of niacin and selenium against UVR induced cytotoxicity and genotoxicity in cultured fish cells.

3. Evaluate the (photo)cytotoxic and (photo)genotoxic potential of TiO_2 nanoparticles as a potential novel source of (oxidative) stress in cultured fish cells.

Following *in vitro* experiments the following aims were established for *in vivo* experiments.

1. Evaluate the effect of husbandry stress on fish health, more specifically effects on antioxidant status, immune function and genomic stability.

2. Evaluate the possible benefits of micronutrient supplements in fish feed, in terms of various health parameters and in relation to husbandry stress.

CHAPTER 2: GENERAL METHODS FOR *IN VITRO* WORK

2.1 Introduction

This chapter outlines common methods used for *in vitro*, cell culture based studies in chapters 3, 4 & 5. In particular the neutral red retention (NRR) assay for the evaluation of cell viability/toxicity and the single cell gel electrophoresis (SCGE) or 'Comet' assay for the detection of DNA damage.

2.1.1 Continuous fish cell lines as in vitro models

In vitro models which are routinely used in biology include organ perfusion, organ/tissue slices (biopsies), tissue explants, primary cell homogenates and continuous cell lines. Of these, continuous cell lines have been established as particularly useful tools in fish biology, especially in the field of aquatic ecotoxicology (Segner, 1998; Castaño *et al.*, 2003). Continuous fish cell lines have also been used for studying the cytotoxic and genotoxic effects of UVR in fish (e.g. Mano *et al.*, 1980; Shima and Setlow, 1984; Ahmed *et al.*, 1993; Uchida *et al.*, 1997).

In studying the effects of physically, chemically and biologically damaging agents the use of *in vitro* models, including cellular ones, is often criticised because they lack the complete defence mechanisms of whole organisms. However, although cell culture models cannot entirely replace experiments

using whole animals, there are a number of ethical, technical and economical reasons that support their use as models (Segner, 1998; Castaño *et al.*, 2003):

1. Cell culture provides one of the best experimental systems for studying damaging mechanisms at molecular and cellular levels, by allowing cells to be studied in a controlled environment and in isolation from the multiple environmental, physiological and behavioural responses which regulate their activity *in vivo*.
2. Cell culture models permit studies on a species that might not otherwise be studied, because that species cannot be maintained in a laboratory setting and/or is not routinely available (e.g. large pelagic marine fish species).
3. Cell culture models permit the direct comparison of species at the cellular or molecular level under equivalent conditions. This is important in comparing relative sensitivities of species to damaging agents (i.e. UVR exposure).
4. Cell culture models allow experiments to be carried out rapidly and relatively inexpensively.
5. The use of animal cell culture models is ethically pleasing, conforming to the three Rs (reduction, replacement and refinement of *in vivo* tests), which serve to reduce the number of whole animals used in experiments involving damaging agents.

Nearly all known fish cell lines are anchorage dependent and require a substrate on which they attach and grow to form a 2-dimensional confluent

monolayer over the surface. (Only a few lines have been adapted to grow in suspension) (Segner, 1998). Once seeded, cells undergo a series of distinct phases. Firstly, the lag phase, where the cell glycolyx attaches to the substrate; secondly, the log phase in which there is exponential cell division; thirdly, the plateaux or stationary phase, where a confluent monolayer is formed. The formation of a confluent monolayer causes 'contact inhibition' as the density of the cells reduces proliferation. This stage is more representative of an *in vivo* tissue as the cell-cell contact may allow expression of specific cell functions. Therefore, confluency is essential prior to experimentation.

2.1.2 Measuring cell viability in fish cells - The Neutral red retention (NRR) assay

The NRR assay was used as a measure of cytotoxicity in cultured fish cells. The assay has been recommended for regulatory cytotoxicity testing, and in particular phototoxicity evaluation, based on its sensitivity, reliability and reproducibility between laboratories (Spielmann *et al.*, 1998). The assay is simplistic to carry out and is based on the evaluation of lysosomal membrane stability, i.e. the ability of cells to accumulate and retain a neutral red dye in lysosomes within the cell. Cells are incubated in medium containing the neutral red dye for a number of hours in which time it accumulates within lysosomes (depending of the viability of the cells). Cells are then washed a number of times with a saline solution in order to remove any excess dye that has not accumulated within lysosomal membranes. If cells are damaged or non-viable then lysosomes lose the ability to retain the dye which is removed

during the washing stage. The final stage is to lyse the cells with acetyl alcohol which releases neutral red dye that has been retained within lysosomes and this is quantified spectrophotometrically. The healthier the cell, the more neutral red dye is retained.

The NRR assay has been extensively used in aquatic ecotoxicology to detect cytotoxicity in vertebrate and invertebrate cells, including fish cell lines (Segner, 1998; Dixon *et al.*, 2002; Castaño *et al.*, 2003). However, it appears as though the NRR is a technique that has not been used to quantify UVR-induced cytotoxicity in fish cells.

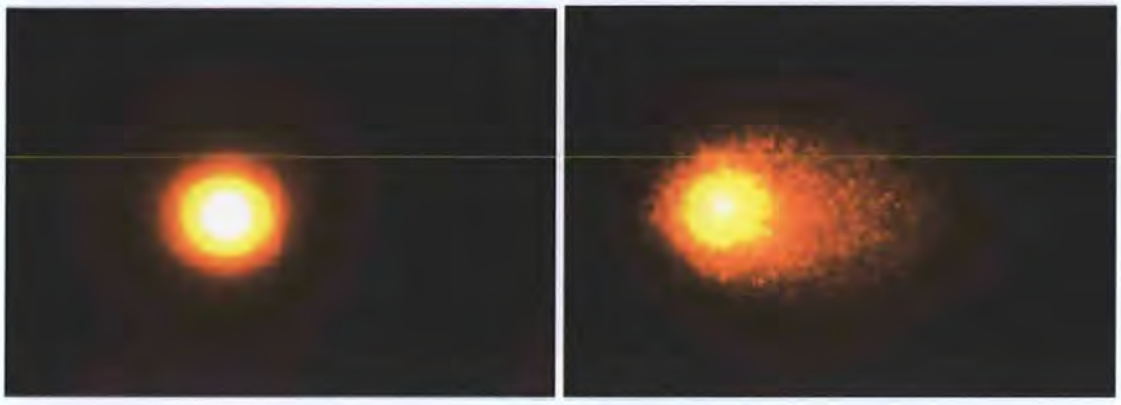
2.1.3 Measuring DNA damage in fish cells – The 'Comet' assay.

The single cell gel electrophoresis (SCGE) assay, more commonly known as the Comet assay, is a widely used method of detecting DNA damage (single strand breaks [SSB], crosslinks, alkali labile lesions) in individual cells in response to chemical or physical agents. It is a rapid, highly sensitive and relatively inexpensive technique for measuring DNA damage, requiring only basic molecular equipment and a small number of cells (10^4 – 10^5) (Dixon *et al.*, 2002; Tice *et al.*, 2000).

The method, first developed by Östling and Johansson (1984), has undergone several modifications, the most significant being the introduction of alkaline conditions to investigate the occurrence of DNA SSB (Singh *et al.*, 1988). Alkaline treatment facilitates the unwinding and denaturation of the DNA

molecules, allowing for the sensitive detection of single-strand damage. Neutral assay conditions, on the other hand, do not induce denaturation and consequently only allow the detection of double-strand damage.

The assay is usually performed to the protocol of Singh *et al.* (1988). More recently, guidelines have been outlined by a panel of leading experts in the use of the Comet assay (Tice *et al.*, 2000). The Comet assay works because DNA strand breaks create fragments or supercoiled DNA-loops that, when embedded in an agarose gel, migrate in an electric field. The strand breaks damage the higher order, tightly-packed structure of DNA, which also allows migration outside the region of the cell nucleus. Slides for the Comet assay are prepared by mixing a suspension of cells with liquid agarose, spreading out the suspension in a thin layer on a microscope slide and allowing the mixture to solidify. Cells are lysed in either neutral or alkaline conditions and DNA-bound proteins are removed so as not to interfere with migration in an electric field. Sufficient washing of the gels in solutions of the appropriate pH help to remove all other ions that might alter the electrophoretic conditions. Electrophoresis at low voltage then allows the migration to occur in a controlled manner, after which the cells are stained with a DNA-binding fluorescent dye for visualization and image analysis of the 'Comets' (Fig. 2.1.). Although image analysis software reports 34 parameters for DNA damage, 2 are commonly used - % tail DNA and Olive tail moment (Tice *et al.*, 2000).



(a) Undamaged DNA (control)

(b) DNA damage due to the formation of strand breaks

Figure 2.1. Visualisation of nuclear DNA damage by the Comet assay. Note how in (b) the formation of single strand breaks has resulting in increased migration of DNA fragments and the manifestation of a 'Comet'.

The Comet assay has been extensively used in aquatic ecotoxicology to detect DNA damage in vertebrate and invertebrate cells, including fish cell lines (Segner, 1998; Dixon *et al.*, 2002; Castaño *et al.*, 2003). However, agents evaluated for genotoxicity using the Comet assay almost exclusively include xenobiotics and toxic metals. It appears as though the Comet assay is a technique that has not been used to quantify UVR-induced DNA damage in fish cells. This is surprising given that it has been extensively employed for investigating UVR induced DNA damage in mammalian cells (Lyons and O'Brien, 2002; Bruge *et al.*, 2003; Salter *et al.*, 2004).

2.2 Materials and methods

2.2.1 Materials

All cell culture materials were purchased from either Invitrogen (Paisley, UK) or Bibby Sterilin (now Barloworld Scientific) (Stone, UK). All other reagents were purchased from Sigma (Poole, UK) unless otherwise stated. Formamidopyrimidine (fapy)-DNA glycosylase (Fpg) was kindly supplied by Professor Andrew Collins (University of Oslo, Norway).

2.2.2 Cell culture

All cell culture procedures were adapted from the methods of Freshnay (2000) and conducted in a class II laminar flow cabinet (Holten, Lamin Air, Model 1.2) under strict aseptic conditions.

2.2.2.1 EPC-A1

EPC-A1 (*Epithelioma Papulosum Cyprini*) cells were obtained from resuscitated stock held at the University of Plymouth, UK, but were first acquired from the European Collection of Cell Cultures (ECACC). The cell line was originally generated from a herpes virus induced skin tumour of a common carp (*Cyprinus carpio* L.) (Fijan *et al.*, 1983). EPCs were routinely sub-cultured fortnightly in Dulbecco's minimal essential medium (MEM) containing 10% fetal

bovine serum (FBS) and 1% non-essential amino acids (NEAA) in a 5% CO_2 atmosphere at 20±1 °C and typically seeded at a density of 1 x 10^6 cells ml^{-1}.

2.2.2.2 GFSk-S1

GFSk-S1 (Goldfish skin) cells were a kind gift of Professor Lucy Lee of Wilfred Laurier University, Canada who developed the primary cell line from the skin of goldfish (*Carassius auratus*) which possess mainly a fibroblast-like morphology (Bols *et al.*, 1994; Lee *et al.* 1997). GFSk's were cultured in Leibovitz's L-15 medium containing 10% FBS at 20±1 °C. Cells were routinely sub-cultured every 2-3 weeks at a density of 1 x 10^5 cells ml^{-1}.

2.2.3 Cytotoxicity Evaluation - Neutral red retention (NRR) assay

The NRR assay protocol was adapted from Babich *et al.* (1993). Where applicable cells were supplemented with micronutrients and/or challenged with chemical or UVR exposures as described in later sections. Cells were incubated in neutral red dye-containing (70 μg ml^{-1}) L-15 medium at 20±1 °C for 3 hours. Post incubation, neutral red was removed and cells washed three times with Dulbecco's phosphate buffered saline (PBS), followed by addition of 200 μl acetyl ethanol (1% acetic acid, 50% ethanol in distilled H_2O [dH_2O]). Plates were briefly shaken and the absorbance of lysates recorded at 540 nm an Optimax microplate reader with Kinetic software SOFTmax® (Molecular Devices, USA). Percentage viability was calculated by dividing the absorbance of the treated wells by the absorbance of the negative control wells, with controls representing 100% viability. Results were obtained from two

independent experiments. For each experiment, 6 replicate wells were used for each treatment.

2.2.4 Genotoxicity Evaluation - Single Cell Gel Electrophoresis (SCGE) – 'Comet' assay

2.2.4.1 Standard protocol for detection of single strand breaks

Where applicable cells were supplemented with micronutrients and/or challenged with chemical or UVR exposures as described in later sections. The Comet assay was then performed as described previously (Tice *et al.*, 2000; others). Briefly, frosted end microscope slides were coated with 1.5% normal melting point (NMP) agarose and allowed to air dry. Trypsinised cells (approximately 2 x 10^4) were centrifuged and the resulting pellet mixed with 170 µl molten 0.75 % low melting point (LMP) agarose. This was then applied to the pre-coated slides as 2 drops of 85 µl. Coverslips were placed over each drop and gels were allowed to set at 4°C for 1 hour. When gels had solidified coverslips were gently removed and slides were immersed in cold (4°C) lysing solution (1% Triton X-100, 10% DMSO, 2.5 mM NaCl, 100 mM Na_2EDTA, 100 mM Tris, 10% sodium lauroyl sarcosinate, pH 10) for at least 1 hour. After the lysing period, slides were placed in a horizontal electrophoresis unit containing freshly prepared electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH, pH > 13). The DNA was allowed to unwind for 20 min before electrophoresis was performed at 25 V, 300 mA for 20 min at 4°C. Afterwards, the slides were gently immersed in neutralization buffer (0.4 M Tris-HCl, pH 7.4) for 10 min,

followed by a 10 minutes wash in dH_2O at 4°C. Gels were fixed by a further 10 minute immersion in cold 100% ethanol. Finally, to visualise Comets 40 µl of 20 $\mu g ml^{-1}$ ethidium bromide stain was applied to each gel. Scoring was achieved using a fluorescence microscope (Leica DMR) and Komet 5.0 image analysis software (Kinetic Imaging Ltd., Merseyside, UK), with 50 cells being scored per gel (100 per slide). Slides were independently coded and scored without knowledge of the code. Results were obtained from two independent experiments. Out of the possible 34 parameters measured, % tail DNA was chosen as it is considered to be the most meaningful (Raisuddin and Jha, 2004) although others parameters, including Olive tail moment, showed similar patterns.

2.2.4.2 Modified protocol for specific detection of oxidised DNA bases with the use of bacterial repair endonucleases

The modified Comet assay protocol for the detection of oxidised bases is essentially identical to the standard protocol (above) but with the addition of an enzyme digestion step after the lysis period and before the electrophoresis step. Nucleoids are treated with lesion-specific endonucleases, in this case formamidopyrimidine DNA glycosylase (Fpg), which converts oxidised purines into DNA single strand breaks. Fpg, and protocol for storage and use, were kindling supplied by Professor Andrew Collins, Department of Nutrition, University of Oslo, Norway (personal communication). First, slides removed from lysing solution were washed three times (for 5 minutes) with enzyme reaction buffer (0.1 M KCl, 0.5 mM Na_2EDTA, 40 mM HEPES, 0.2 mg/ml

bovine serum albumin, pH 8.0). Then, 50 µl of buffer alone (reference slides), or 1.5 µg ml^{-1} Fpg were applied to each gel along with a coverslip prior to incubation at 37 °C for 45 minutes in a humidified atmosphere. Slides were then subjected to the remaining stages of the standard Comet assay protocol.

2.2.5. UVR exposure

Cell monolayers were exposed to UVR in PBS (2 ml for 6-well plates; 200 µL for 96-well plates) to prevent desiccation during exposure. Cells were irradiated on ice (to prevent overheating) with the plate lids removed. Control cells were sham irradiated, i.e. were placed under the lamp for the same time as exposed cells, but were completely covered with aluminium foil to keep them in the dark. UVB radiation was provided by a bench lamp containing two 20 W ultraviolet-B bulbs (Phillips TL 20W/12 RS, Holland) emitting a broad spectrum of 280–370 nm (peak 310 nm). UVA was delivered from a bench lamp containing two 40 Watt bulbs (Spectroline XX-40, USA) with an output of 340-420 nm (peak 370 nm). Radiation intensity was quantified using a spectroradiometer (Macam Photometrics, Scotland, model SR9910) sensitive to wavelengths 240-800 nm.

At a distance of 10 cm, irradiation intensities were typically 0.32 and 2.34 $Wm^{-2}s^{-1}$ for UVB and UVA lamps, respectively (Fig. 2.2). Radiation intensity tended to decrease at either end of the bulbs (data not shown), so to minimise error irradiation only took place in the centre of the lamp. Exposure time was adjusted based on radiation intensity readings to yield the doses required.

UVR doses administered where based on previous studies using fish cells for cytotoxicity and genotoxicity testing (Table 2.1). Doses were calculated as follows:

$$\text{Dose } (Jm^{-2}) = \text{Peak radiation intensity } (Wm^{-2}s^{-1}) \times \text{Duration of exposure (s)}$$

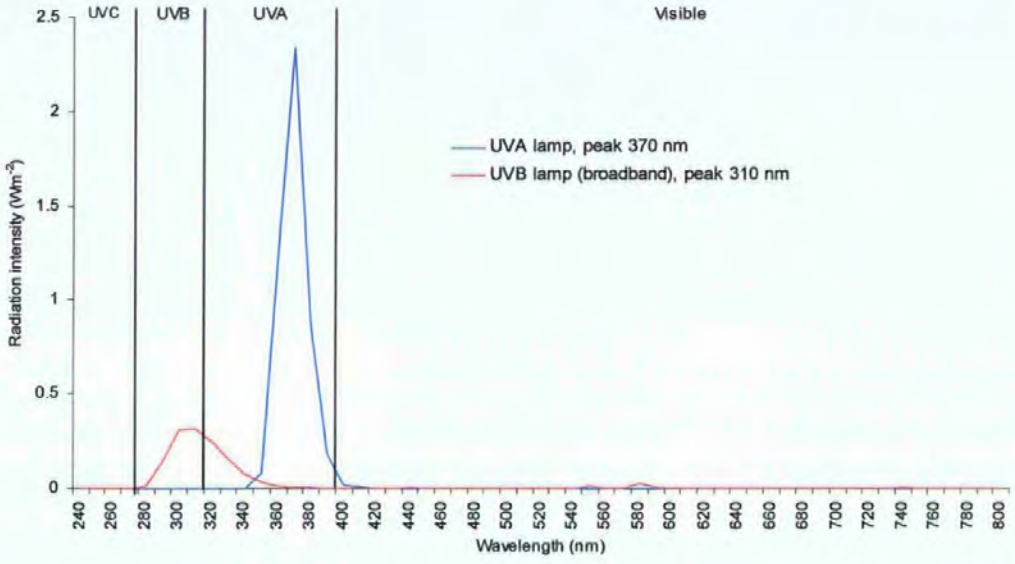


Figure 2.2 Spectral distribution of UVB and UVA lamps.

2.2.6 Statistical analysis

For NRR assay data, differences between means were analysed using the Student's *t*-test. The Mann–Whitney *U*-test was performed on Comet data to compare medium % tail DNA values. Analyses were performed using Minitab V.13.

Table 2.1. UVR exposure methods using cultured fish cells. * UVA: 320-400 nm, UVB: 280-320 nm, UVC: 100-280 nm.

Assay(s) used	Cell line/ primary culture	Exposure vehicle	Exposure conditions	Wavelength*	Intensity ($J/m^{-2}s^{-1}$)	Dose (J/m^{-2})	Duration of exposure (max)	Reference(s)
Colony formation, thymine dimmer formation	CAF-MM1 (goldfish)	60 mm dish	In PBS (2.5 ml, forming 1 mm layer)	UVC	0.44	0-8	18 s	Mano *et al.* (1980, 1982)
Colony formation, ESS	RBCF-1 (goldfish)	60/100 mm dish	In PBS	UVC	0.41	0-20	48 s	Yashuri, *et al.* (1992)
Colony formation, ESS	GEM-218 (goldfish)	60/100 mm dish	In PBS (2 ml/5 ml), on ice	UVC UVB /UVA/Vis UVA/Vis	0.24-0.76 1.0 0.3-0.75	0-8 0-2500 0-1800	33 s 45 mins 1.5 hrs	Ahmed *et al.* (1993)
Colony formation, ESS; ELISA	RBCF-1 (goldfish)	100 mm dish	Rinsed with HBSS	UVC	DNS	0-20	DNS	Uchida *et al.* (1995)
Colony formation, ESS	OL32 (medaka)	100 mm dish	In saline	Mostly UVB (270-330 nm)	12.5	400	48 s	Funayama *et al.* (1996)
Colony formation	EPC-A1 (carp)	60 mm dish	In 2 ml PBS for long exposures (i.e. UVA), lid on.	UVB UVA	2.15 3.15	0-450 0-30k	4 mins 2.5 hrs	O'Reilly & Mothersill (1997)
Histological methods	Primary lens epithelial cells (skate)	Coverslips in 35 mm dishes	In medium, lid on, temperature controlled by flowing tap water	UVA	25	135k-545k	6 hrs	Rafferty *et al.* (1997)
ELISA, gel shift assay	RBCF-1 (goldfish)	100 mm dish	Rinsed with HBSS	UVC	0.67	10	15 s	Uchida *et al.* (1997)
ELISA	RBCF-1 (goldfish)	100 mm dish	Rinsed with HBSS	UVC	0.67	10	15 s	Uchida *et al.* (1998)
Cell morphology, colony formation, DNA fragmentation, ESS, caspase activity assay.	OCP13 (medaka)	100 mm dish	In saline	UVA UVB UVC	14 22 0.6	0-50k 0-400 0-30	4 s 18 s 50 s	Nishigaki *et al.* (1999)
ESS	Primary hepatocytes (catfish)	100 x 15 mm dish	Rinsed with PBS	UVC	1.5	0-40	26 s	Willet *et al.* (2001)
Growth, colony formation, radioimmunoassay of DNA photoproducts, DNA synthesis.	A2 & PSM (*Xiphophorus*)	60/100 mm dish	Cells @ 90% confluence, in HBSS, lid on.	UVA/UVB (280-360 nm)	DNS	0-250	DNS	Moredock *et al.* (2003)

CHAPTER 3: EFFECTS OF NIACIN SUPPLEMENTATION ON UVB-INDUCED GENOTOXICITY IN CULTURED CARP CELLS *IN VITRO*

3.1 Introduction

As outlined in chapter 1, mammalian research has demonstrated that a sufficient supply of dietary niacin (vitamin B_3) is important for the synthesis of the co-enzymes NAD and NADP which are involved in vital redox reactions in normal cellular metabolism. NAD is also important for maintenance of genomic stability, both in terms of DNA repair and prevention of oxidative DNA damage, as well as for the suppression of carcinogenesis and the modulation of immunosupression (Murray, 1995; De Murcia *et al.*, 1997; Gensler, 1997; Sheng *et al.* 1998; Gensler *et al.*, 1999; Hageman and Stierum, 2001; Spronck, 2002; Kirkland, 2003).

For fish, niacin is recognised as an important vitamin that must be supplemented in formulated feeds (Lovell, 1998) because, although it can be synthesised from the amino acid tryptophan, the utilisation of dietary tryptophan as a precursor of niacin is not entirely effective. Moreover, niacin deficiency can lead to poor growth and feed utilization, anaemia and gill swellings (Lovell, 1998). Additionally in the rainbow trout, an adequate supply of niacin has been shown to be necessary for the prevention of fin damage due to ultraviolet radiation ('sunburn') (Poston and Wolfe, 1985). Insufficient niacin supply resulted in severe erosion of both the pectoral fins and the caudal fin when fish were subsequently exposed on two consecutive days to

UV radiation. This was attributed to the lack of cellular NAD, which as mentioned earlier (section 1.2.1), has significant antioxidant properties and may remove reactive oxygen species that are generated by UVR. However, in contrast to mammalian research no study has sought to evaluate the role of niacin in maintaining genomic stability in fish and in particular whether it can modulate UVR-induced DNA damage. Therefore, this chapter investigates the potential of niacin supplementation to reduce UVB-induced DNA damage in a carp skin cell line (EPC-A1) *in vitro*.

3.2 Materials and methods

3.2.1 Materials

All cell culture materials were purchased from either Invitrogen (Paisley, UK) or Bibby Sterilin (now Barloworld Scientific) (Stone, UK). All other reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

3.2.2 Cell culture

EPC-A1 cells were routinely passaged as described in section 2.2.1.

3.2.3 Validation of the standard Comet assay protocol for detection of single strand breaks

For validation experiments with ethyl methanesulphonate (EMS), H_2O_2 and UVB EPC-A1 cells were seeded in 6 well multi-plates (35 mm ∅ wells) at a density of 1 x 10^6 cells ml^{-1} (total volume of 2 ml per well) and allowed to attach overnight. To prepare cells for chemical or UVB exposure, old culture medium was removed, and cells washed twice with PBS (2 x 2 ml wash per well). Following exposures, the standard Comet assay protocol for the detection of single strand DNA breaks was then performed as described in section 2.4.2.

3.2.3.1 EMS exposure

EPC-A1 cells were incubated in complete culture medium containing 0.8-10 mM EMS for 6 hours at 20±1 °C. Initially EMS was dissolved in sterile DMSO at a concentration of 100 mM, which was kept as a stock solution at 4°C. Subsequent dilutions were made fresh in complete culture medium. After the EMS exposure old medium was removed and cells washed twice with PBS to remove any remaining extra-cellular EMS.

3.2.3.2 H_2O_2 exposure

EPC-A1 cells were incubated in complete culture medium containing 5-45 μM H_2O_2 for 10 minutes at 20±1 °C. Immediately before each experiment a 100

μM H_2O_2 stock solution was freshly prepared in PBS with subsequent dilutions being made in complete culture medium. After the H_2O_2 exposure old medium was removed and cells were washed twice with PBS to remove any remaining extra-cellular H_2O_2.

3.2.3.3 UVB exposure

UVB exposure was performed as described in section 2.6. For validation experiments EPC-A1 cells were exposed to a range of UVB doses (0 – 600 Jm^{-2}) by varying the duration of exposure under the lamp (section 2.4.1).

3.2.4 Niacin supplementation experiments

EPC-A1 cells were seeded in 6 well multi-plates (35 mm ∅ wells) at a density of 1 x 10^6 cells ml^{-1} (total volume of 2 ml per well) and allowed to attach overnight. To prepare cells for niacin supplementation, old culture medium was removed, and cells washed twice with PBS (2 x 2 ml wash per well). Cells were then incubated in complete culture medium containing 0.18-1.0 mM niacin (nicotinamide; nicotinic acid amide [NAM]) at 20±1 °C for 48 hours. The basal level of NAM in the culture medium was 0.01 mM based on information given on the MEM ingredients given by Invitrogen. A stock solution of 10 mM cell culture tested NAM was prepared in sterile PBS and subsequent dilutions made in complete culture medium to working concentrations. Following the incubation with niacin, cells were exposed to a single dose of UVB at 450 Jm^{-2} as described in section 2.6. For experiments with UVB and niacin two controls

were used – a negative control (no UVB exposure and no niacin supplementation) and a positive control (UVB exposure with no niacin supplementation).

3.3 Results

3.3.1 Comet assay validation

Validation experiments for the Comet assay showed clear dose-response relationships for reference genotoxins EMS and H_2O_2 and for UVB (Fig. 3.1). From UVB validation experiments it was decided that during irradiation experiments with niacin, a UVB dose of 450 Jm^{-2} would be used as at this level DNA damage was highly significant although not the highest level of DNA damage to be measured.

3.3.2 Niacin supplementation experiments

Niacin supplementations of 0.56 and 1.0 mM significantly reduced UVB induced DNA damage in EPC-A1 cells (Mann-Whitney U, $p < 0.05$ and < 0.001 respectively, Fig. 3.2) This represents a 16% and 24% reduction in DNA damage compared to the positive control.

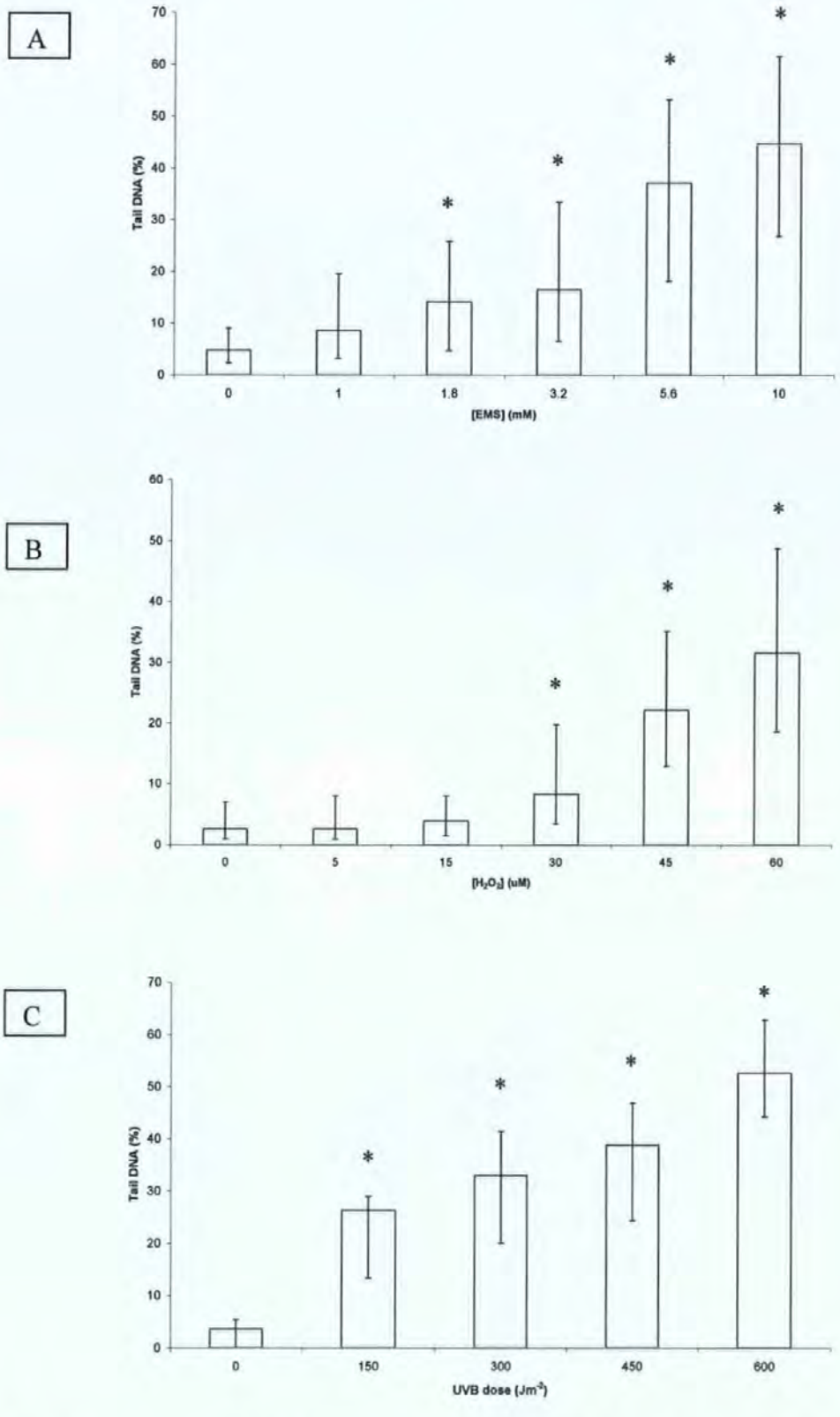


Fig. 3.1 Validation of the standard Comet assay. EPC-A1 cells exposed to (a) EMS (b) H_2O_2 and (c) UVB. Data points represented as median values. Interquartile ranges give a measure of data spread. * denotes significant difference versus negative control (P <0.05).

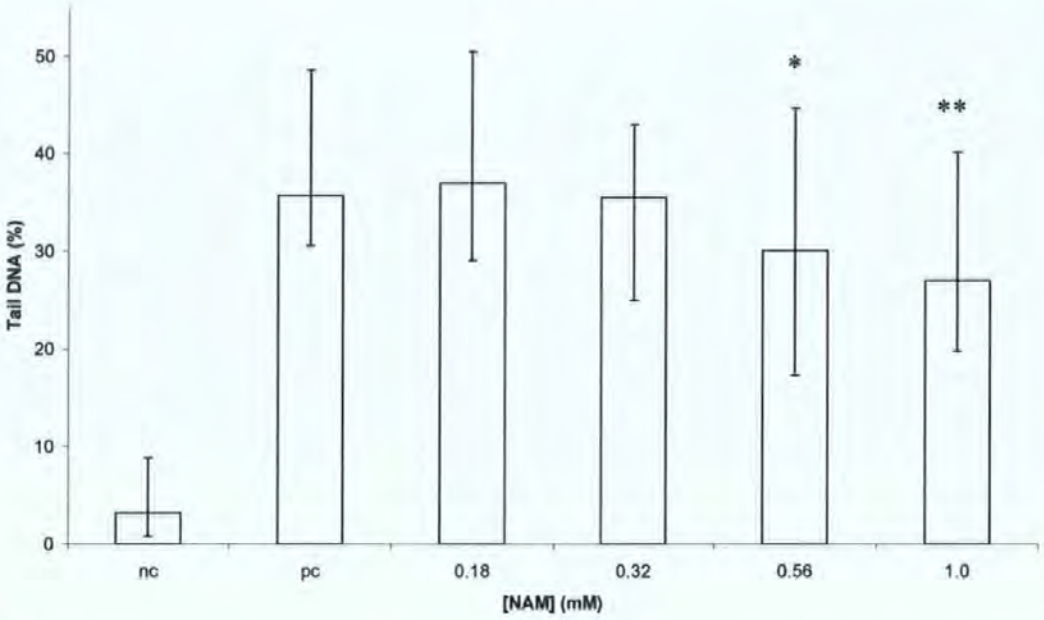


Fig. 3.2 Effect of niacin (NAM) supplementation on UVB-induced DNA damage in EPC-A1 cells as measured by the Comet assay. Data points represented as median values. nc = negative control – sham irradiated (no UVB) and no NAM supplementation; pc = positive control – UVB irradiated and no NAM supplementation. Interquartile ranges give a measure of data spread. Significant differences versus positive control indicated by * (P <0.05) and ** (p <0.01).

3.4. Discussion

The Comet assay was validated with the two reference genotoxins, hydrogen peroxide (H_2O_2) and ethyl methanesulphonate (EMS) which cause DNA SSB via different mechanisms. Comet assay validation is recommended in order to confirm it's sensitivity and ability to distinguish between DNA damage levels

over a range of genotoxin concentrations/doses (Tice *et al.*, 2000). In this investigation, validation experiments clearly show that H_2O_2 and EMS were able to induce increased levels of DNA damage (SSBs) compared to control cells, in a dose-dependent manner. Therefore, H_2O_2 and EMS fulfil the criteria of positive controls in this study by generating reliable, sensitive and reproducible responses (Tice *et al.*, 2000).

Hydrogen peroxide (H_2O_2) was used as a reference genotoxin because of its action in producing cellular DNA damage due to the formation of single strand breaks (SSBs). Although H_2O_2 does not directly interact with DNA, it is converted to hydroxyl radicals (OH) through the metal-catalysed (Cu, Fe) Fenton reaction (Cotelle & Ferard, 1999). Therefore, H_2O_2 can be considered as a reactive intermediate that is involved in oxidative DNA damage induced by OH radicals. Although the induction of SSBs by H_2O_2 was probably due to cellular oxidative stress, in the form of increased OH production, the exact origin of the SSBs is unclear. In response to an increased level of ROS, SSBs may occur in DNA due to a number of reactions. For example, SSBs may arise from direct scission of the DNA phosphodiester backbone by radical attack (Horvathova *et al.*, 1998). They may also result from incomplete base excision repair (BER), which deals with small alterations to DNA bases such as oxidation products (e.g. 8-OHdG) (Horvathova *et al.*, 1998; Collins and Harrington, 2002). The simplistic version of the Comet assay used in this investigation was not designed to discern between the different reactions that lead to the formation of SSBs via H_2O_2.

EMS does not directly induce SSBs in DNA, as it does not induce direct scission of the DNA backbone. Instead EMS acts through alkylation of several positions on DNA molecules (Horvathova *et al.*, 1998). Base modification from alkylation causes weakening of the *N*-glycosidic bond which leads to depurination/depyrimidination and the appearance of alkali-labile sites (ALS). ALS are removed by ALS endonucleases, which cleave DNA adjacent to the ALS, creating SSBs. Again, the standard version of the Comet assay used in this investigation was not designed to distinguish between different reactions that lead to SSBs, including those alkylation reactions that may have lead to EMS induced SSBs.

Exposure of EPC-A1 cells to UVB caused a dose dependent increase in DNA damage, in the form of DNA SSB, as evaluated by the Comet assay. Previously, the Comet assay has been extensively used in investigating the DNA-damaging effects of UVR on mammalian cells (Lyons and O'Brien, 2002; Bruge *et al.*, 2003; Salter *et al.*, 2004) but apparently has not been applied to investigating UVR-induced DNA damage in fish cells before. Therefore, the measurement of UVR-induced SSBs by the Comet assay in the EPC-A1 cell line has been achieved for the first time in this investigation. Previously, ecotoxicological studies have reported strand breaks, as measured by the Comet assay, solely in response to xenobiotic or metal exposure (Mitchelmore and Chipman, 1998; Kamar, 2002; de Andrade and de Freitas, 2004).

It is well known that ultraviolet radiation is able to induce several classes of photochemical change in DNA, either through direct absorption of incident

photons or through the generation of reactive oxygen species (see section 1.1.1) (Cridland and Saunders, 1994; Kielbassa, *et al.*, 1997; Gruijl, 2002; Ichihashi *et al.*, 2003). The standard version of the Comet assay used in this investigation could only measure the ultimate formation of SSBs in response to UVB exposure. The exact origin of these breaks, and hence the mechanism of DNA photodamage is not known, and can only be postulated. Reportedly, the main DNA adducts formed in animal cells during ultraviolet radiation exposure are cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts, which is due mainly to the direct absorption of incident photons during UVB exposure (Kielbassa, *et al.*, 1997; Meador *et al.*, 2000; Ichihashi *et al.*, 2003). The standard version of the Comet assay is not capable of detecting these. However, incomplete nucleotide excision repair (NER) of these dimers may result in the formation of DNA single strand breaks, which would be detected by the standard Comet assay. The manifestation of this type of DNA damage in this study is unlikely as cells were not afforded a period of recovery time post UVB exposure, before performing the Comet assay, to allow repair mechanisms to be induced. Furthermore, many studies have reported fish cells to have limited capacity for DNA excision repair, if present at all (Bailey *et al.*, 1996, Willet *et al.*, 2001).

The DNA damage detected in EPC-A1 cells is more likely to be due to the production of ROS and oxidative stress. For example, singlet molecular oxygen ($^1O_2$) and hydroxyl radicals (OH) are produced by UVB radiation, which may cause direct scission of the DNA backbone, causing SSBs (Cridland and Saunders, 1994; Kielbassa, *et al.*, 1997; Ichihashi *et al.*, 2003).

Results from this study show that niacin (NAM) supplements of 0.56 and 1.0 mM significantly reduced UVB-induced DNA damage in EPC-A1 cells compared to the positive control (irradiated cells with no NAM supplementation). In fish, sufficient dietary niacin is necessary for preventing gross pathologies that may lead to reduced growth and feed utilization (Lovell, 1998). However, there is no available literature on the influence of dietary niacin on genomic stability in fish. Therefore this study is the first to report the protective effect of niacin on UVB-induced DNA damage in fish. However, in terms of preventing the detrimental effects of UVR, this study support the findings of Poston & Wolfe (1985) who found that an adequate supply of dietary niacin was necessary to prevent erosion of both the pectoral fins the caudal fin in UV exposed rainbow trout.

It is possible that a niacin-dependent decrease in UVB-induced DNA damage could be due to an increase in the synthesis of NAD, which is the sole substrate for the enzyme poly (ADP-ribose) polymerase-1 (PARP-1), which is involved in DNA excision repair (Hageman and Stierum, 2001). NAD is also required for nucleotide and DNA synthesis. Increasing the cellular pools of available NAD could have resulted in increased levels of PARP-1 expression and activity, and hence repair of SSBs induced by UVB. Other studies have shown that mice and cells defective in PARP-1, or with low or depleted intracellular NAD levels, had elevated spontaneous genetic rearrangements and abnormal responses to DNA damaging agents (de Murcia *et al.*, 1997; Wang *et al.*, 1997; LeRhun *et al.*, 1998; Spronck & Kirkland, 2002). However, as discussed in the section above (3.4.2), cells were processed for the Comet

assay immediately after UVB exposure, so any DNA repair mechanisms, including those involving PARP-1, would have had little time to complete the excision repair of damaged DNA strands. Also as discussed previously, in some studies fish have been shown to have little or no capacity for DNA excision repair pathways (Bailey *et al.*, 1996, Willet *et al.*, 2001).

A more likely explanation is an increased resistance to oxidative stress induced by UVB in cells supplemented with niacin. Niacin has been reported to have a strong antioxidant capacity *in vitro*, greater than that of ascorbic acid (Kamat and Devasagayam, 1996). Increasing the antioxidant capacity of EPC-A1 cells with niacin supplements may have enhanced their ability to scavenge reactive oxygen species ($^1O_2$ and OH) produced by UVB that attack DNA strands. Indeed, in rats niacin deficiency has been shown to increase susceptibility of DNA to oxidising agents (Zhang *et al.*, 1993; Rawling *et al.*, 1994; Boyonoski, *et al.*, 1999). Furthermore, in human trials niacin supplementation has been reported to increase resistance against oxidative DNA damage in peripheral lymphocytes (Weitberg, 1989; Sheng *et al.*, 1998).

Overall, these results suggest that niacin may be important in maintaining genomic stability in fish, especially during exposure to environmental genotoxins such as UVR, by increasing the antioxidant potential of cells. The possible role of niacin-dependent PARP-1 and DNA repair in protecting fish from genomic instability remains to be defined. Further work must be done to establish whether dietary niacin supplements may help protect fish from

environmental and anthropogenic stress factors which may result in genomic instability *in vivo*.

CHAPTER 4: EFFECTS SELENIUM SUPPLEMENTATION ON H_2O_2 and UVR-INDUCED CYTOTOXOCITY AND GENOTOXICITY IN CULTURED GOLDFISH CELLS *IN VITRO*

4.1 Introduction

As outlined in chapter 1, selenium is an essential trace element for animal nutrition. In fish optimal levels of selenium in the diet are required for optimal growth, protection of cells and tissues from oxidative injury and disease resistance. Many of the nutritional roles of selenium that have been investigated in mammal research have also been studied in fish. However, there is a lack of information on the role of selenium in the maintenance of genomic stability in fish, which is surprising given the considerable attention this area of research has received in mammalian studies (McKenzie, 2000; Rayman, 2000; El-Bayoumy, 2001).

Also, considering the increasing awareness of the detrimental effects of UVR exposure for fish, there is a requirement to fill the knowledge gap on the possible beneficial effects of selenium supplementation on UVR induced oxidative stress (and other sourses of oxidative stress) and genomic instability. Indeed, selenium has been shown to protect against the many deleterious effects of UVR exposure both *in vitro* and *in vivo,* in mammalian studies including DNA damage and carcinogenesis.

Table 4.1 summarises selected studies on the effects of selenium supplementation on UVR exposure in cultured cells *in vitro*. Supplementation with sodium selenite and/or selenomethionine has been shown to reduce UVA/UVB-induced apoptosis and cell death in human keratinoctyes and melanocytes (Rafferty *et al.*, 1998, 2003). Selenium supplements in selenium-depleted human fibroblasts also protected against UVA and UVB induced cytotoxicity and lipid peroxidation, which was suggested to be mediated by a concurrent increase in glutathione peroxidase activity (Moysan *et al.*, 1995). A combination of thiols and selenium protected human skin fibroblasts against UVA induced cytotoxicity, reportedly through the up regulation of glutathione (GSH) synthesis. GSH is not only a radical scavenger itself, but also a co-factor for the antioxidant enzyme glutathione peroxidase (Emonet *et al.*, 1997). As well as selenites and seleno-amino acids, glutathione peroxidase mimetics, containing selenium, have been shown to be protective against UVR. UVB-induced keratinocyte apoptosis was inhibited by the GPX mimic, 2-selenium-bridged β-cyclodextrin (2-SeCD), and was attributed to the elimination of H_2O_2 (Mu *et al.*, 2003). Another GPX mimic, selenium-containing single-chain Fv catalytic antibody (Se-scFv2F3) protected rat epidermal cells from UVB induced cell death characterised by decreases in lipid peroxidation (malondialdehyde) and H_2O_2, and the normalisation of lactate dehydrogenase activity (Sun *et al*, 2003).

In vitro selenium supplements also protected against broadband UVR induced oxidative DNA damage in human keratinoctyes, as measured by a modified Comet assay, utilising lesion specific enzymes to detect oxidised DNA bases

Table 4.1. Selected studies on the effects of *in vitro* selenium supplementation on UVR exposure

Study #	Form of selenium	Cells used	Damaging agent	Level Se used (in addition to Se in serum/medium)	Se exposure period	Parameter measured (outcome)	Ref. #
1	Sodium selenite, selenomethionine	Keratinocytes and melanocytes	UVB radiation	*Culture medium analysed:* Melanocyte medium – 28 nM Keratinocyte medium – 8.9 nM DMEM + 10 % FBS – No se detected *UV experiments:* 1nM to 1 µM for both	24 hrs	Cell death - apoptosis (R)	Rafferty *et al* (1998)
2	Sodium selenite, DL selenomethionine	Human keratinocytes	UVB radiation	Selenite – 50 nM L^{-1} Selenomethionine – 200 nM L^{-1}	24 hrs	Oxidative DNA damage – Comet (R). CPD formation and excision repair (NE).	Rafferty *et al* (2003) (a)
3	2-SeCD (GSHPx mimic)	NIH3T3 cells	UVB radiation	8-64 µM	4 hrs	DNA damage (R) Lipid peroxidation (R) H_2O_2 content (R) Cell viability (I) P53 and Bcl-2 expression (R & I respectively)	Sun *et al* (2005)
4	Sodium selenite, selenomethionine	Human keratinocytes	UVB radiation	Selenite – 10-1000 nM Selenomethionine – 50-1000 nM	24 hrs	Apoptosis (R) p53 expression (NE)	Rafferty *et al* (2003) (b)
5	Sodium selenite	Human skin fibroblasts	UVA radiation	600 nM	3 weeks	DNA damage – Comet (R)	Emonet-Piccardi *et al* (1998)
6	Sodium selenite	Mouse keratinocytes	UVB radiation	5 or 12.5 µM	2 days	8OHdG measured by HPLC (R)	Stewart *et al* (1996)
7	Sodium selenite, selenomethionine	PAM 212 cells	UVB radiation	Selenite – 1-50 nM L^{-1} Selenomethionine – 50-200 nM L^{-1}	24 hrs	Interleukin 10 expression (R)	Rafferty *et al.* (2002)
8	Se-scFv2F3 (Se containing catalytic antibody, mimic of GSHPx)	Rat epidermal cells	UVB radiation	0.4 U ml^{-1}	4hrs	Cell viability (I) Lipid peroxidation (R) H_2O_2 content (R)	Emonet *et al* (1997)
9	Selenium yeast, as part of antioxidant combination	Human skin fibroblasts and HaCaT cells	UVA radiation	2.5-50 µg ml^{-1}	3 hrs	Cell viability (I) Antioxidant capacity (I) Histological recordings	El Hindi *et al* (2004)
10	Spring water containing elemental Se, Sodium selenite	Human skin fibroblasts	UVA/UVB radiation	320 nM	3 or 10 days	Cell viability (I) Lipid peroxidation (R) GSHPx activity (I)	Moysan *et al* (2000)
11	Sodium selenite	Human skin fibroblasts	UVA radiation	1.27 µM	3 sub cultures	GSHPx (I) Cell viability (I) Lipid peroxidation (R)	Leccia *et al* (1993)

(Rafferty *et al.*, 2003). Furthermore, a reduced number of oxidised DNA base adducts (8-hydroxydeoxyguanosine), evaluated by HPLC, was reported in UVB-exposed mouse keratinoctyes supplemented with selenite (Stewart *et al.*, 1996). Selenium also reduces UVB-induced oxidative stress mediated release of cytokines such as interleukin-10, which could suppress cell mediated immunity (photoimmunesupression) (Rafferty *et al.*, 2002).

In vivo studies show that dietary and topically applied selenium protects against UVB-induced skin cancers in mice (Burke *et al.*, 1992, Pence *et al.*, 1994), although it is not yet clear whether selenium protects against skin cancer in humans. The mechanisms of the anticancer properties of selenium are not fully understood but the antioxidant properties of selenium and selenoproteins may be partly responsible.

In light of this information the aims of this chapter were to:

(a) develop sensitive and reproducible assays for the detection of H_2O_2, UVA and UVB induced cytotoxicity and oxidative DNA damage in cultured goldfish skin cells. In the previous chapter (3.0), UVB-induced DNA damage, in the form of SSB, was determined using the standard version of the Comet assay. However, in this chapter this assay was modified to include the use of the lesion specific enzyme Fpg which increases the assays sensitivity and specificity for the detection of oxidised DNA bases.

(b) evaluate the potential protective effects of two sources of selenium against H_2O_2, UVA and UVB induced cytotoxicity and oxidative DNA damage in cultured goldfish skin cells.

4.2 Materials and methods

4.2.1 Cell culture

GFSk-S1 cells were routinely passaged fortnightly as described in section 2.2.1.

4.2.2 Validation of Neutral Red Retention (NRR) cytotoxicity assay

GFSK-S1 cells were seeded in 96 well tissue culture treated multi-plates at a density of 1 x 10^5 cells ml^{-1} (total volume of 200 µl per well) and allowed to attach overnight. To prepare cells for H_2O_2 and UVA/UVB exposure, old culture medium was removed, and cells washed twice with PBS (2 x 200 µl wash per well). Following exposures, the NRR assay was performed as described in 2.3.

4.2.2.1 H_2O_2 exposure

GFSK-S1 cells were then incubated in complete culture medium containing 1-5 mM H_2O_2 for 24 hours at 20±1 °C. Immediately before each experiment a 100 mM H_2O_2 stock solution was freshly prepared in PBS with subsequent dilutions

being made in complete culture medium. After H_2O_2 exposure, medium was removed and cells washed twice with PBS to remove any remaining extra-cellular H_2O_2.

4.2.2.2 UVA/UVB exposure

UVA and UVB exposures were performed as described in section 2.6. For NRR assay validation experiments with UVA, GFSk-S1 cells were exposed to doses of 0 – 10 kJm^{-2} by varying the duration of exposure under the lamp. After the exposure period PBS was removed and cells were re-incubated in fresh complete culture medium for a further 24 hours, before commencing the NRR protocol. For validation experiments with UVB cells were exposed to doses 0 – 60 Jm^{-2} in the same way.

4.2.3. Validation of the modified Comet assay protocol for the detection of oxidised bases

GFSK-S1 cells were seeded in 6 well tissue culture treated multi-plates (35 mm ∅ wells) at a density of 1 x 10^5 cells ml^{-1} (total volume of 2 ml per well) and allowed to attach overnight. To prepare cells for H_2O_2 and UVA/UVB exposure, old culture medium was removed, and cells washed twice with PBS (2 x 2 ml wash per well). Following exposures, the modified Comet assay protocol for the detection of oxidised bases was then performed as described in section 2.4.3.

4.2.3.1 H_2O_2 exposure

GFSK-S1 cells were incubated in complete culture medium containing 1-20 μM H_2O_2 for 10 minutes at 20±1 °C. Immediately before each experiment a 100 μM H_2O_2 stock solution was freshly prepared in PBS with subsequent dilutions being made in complete culture medium. After H_2O_2 exposure, medium was removed and cells washed twice with PBS to remove any remaining extra-cellular H_2O_2.

4.2.3.2 UVA/UVB exposure

UVA and UVB exposures were performed as described in section 2.6. For Comet assay validation experiments with UVA GFSk-S1 cells were exposed to a doses of 0 – 10 kJm^{-2} by varying the duration of exposure under the lamp. For validation experiments with UVB cells were exposed to doses 0 – 60 Jm^{-2} in the same way.

4.2.4 Selenium supplementation experiments

Selenium was supplemented to cells in the form of an inorganic salt (sodium selenite) and in an organically bound form as the amino acid seleno-L-methionine. Both compounds were dissolved in sterile PBS to make 1000 μM stock solutions which were then aliquoted and stored at -20°C. Further dilutions were made in complete culture medium to working concentrations. In the case of selenomethionine, where concentrations up to 1000 μM were used

for toxicity testing, initial dilutions were made in culture medium and not PBS. The background selenium concentration in the complete culture medium was determined by ICP-MS and found to be 32 ± 5 nM (assuming 10% supplementation of FBS).

Relative toxicities of the two compounds were initially investigated over a wide range of concentrations using the NRR assay. GFSK-S1 cells were seeded in 96 well tissue culture treated multi-plates at a density of 1 x 10^5 cells ml^{-1} (total volume of 200 µl per well) and allowed to attach overnight. Old medium was removed and cells incubated in fresh complete culture medium containing 2-100 µM for sodium selenite and 2-1000 µM for selenomethionine at 20±1 °C for a period of 96 hours. Data obtained from these experiments formed the rationale for selenium supplementation levels used in further experiments. The levels used are also similar to those used in fish and mammalian cell culture studies cited in the literature (Table 4.1.).

4.2.4.1. Effect of selenium supplementation on H_2O_2, UVB and UVA-induced cytotoxicity

GFSk-S1 cells were seeded in 96 well multi-plates at a density of 1 x 10^6 cells ml^{-1} (total volume of 200 µl per well) and allowed to attach overnight. Cells were then incubated in fresh complete culture medium containing 2-10 µM sodium selenite or selenomethionine at 20±1 °C for a period of 96 hours. Following the selenium supplementation period, old medium was removed and cells were challenged with either H_2O_2, UVA or UVB.

For H_2O_2 exposure, cells were incubated with complete culture medium (supplemented with selenium as before) containing 330 μM H_2O_2 at 20±1 °C for 24 hours. Post exposure cells were washed twice with PBS and the NRR assay performed as described in section 2.3.

UVA and UVB exposures were administered as described in section 2.6. For UVA experiments cells were exposed to 7.5 kJm^{-2}. Two controls were used: a negative control (no UVA exposure and no selenium supplementation) and a positive control (UVA exposure with no selenium supplementation). After the exposure period PBS was removed and cells were re-incubated in fresh selenium supplemented culture medium for a further 24 hours, before commencing the NRR protocol as described in section 2.3. For UVB experiments cells were exposed to 45 Jm^{-2} in the same way.

4.2.4.2. Effect of selenium supplementation on H_2O_2, UVB and UVA-induced oxidative DNA damage

GFSk-S1 cells were seeded in 6 well multi-plates at a density of 1 x 10^6 cells ml^{-1} (total volume of 2 ml per well) and allowed to attach overnight. Cells were then incubated in fresh complete culture medium containing 2 μM sodium selenite or selenomethionine at 20±1 °C for a period of 96 hours. Following the selenium supplementation period, old medium was removed and cells were challenged with H_2O_2, UVA or UVB.

For H_2O_2 exposure, cells were incubated with complete culture medium containing 5 μM H_2O_2 at 20±1 °C for 10 minutes. Post exposure cells were washed twice with PBS and the modified Comet assay performed as described in section 2.4.3.

UVA and UVB exposures were administered as described in section 2.6. For UVA experiments cells were exposed to 5.0 kJm^{-2} with positive and negative controls. The modified Comet assay was then performed as described in section 2.4.3. For UVB experiments cells were exposed to 15 Jm^{-2} in the same way.

4.3. Results

4.3.1 Assay validation

Validation experiments for the NRR assay showed clear dose–response relationships for H_2O_2, UVA and UVB exposure (Fig. 4.1). Significant decreases ($P < 0.05$) in cell viability were observed after exposure to 3-5 mM H_2O_2 , 2.5-7.5 kJm^{-2} UVA and 15-60 Jm^{-2} UVB.

Validation of the modified Comet assay also showed clear dose–response relationships for H_2O_2, UVA and UVB, particularly for Fpg-sensitive sites which greatly increased the sensitivity of the assay (Fig. 4.2). Concentrations of H_2O_2 ≥5 μM produced significant increases ($P < 0.05$) in direct SSB and Fpg-sensitive sites. For UVB, significant increases were also observed in direct

SSB and Fpg-sensitive sites after exposure to doses ≥ 15 kJm^{-2}. For UVA, significant increases were only observed in Fpg-sensitive sites (≥2.5 kJm^{-2}).

From these results, 330 μM H_2O_2, 7.5 kJm^{-2} UVA and 45 Jm^{-2} UVB were chosen as positive controls in the NRR assay for further experiments involving selenium supplementation. For the modified Comet assay, 5 μM H_2O_2, 5.0 kJm^{-2} UVA and 15 Jm^{-2} UVB were chosen as positive controls. These doses were considered optimal as they were the lowest doses tested that caused highly significant increases in cytotoxicity and oxidative DNA damage (Fpg-sensitive sites).

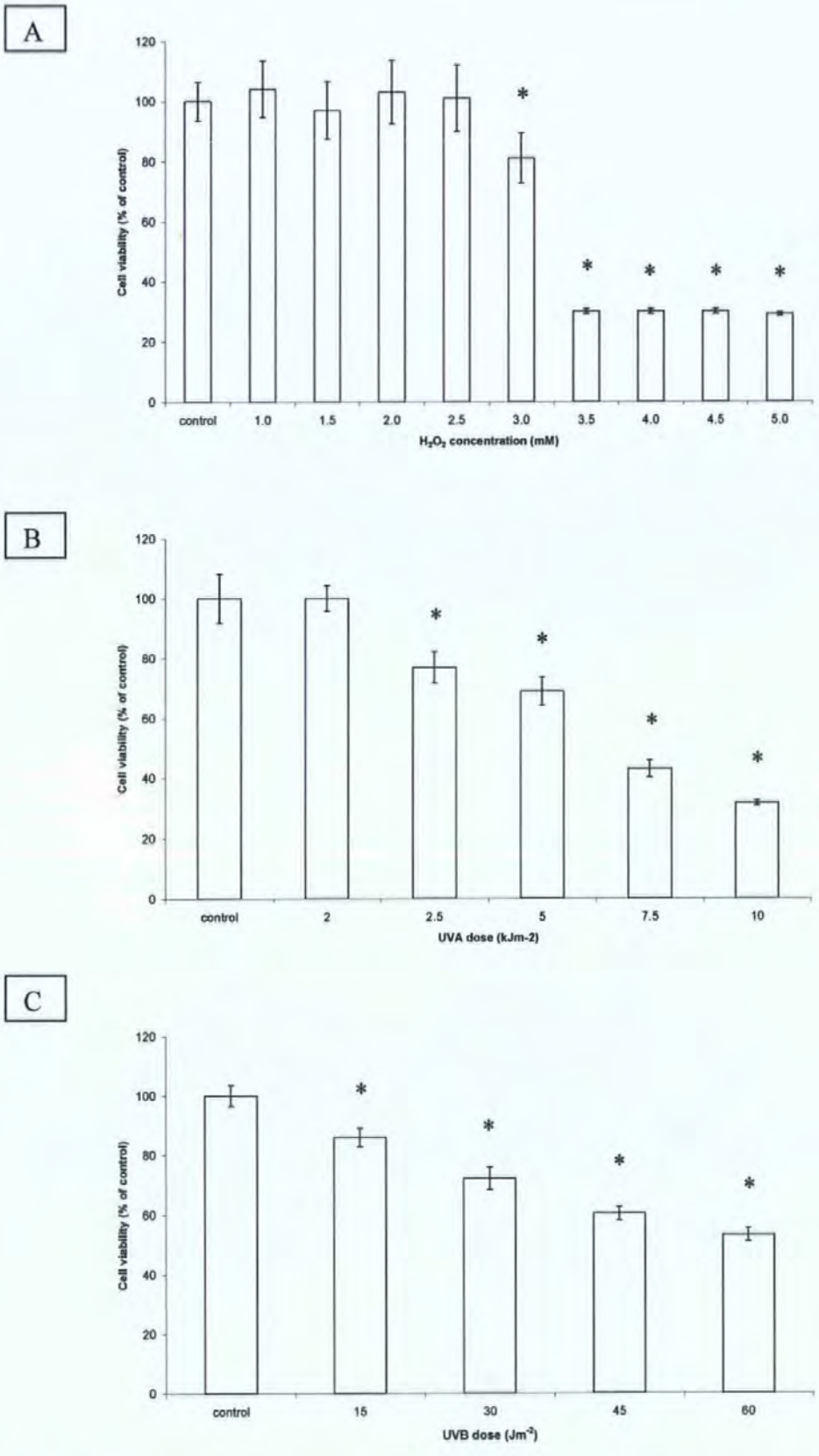


Fig. 4.1. Validation of the NRR assay. GFSk-S1 cells exposed to (a) H_2O_2, (b) UVA and (c) UVB. Data represented as means ± S.E. * denotes significant difference versus control (P <0.05).

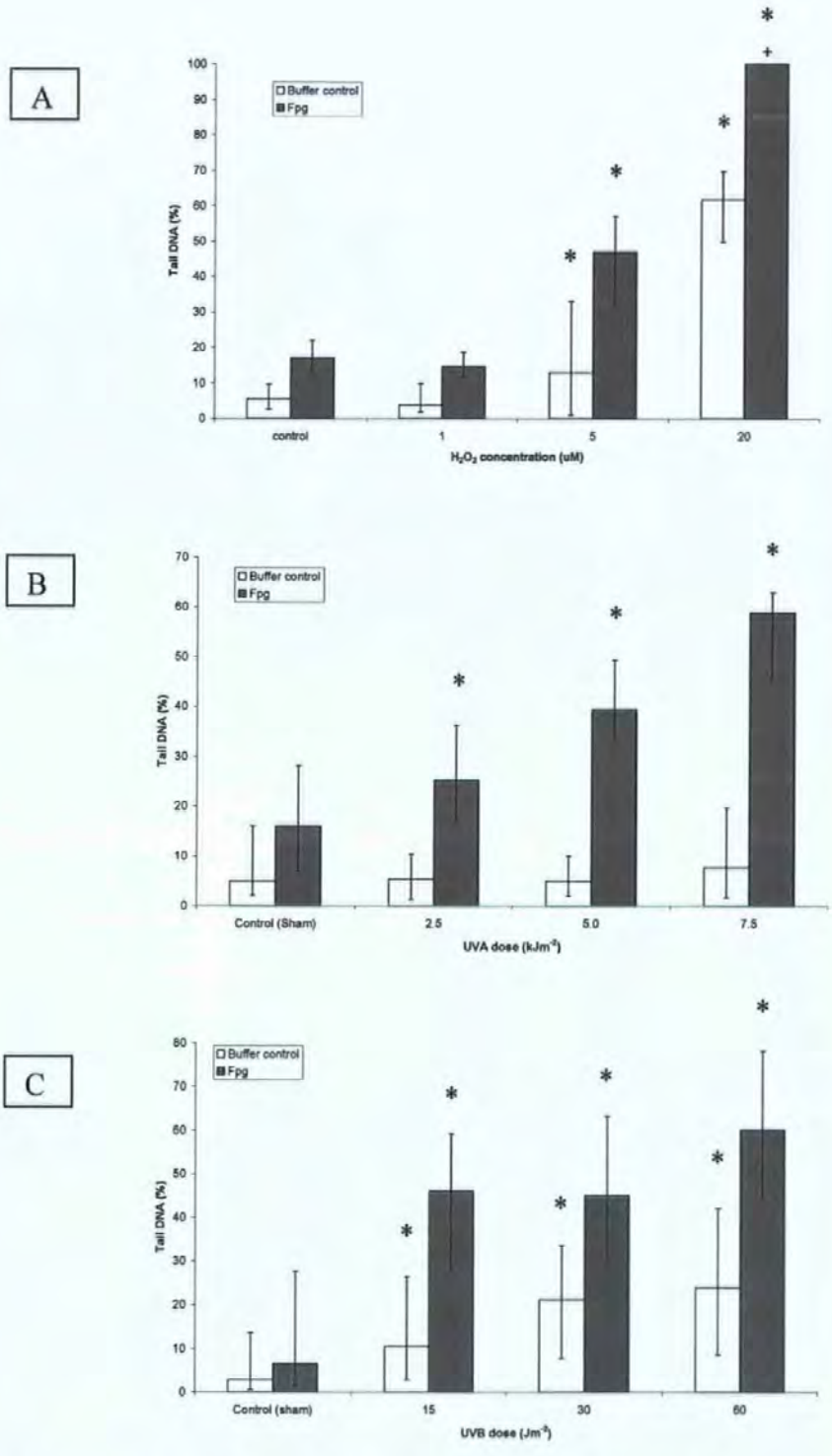


Fig. 4.2. Validation of the modified Comet assay. GFSk-S1 cells exposed to (a) H_2O_2 and (b) UVA and (c) UVB. Tail DNA (%) expressed as median values. Interquartile ranges give a measure of data spread. * denotes significant difference versus control (P <0.05).

4.3.2. Relative toxicity of selenium compounds to GFSk-S1 cells

Supplementation with both selenite and selenomethionine at low concentrations (2-20 μM) significantly improved cell viability compared to control cells after a 4 day incubation period ($P < 0.05$) (Fig. 4.3). At higher concentrations sodium selenite became extremely toxic, reducing viability to ~30 % of control. Contrastingly though, for selenomethionine concentrations up to 1000 μM showed no signs of toxicity with cell viability maintained at > 90%, although the beneficial effects of supplementation gradually diminished at concentrations > 100 μM. Based on these data, under normal conditions, the optimal concentration of selenium for maintaining cell viability appears to be 5-20 μM sodium selenite (18% increase in viability) and 100 μM selenomethionine (30% increase in viability). For further experiments involving H_2O_2 and UVR exposures, selenium concentrations were based on these results as well as those previously used in other *in vitro* studies (Table 4.1).

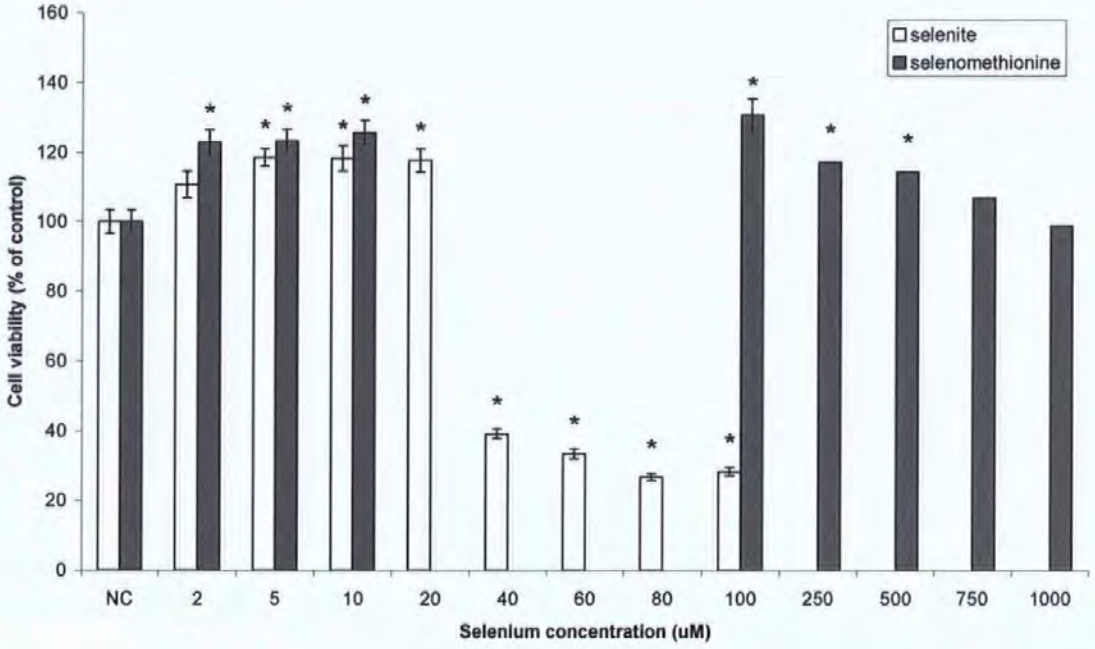


Fig. 4.3 Effect of inorganic (sodium selenite) and organic (selenomethionine) selenium supplementation (96 hours) on viability of GFSk-S1 cells as determined by the NRR assay. Data represented as means ± S.E. * denotes significant differece ($P < 0.05$) vs. negative control (NC).

4.3.3 Effect of selenium supplementation on H_2O_2, UVA & UVB induced cytotoxicity

Exposure of GFSk-S1 cells to H_2O_2, UVA and UVB caused decreases of ~50% in viability compared to non-irrdiated controls. Pre-treatment for 96 hours with selenite or selenomethionine prevented cytotoxicity induced by all three stressors, to varying degrees ($P < 0.05$, Figs. 4.4, 4.5, 4.6). For H_2O_2-induced cytotoxicity, 2 and 5 μM selenium supplements restored cell viability to almost that of negative control levels (90-98% of control), although 10 μM selenium had no such protective effect. Selenium supplements of 2 and 5 μM also

increased cell viability after exposure of cells to UVA, from 55% to 70-78%. For UVB-induced cytotoxicity only the 2 μM selenium supplementation level was shown to have a protective effect, increasing viability from 47% to ~60% of the negative control cells. The source of selenium supplement (sodium selenite or selenomethionine) appeared to have no significant effect on any of the cell viability responses measured.

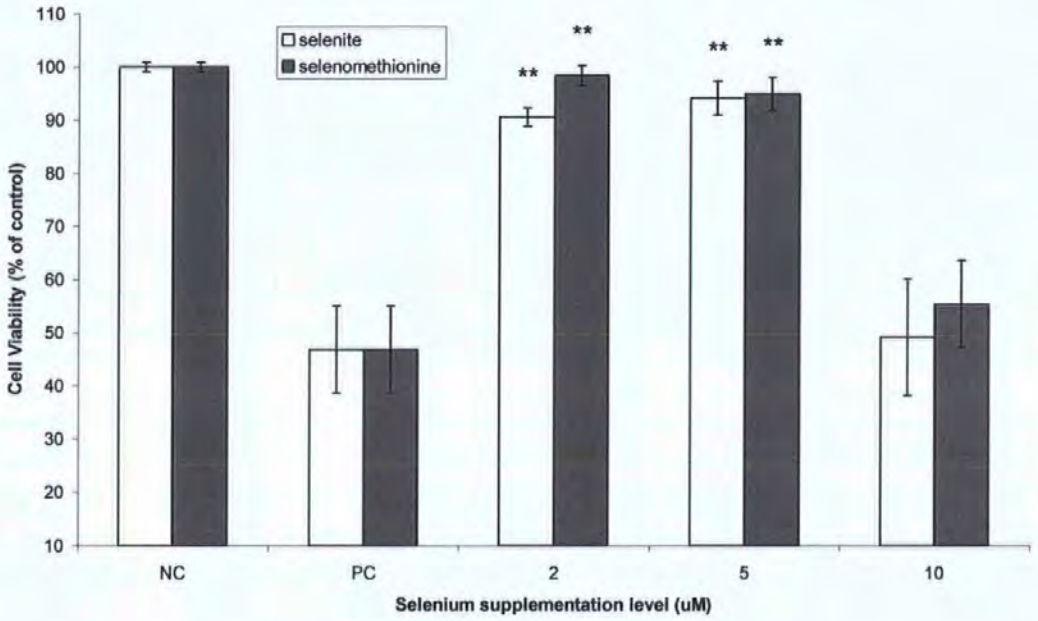


Fig. 4.4 Effect of inorganic (sodium selenite) and organic (selenomethionine) selenium supplementation (96 hours) on viability of GFSk-S1 cells when challenged with H_2O_2 as determined by the NRR assay. Data represented as means ± S.E. ** denotes significant difference (P<0.001) vs positive control (PC) cells. Positive control = 330 μM H_2O_2 treatment (24 hours).

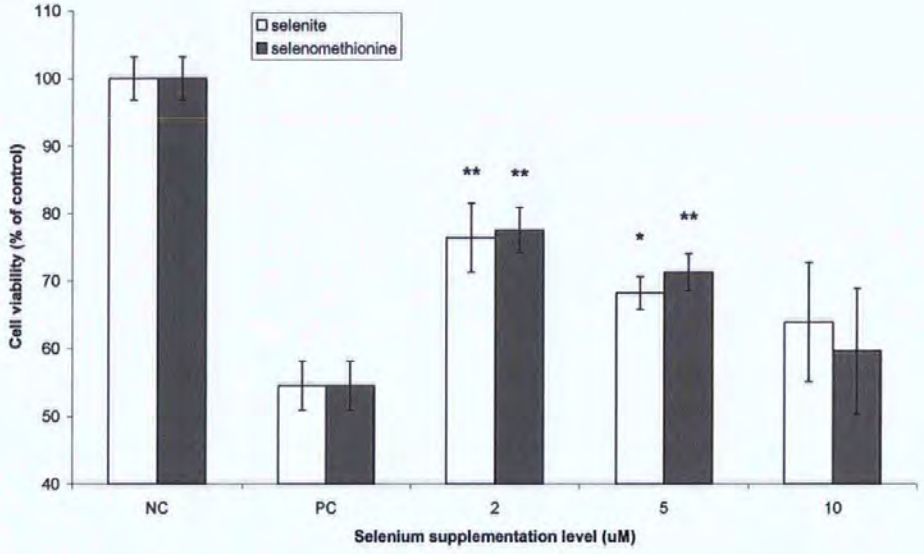


Fig. 4.5 Effect of inorganic (sodium selenite) and organic (selenomethionine) selenium supplementation (96 hours) on viability of GFSk-S1 cells when challenged with UVA as determined by the NRR assay. Data represented as means ± S.E. ** denotes significant difference (P<0.001) vs positive control (PC) cells. Positive control = 7.5 kJm^{-2} UVA dose.

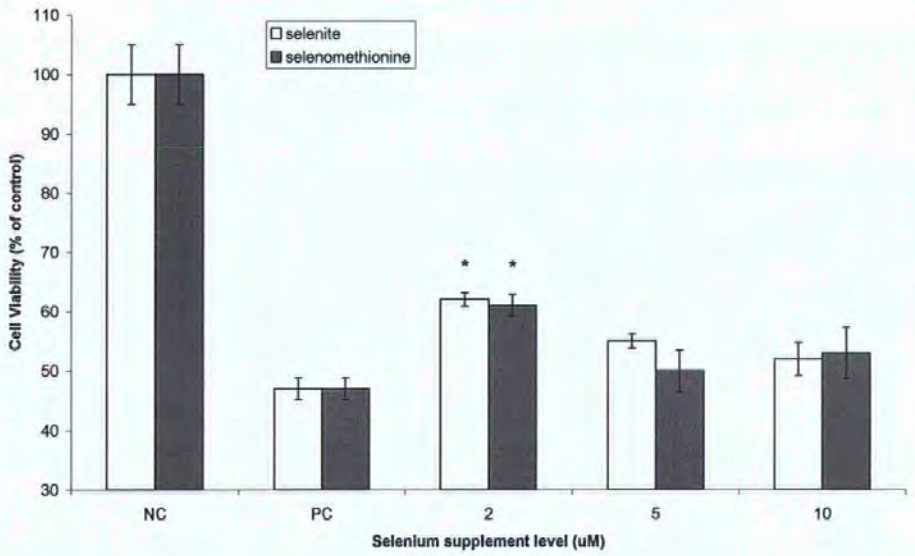


Fig. 4.6 Effect of inorganic (sodium selenite) and organic (selenomethionine) selenium supplementation (96 hours) on viability of GFSk-S1 cells when challenged with UVB as determined by the NRR assay. Data represented as means ± S.E. Positive control = 45 Jm^{-2} UVB dose. * denotes significant difference versus control.

4.3.5. Effect of selenium supplementation on H_2O_2, UVA & UVB induced genotoxicity

Exposure of GFSk-S1 cells to H_2O_2, UVA and UVB caused significant increases in oxidative DNA damage, represented by Fpg-sensitive sites, compared to non-irrdadiated controls. Pre-treatment for 96 hours with 2 μM selenite or selenomethionine prevented oxidative DNA damage induced by all three stressors, again to varying degrees ($P < 0.05$, Fig. 4.7, 4.8, 4.9). For H_2O_2-indcued DNA damage, selenium reduced Fpg-sensitive sites by 56 – 67 % compared to non-irradiated controls. Direct SSB breaks induced by H_2O_2 were also reduced by selenium supplementation. UVA- and UVB-induced oxidative DNA damage was reduced by selenium pre-treatment by ~ 40% and 25%, respectively. The source of selenium supplement (sodium selenite or selenomethionine) appeared to have no significant effect on any of the DNA damage responses measured.

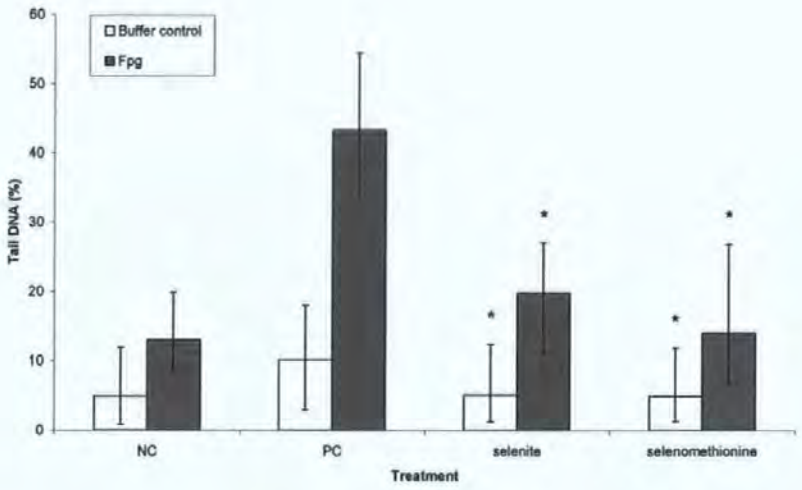


Fig. 4.7. Effect of inorganic (sodium selenite) and organic (selenomethionine) selenium supplementation (96 hours) on H_2O_2 induced DNA damage in GFSk-S1 cells as measured by the modified Comet assay. Tail DNA (%) expressed as median values with bars indicating the 25th and 75th percentile range to give an appreciation of spread. * denotes significant versus control ($P < 0.05$).

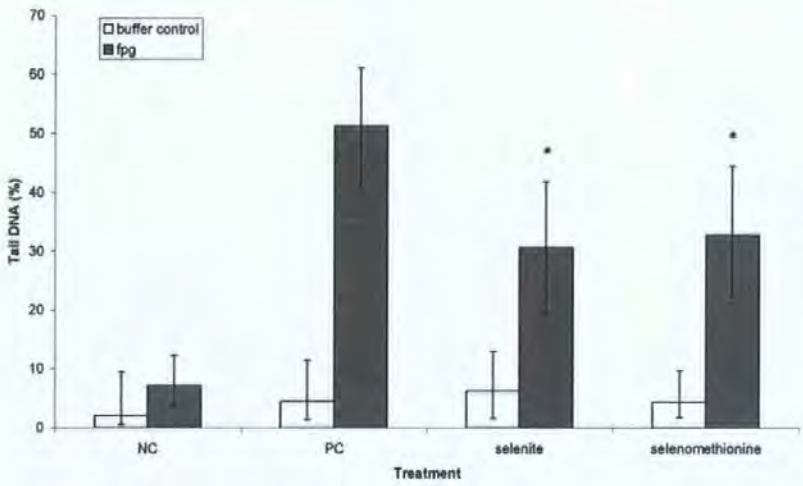


Fig. 4.8 Effect of inorganic (sodium selenite) and organic (selenomethionine) selenium supplementation (2 μM, 96 hours) on UVA induced DNA damage in GFSk-S1 cells as measured by the modified Comet assay. Tail DNA (%) expressed as median values with bars indicating the 25th and 75th percentile range to give an appreciation of spread. * denotes significant versus control ($P < 0.05$).

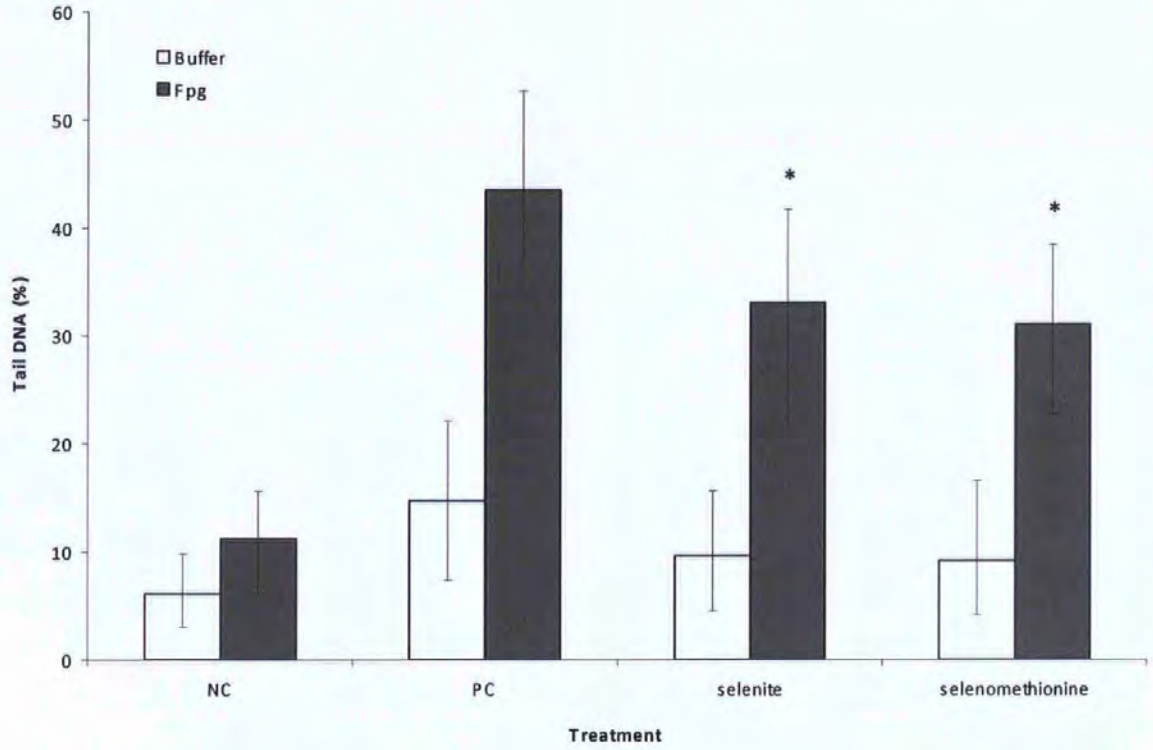


Fig. 4.9. Effect of inorganic (sodium selenite) and organic (selenomethionine) selenium supplementation (96 hours) on UVB induced DNA damage in GFSk-S1 cells as measured by the modified Comet assay. Tail DNA (%) expressed as median values with bars indicating the 25th and 75th percentile range to give an appreciation of spread. * denotes significant versus control ($P < 0.05$).

4.4 Discussion

Validation experiments clearly showed, for the first time, that both the NRR assay and the modified Comet assay are sensitive assays for detecting H_2O_2 and UVR-induced cytotoxicity and genotoxicity, respectively, in the GFSk-S1 cell line. For all stress treatments cytotoxicity and oxidative DNA damage was induced in a dose-dependent manor.

The NRR retention assay has previously been used in fish cell lines to detect chemical (including H_2O_2) induced cytotoxicity (Wright *et al.*, 2000; Davoren & Fogerty, 2006). However, this is the first study to use the NRR assay to evaluate UVR induced cytotoxicity in any fish cell line.

Previously, the modification of the Comet assay protocol, which includes a digestion step with bacterial repair endonucleases to target oxidised DNA bases, has been shown to greatly enhance the sensitivity and specificity of the assay, compared to the standard protocol (Collins *et al.*, 1993). In the present study, this is highlighted by the fact that the level of Fpg-sensitive sites (oxidised purines) increased to a greater degree than the level of direct SSB in all stress treatments (H_2O_2, UVA & UVB). The increase in Fpg-sensitive sites compared to direct SSB suggests that 8-hydroxyguanine (8-OH-G) was probably the most likely adduct induced, although formamidopyrimidines (imidazole ring-opened purines) are also possible substrates for Fpg (Kielbassa *et al.*, 1997). H_2O_2, UVA & UVB have all previously been shown to cause oxidative DNA damage, as evaluated by the modified Comet assay and increased 8-OH-G formation measured by HPLC methods (Stewart *et al.*, 1996; Thomas *et al.*, 1998; Rafferty *et al.*, 2003; Wu *et al.*, 2004).

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Although the standard Comet assay has been used extensively for evaluating the genotoxic potential of contaminants in fish (see reviews Mitchelmore & Chipman, 1998; Cottelle & Ferard, 1999), there are only two other studies that have specifically evaluated oxidative DNA damage in fish cells using the modified Comet assay (Akcha *et al.*, 2003; Aniagu *et al.*, 2006). Furthermore,

this is the only study using fish cells to have validated the modified Comet assay protocol with a reference genotoxin and UVR.

Prior to challenging cells with H_2O_2 or UVR, the relative toxicity of sodium selenite and selenomethionine was evaluated with the NRR assay using a wide range of selenium concentrations (2-100 μM for sodium selenite and 2-1000 μM for selenomethionine). Both sodium selenite and selenomethionine caused significant increases in 'normal' levels (unchallenged with stressors) of cell viability. This is not surprising as previous studies have shown standard cell culture media is inadequate in terms of selenium content (Leist *et al.*, 1996). Although sodium selenite caused increases in levels of cell viability at low doses (2-20 μM), further increases in concentration (≥ 40 μM) were highly toxic. This is in contrast to selenomethionine which, even at the highest dose (1000 μM), showed no cytotoxic effect. In fish organically bound forms of selenium have previously been shown to be considerably less toxic and more bioavailable in supplemented diets (Lorentzen *et al.*, 1994; Jovanovic *et al.*, 1997; Wang *et al.*, 1997; Wang and Lovell, 1997), and are also preferred in human interventions because they are less acutely toxic (Rayman, 2000). However, as selenomethionine is non-specifically incorporated into proteins (in place of methionine) it has not catalytic activity and therefore must be catabolised into an organic precursor before entering the available selenium pool. Inorganic sources only need be reduced to selenide to provide selenophosphate, the precursor of selenocysteine, the active form of selenium in selenoproteins. Furthermore, non-specific retention of selenium as selenomethionine in proteins may lead to long term toxicity.

The optimal dose for both forms of selenium appeared to be in the 2-10 μM range, and therefore these doses were used in supplement studies with H_2O_2 or UVR.

Selenium supplements, as sodium selenite and selenomethionine, significantly reduced H_2O_2, UVA & UVB induced cytotoxicity and oxidative DNA damage in GFSk-S1 cells compared to positive controls (irradiated cells with no selenium supplementation). Selenium showed greater protection against H_2O_2 and UVA-induced oxidative stress than for UVB, which may be explained by the fact that UVB is much more energetic than UVA (de Gruijl *et al.*, 2002) and thus the production of reaction oxygen species may have had a more overwhelming effect on the protection afforded by selenium. Indeed, studies have shown that a dose of just 0.6 J/cm^2 UVB can elevate the formation of 8-OH-G by 280% in keratinocytes (Beehler *et al.*, 1992). In the present study the minimum dose required to cause increases in cytotoxicity and oxidative DNA damage was 15 J/cm^2 UVB compared to 2500 J/cm^2 for UVA. Previous studies with mammalian cells have shown the protective effect of selenium on UVR induced cytotoxicity (Moysan *et al.*, 1995; Emonet *et al.*, 1997; Rafferty *et al.*, 1998, 2003) and oxidative DNA damage (Stewart *et al.*, 1996; Rafferty et al., 2003). However, this is the first study to show these phenomena in fish cells.

It is most likely that the protective effect of selenium was due to the increased activity of the antioxidant selenoenzymes. The most familiar of these are the glutathione peroxidases (Rotruck *et al.*, 1973) - cytosolic and membrane-bound antioxidant enzymes, which remove H_2O_2, and lipid and phospholipids

hydroperoxides, respectively. This limits oxidative damage to important macromolecules such as lipids, lipoproteins and DNA, the net effect being cell death. GSHPx and GSHPx mimics have been implicated in selenium dependent protection against UVR *in vitro* (Moysan *et al.,* 1995; Emonet *et al.*, 1997; Mu *et al.*, 2003; Sun *et al.*, 2003). Although no studies have looked at GSHPx dependent protection against UVR in fish, the beneficial effects of selenium supplementation in diets on GSHPx activity have been confirmed (Bell *et al.*, 1986; Jovanovic *et al.*, 1997; Diminov *et al.*, 1998).

More recently thioredoxin reductase (TR) has been confirmed as another important antioxidant enzyme that may also reduce harmful peroxides such as H_2O_2 and peroxynitrite (Miller *et al.*, 2001; Saito *et al.*, 2003; Ebert *et al.* 2006). As yet, there appears to be little information, if any, on the role or TR in protecting cells from UVR-induced oxidative stress, but this may present interesting results in future studies. Furthermore, there appears to be no published studies which have measured TR activity in fish.

Apart from the incorporation of selenium into antioxidant enzymes, selenium may have other mechanisms of protecting cells and DNA from oxidative stress induced by agents such as UVR. For example, metal sequestration has been proposed as a alternative mechanism for selenium antioxidant activity (Battin *et al.*, 2006). Metals, including copper and iron, are involved in generating the highly reactive hydroxyl radical (OH) from H_2O_2 under oxidative stress conditions, via Fenton-type reactions. Selenium compounds readily coordinate with such metal ions thus preventing the formation of OH and subsequent

oxidative stress. Battin *et al.* (2006) found that supplementation with selenocysteine and selenomethionine inhibited oxidative DNA damage in plasmid DNA due to selenium-copper coordination. Selenium also protected against cadmium chloride induced chromosomal damage (micronucleus formation) in Chinese hamster lung fibroblasts (Hurna *et al.*, 1997). The cadmium detoxification by selenium is thought to be related to the formation of selenium-cadmium complexes (Sugiama, 1994). Furthermore, in fish hepatocytes *in vitro*, micronucleus formation by exposure to different types of mercury was also inhibited by addition of selenium, possibly by a similar mechanism (Al-Sabti, 1995).

Overall results from this study for the first time show that selenium may have a role in protecting fish from the oxidative stress induced DNA damage and cytotoxicity in general and from environmental UVR exposure. It is not possible to know the exact mechanism of selenium dependent UVR protection of GFSk-S1 cells in this study as, for one thing, the activity of GSHPx and TR were not measured. Further experiments are required to evaluate how these enzymes respond to UVR-induced stress and selenium supplementation regimes, both *in vitro* and *in vivo*.

CHAPTER 5: OXIDATIVE STRESS EFFECTS OF TANIUM DIOXIDE (TIO_2) NANOPARTICLE EXPOSURE ON CULTURED FISH CELLS *IN VITRO*

5.1 Introduction

Nanotechnology concerns the development and use of substances which have a particle size <100 nm, the aim being to significantly increase the surface area to mass ratio, thereby greatly enhancing chemical/catalytic reactivity (amongst other properties), compared to normal-sized particles of the same substance. Although this provides many benefits for industry, there is increasing concern that substances previously considered biologically inert may indeed become toxic in a nanoparticulate state due to their increased reactivity and possible easier penetration of cells.

As discussed in chapter 1, titanium dioxide (TiO_2) nanoparticles have been shown to increase production of ROS, causing oxidative stress which may lead to cytotoxic and genotoxic effects in cultured cells. These effects may be exacerbated by the photosensitisation of TiO_2 nanoparticles during UVR exposure.

Possible ROS that could be formed are hydroxyl radicals (OH), superoxide radical anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) which may be identified by means of electron spin resonance (ESR), using the technique of spin trapping.

Despite growing concern over the potential risk of manufactured nanoparticles to the environment, there is a paucity of information on their potential toxic effects on natural biota, including aquatic organisms such as fish. Therefore, the aim of this chapter is to evaluate whether TiO_2 nanoparticles are capable of inducing cytotoxicity and genotoxicity in a primary fish cell line, either alone or in combination with UVA radiation. Also, to evaluate what types of free radicals are generated and may be responsible for any observed toxic effects. While cytotoxic effects were determined using neutral red retention (NRR) assay, a modified Single Cell Gel Electrophoresis (SCGE) or Comet assay, using a lesions-specific bacterial repair enzyme (Fpg), was used to determine oxidative DNA damage. Electron Spin Resonance (ESR), using a range of spin traps, was used to qualitatively determine the generation of free radicals.

5.2 Materials and methods

5.2.1 Materials

All cell culture materials were purchased from either Invitrogen (Paisley, UK) or Bibby Sterilin (now Barloworld Scientific) (Stone, UK). All other reagents were purchased from Sigma (Poole, UK) unless otherwise stated. Fpg was kindly supplied by Professor Andrew Collins (University of Oslo, Norway). Teflon tubing for ESR analysis was kindly donated by Zeus Industrial Products Inc., Orangeburg SC, USA. DEPMPO was from Axxora Ltd. (Nottingham, UK).

5.2.2 Cell culture

GFSk-S1 cells were routinely passaged as described in section 2.2.1.

5.2.2 Validation of Neutral Red Retention (NRR) cytotoxicity assay and the modified Comet assay.

The NRR assay and modified Comet assay were previously validated with H_2O_2 and UVA and results discussed in sections 3.2.2 and 3.2.3.

5.2.3 Titanium dioxide (TiO_2) and UVA treatments

TiO_2 was used in the anatase form, with an average particle size of 5 nm. For low concentrations (0.1 - 100 μg ml^{-1}), TiO_2 nanoparticles were suspended in sterilized PBS, as recommended (Gurr *et al.*, 2005), to an initial working concentration of 1000 μg ml^{-1}. Final dilutions were made in complete culture medium to desired concentrations. For concentrations >100 μg ml^{-1} initial dilutions were made in complete culture medium. Although, light was excluded during incubation, there was some unavoidable exposure to light during handling as the experiments involving addition of accurate concentrations of the nanoparticles to the cells could not be carried out in darkness. Therefore, the possibility of some damage due to exposure to ambient light, compared to concurrent controls, cannot be totally excluded.

5.2.3.1 Cytotoxicity evaluation – Neutral Red Retention (NRR) assay

GFSk-S1 cells were seeded in 96 well tissue culture treated multi-plates at a density of 1 x 10^5 cells ml^{-1} (total volume of 200 µl per well) and allowed to attach overnight. Cells were then incubated in fresh culture medium containing 0.1 - 1000 µg ml^{-1} (0.06–625 µg cm^{-2}) TiO_2 for 24 hours (no UV) or 2 hours (+UV) at 20±1 °C (Zhang and Sun, 2004). For combined exposures with UVA and TiO_2, UVA exposure was performed as described in section 2.6 and delivered at a dose of either 500 or 2000 Jm^{-2}. After the exposure period cells were washed twice with PBS and the NRR assay protocol was performed as outlined in section 2.3.

5.2.3.2 Genotoxicity evaluation – The modified Comet assay

GFSK-S1 cells were seeded in 6 well tissue culture treated multi-plates (35 mm ∅ wells) at a density of 1 x 10^5 cells ml^{-1} (total volume of 2 ml per well) and allowed to attach overnight. Cells were then incubated in fresh culture medium containing TiO_2 for 2 or 24 hours at 20±1 °C at concentrations ranging from 1 to 100 µg ml^{-1} (0.2–20.8 µg cm^{-2}) (Gurr *et al.*, 2005; Wang *et al.*, 2007). For combined exposures with UVA and TiO_2, UVA was exposure was performed as described in section 2.6 and delivered at a dose of 2.5 kJm^{-2}. After the exposure period cells were washed twice with PBS and the modified Comet assay performed as described above section 2.4.3.

5.2.3.3 Free radical detection – Electron Spin Resonance (ESR)

Electron spin (or paramagnetic) resonance (ESR or EPR) is a spectroscopic technique that detects the unpaired electron present in a free radical (Halliwell and Gutteridge, 1997). As such it is the only general approach that can provide direct evidence for the presence of a free radical. In addition, analysis of the ESR spectrum generally enables determination of the identity of the free radical.

GFSK-S1 cells were seeded in 75 cm^2 tissue culture flasks at a density of 1 x 10^5 cells ml^{-1} (total volume of 20 ml per flask) and allowed to attach overnight. Cells were then treated with TiO_2 (10 - 500 $\mu g\ ml^{-1}$ [2.6 - 133 $\mu g\ cm^{-2}$]) for 24 hours at 20±1°C.

Spectra were obtained at room temperature using a Bruker ECS 106, X band spectrometer. Typical spectrometer settings were: magnetic field centre 335.9 mT, field scan 10–12 mT, microwave frequency 9.43 GHz, microwave power 10 mW, modulation frequency 100 kHz, modulation amplitude 0.1mT, time constant 0.3 s, sweep time 168 s. Briefly, trypsinised cells were washed three times in PBS and cell pellets, containing approximately 2 x 10^6 cells were resuspended in 25 µl of spin trap in PBS, which was drawn into a 3 cm length of thin-walled 0.8 mm diameter Teflon tubing (Zeus Industrial Products Inc., Orangeburg SC, USA). The Teflon tubing was folded in half and placed inside a 4 mm diameter quartz tube which allowed air to circulate and prevented the cells becoming anoxic. Spectra were recorded before and after exposure to

UVA. Cell samples were exposed to 2.5 Jm^{-2} UVA outside the spectrometer and then spectra were recorded, starting 1 min after the end of irradiation. The spin traps used were 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), alone or in combination with superoxide dismutase (SOD) (Thornalley & Dodd, 1985); α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN); 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) and, for detection of singlet oxygen (Lion *et al.*, 1980), 2,2,6,6-tetramethyl-4-piperidinol. Any spin traps that showed free radical signals before treatment were shaken with activated charcoal which was then removed by centrifugation.

5.3 Results

5.3.1 Cytotoxicity of TiO2 (NRR assay)

Exposure of goldfish cells to TiO_2 nanoparticles for 24 hours, in the absence of UVA, caused only a slight decrease in cell viability as measured by the NRR assay. Even at the highest concentration (1000 μg ml^{-1}), viability was still ~80% of control, although the decrease was statistically significant vs. the negative control (*t*-test, $P < 0.05$) (Fig. 5.1). However, when TiO_2-treated cells were co-exposed with UVA (2.0 kJm^{-2}), significant dose-dependent decreases in cell viability was observed for TiO_2 concentrations ≥ 50 μg ml^{-1} ($P < 0.01$) (Fig. 5.1). At the highest concentration of TiO_2 (1000 μg ml^{-1}) cell viability dropped 2-fold (compared to non-irradiated cells) to ~40% of control. No such photosensitisation was observed at a lower dose of UVA (0.5 kJm^{-2}),

therefore, the effect was dependent on both the concentration of TiO_2 and the dose of UVA administered.

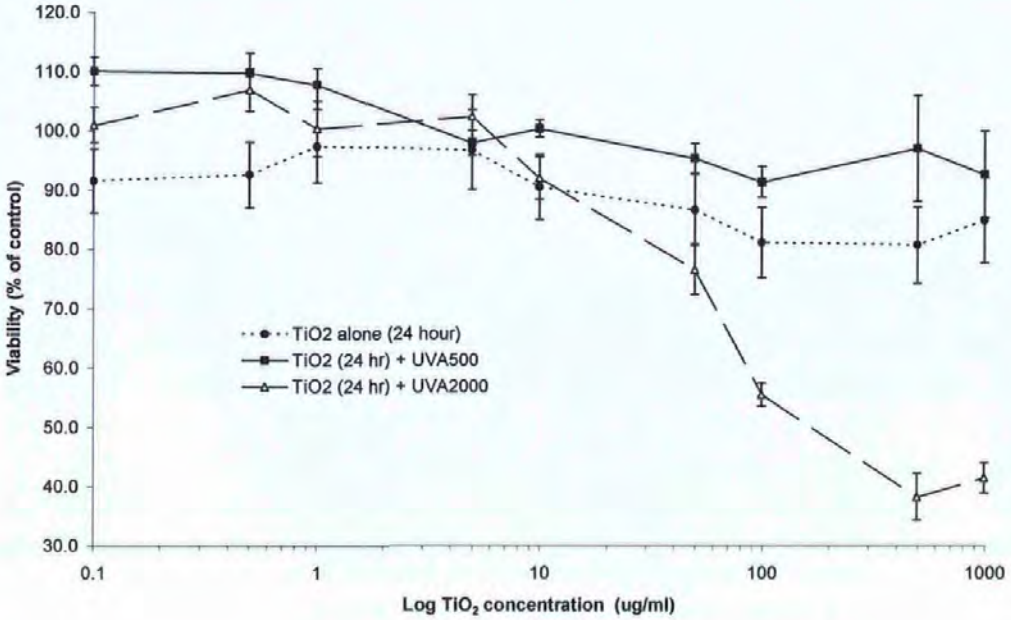

Fig. 5.1 Effect of TiO_2 concentration and UVA exposure on viability of GFSk-S1 cells, as evaluated by NRR assay. Cells were treated with TiO_2 for 24 hours (no UVR) or 2 hours (+UVR) at concentrations ranging from 0.1 to 1000 μ gml^{-1}. UVA was administered at two levels - 500 and 2000 Jm^{-2}. Data represented as means ± S.E.

5.3.2 Genotoxicity of TiO_2 (Comet assay)

Cells were treated with 1, 10 and 100 μg ml^{-1} TiO_2 nanoparticles for 24 hours in the absence of UV. All dose levels caused significant increases in oxidative DNA damage, as represented by increased levels of Fpg-sensitive sites (Man–

Whitney *U*, $P < 0.001$) (Fig. 5.2). A significant increase in background SSB was only observed at the highest concentration (100 μg ml^{-1}) (Mann–Whitney *U*, $P < 0.001$). A significant level of oxidative DNA damage (Fpg sensitive sites) was also detected in cells incubated with 10 μg ml^{-1} TiO_2 for a shorter incubation period (2 h) ($P < 0.05$, Fig. 5.3). When TiO_2-treated cells were irradiated with UVA, a further increase in oxidative DNA damage was evident, compared to TiO_2 or UV alone (Mann–Whitney *U*, $P < 0.001$) (Fig. 5.3).

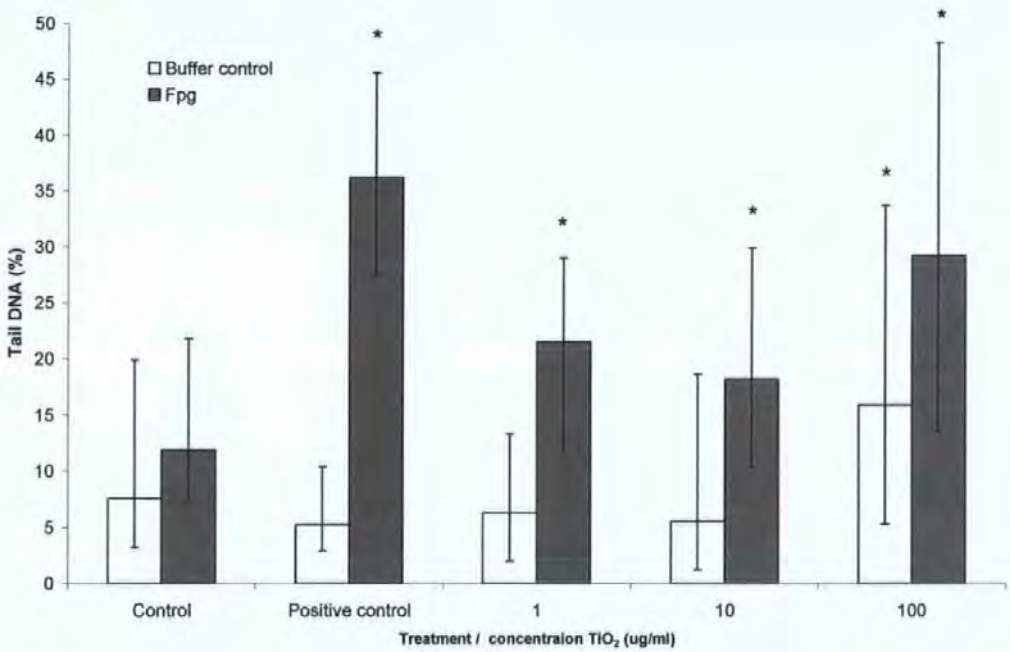

Fig. 5.2 Effect of TiO_2 nanoparticle exposure (in the absence of UVA) on oxidative DNA damage as measured by the modified Comet assay. For TiO_2 treatment cells were exposed at concentrations of 1, 10 and 100 μg ml^{-1} for 24 hours. The positive control was 5 μM H_2O_2 for 10 min. Data points represented as median values. Interquartile ranges give a measure of data spread. * denotes significant verses control ($P < 0.001$).

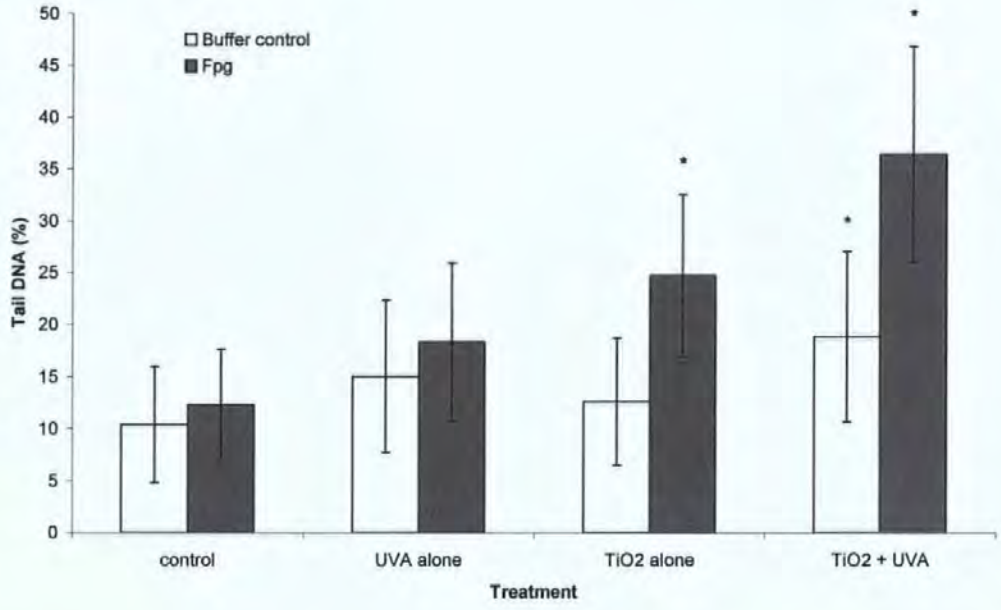

Fig. 5.3 Effect of UVA irradiation of TiO_2 nanoparticle-treated GFSk-S1 cells on oxidative DNA damage as measured by the modified Comet assay. UVA alone was a single UVA dose of 2.5 kJm^{-2}. TiO_2 alone was 10 $\mu g\ ml^{-1}$ for 2 hours. The combined exposure (TiO_2 +UVA) was 10 $\mu g\ ml^{-1}$ TiO_2 for 2 hours followed by a UVA dose of 2.5 kJm^{-2}. Data points represent as median values. Interquartile ranges give a measure of data spread. * denotes significant versus control ($P < 0.001$).

5.3.3. ESR

UVA irradiation of an aqueous suspension of TiO_2 in the presence of DMPO produced the DMPO–OH adduct (Fig. 5.4a), which was unaffected by the prior addition of SOD. Using POBN as the spin trap, no adduct was observed after UVA irradiation unless a trace of ethanol was added before irradiation, when the POBN–$CH(OH)CH_3$ adduct was formed on irradiation (Fig. 5.4b). The spin

trap DEPMPO showed the formation of the DEPMPO–OH adduct after UV irradiation of aqueous TiO_2, but in the presence of 100 mM mannitol, the OH adduct was reduced in intensity and another spectrum, thought to be a DEPMPO–mannitol radical adduct was also formed (Fig. 5.4c and d). No signals were observed when 2,2,6,6-tetramethyl-4-piperidinol was used as the trap, although an intense 3-line spectrum of the 4- hydroxy-2,2,6,6-piperidine-1-oxyl radical was observed when tetrasodium-meso-tetraphenylporphine sulphonate (TPPS) and white light was used as a positive control for this trap. Storage of an aerated solution of TiO_2 and DEPMPO in PBS for 3–4 hours produced a well-defined DEPMPO–OH adduct, in the absence of UVA. However, subsequent experiments have shown that DEPMPO–OH is also formed on exposure of aqueous TiO_2 to white light. UVA irradiation of TiO_2-treated cells, in the presence of DMPO, produced the DMPO–OH adduct (Fig. 5.5a), whilst control cells showed no such formation of radicals. Addition of SOD to the TiO_2-treated cells appeared to have no effect on the formation of the DMPO–OH adduct. UVA irradiation of TiO_2-treated cells in the presence of the spin trap POBN produced a spin adduct with a 6-line spectrum, characterised by aN = 1.56 mT and aH = 0.33 mT. Using DEPMPO as the spin trap, the ESR spectra of TiO_2-treated cells, observed after UVA irradiation (Fig. 5.5b), showed three different species, DEPMPO–OH, an unidentified adduct, DEPMPO–X (Fig. 5.5c) and a very small contribution from DEPMPO–OOH. The presence of the latter component was confirmed by addition of SOD, prior to UV irradiation, which suppressed two small peaks near the centre of the spectrum, whilst having a negligible effect on the rest of the spectrum. Addition of 500 U ml^{-1} catalase prior to irradiation had no detectable effect on the

resulting spectrum. The ratio of DEPMPO–OH:DEPMPO–X was found to be dependent on the concentration of TiO_2. Cells treated with 10 or 50 μg ml^{-1} showed DEPMPO–OH as the major component, while with 100 or 500 μg ml^{-1} DEPMPO–X was equal to or greater than DEPMPO–OH. The overall radical concentration increased by an order of magnitude between 10 and 500 μg ml^{-1} TiO_2. Storage of TiO_2-treated cells in the presence of DEPMPO for several hours showed the formation of a low concentration of the mixed DEPMPO adducts, in the absence of UVA.

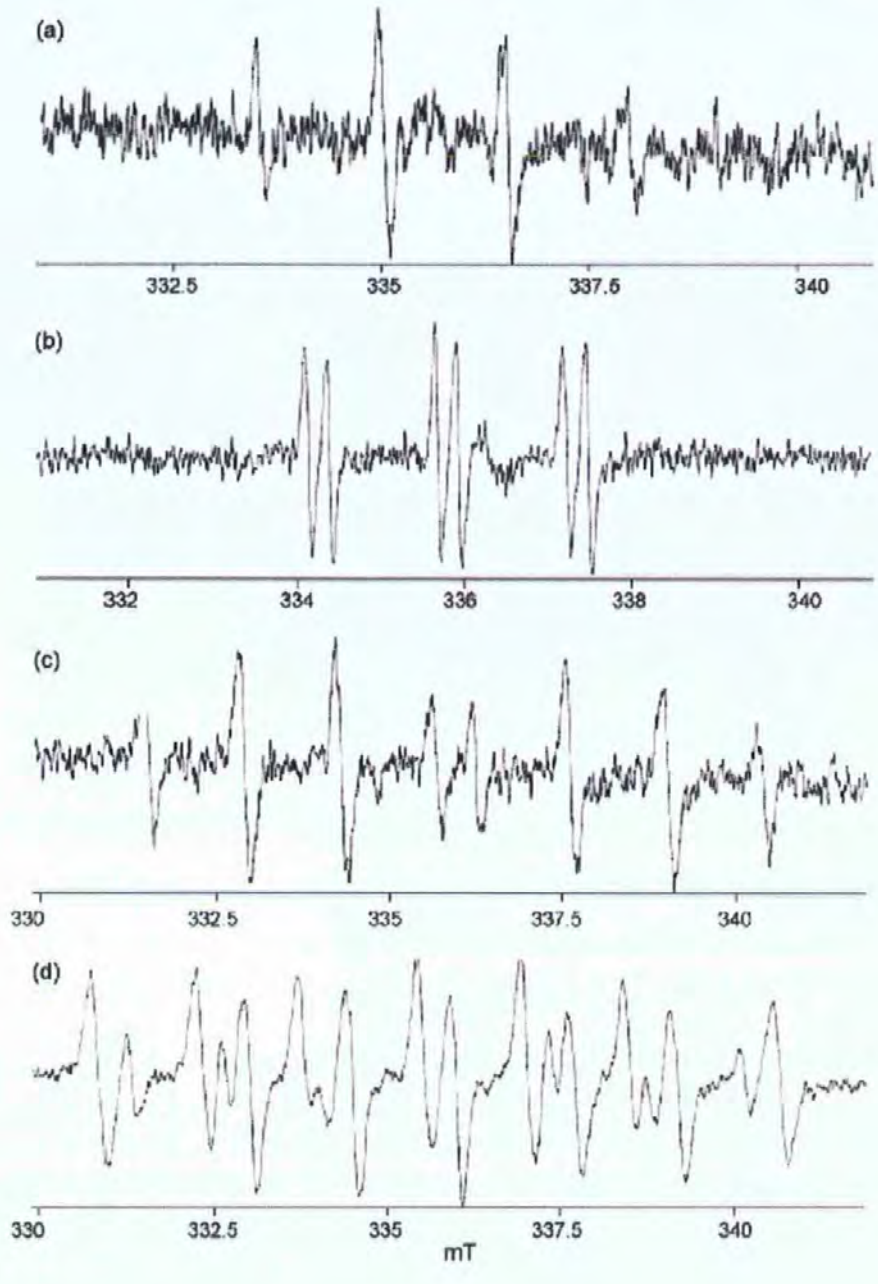

Fig. 5.4. ESR spectra of an aqueous suspension of TiO_2 after irradiation with UVA (a) in the presence of DMPO ($aN = a^{\beta}_{H} = 1.49$ mT); (b) in the presence of POBN and ethanol ($aN = 1.57$mT, $aH = 0.24$ mT); (c) in the presence of DEPMPO ($aH = aN = 1.37$mT, $aP = 4.71$ mT); (d) in the presence of DEPMPO and mannitol (additional signal from a mannitol radical: $aH = 2.14$mT, $aN = 1.48$mT, $aP = 4.68$ mT).

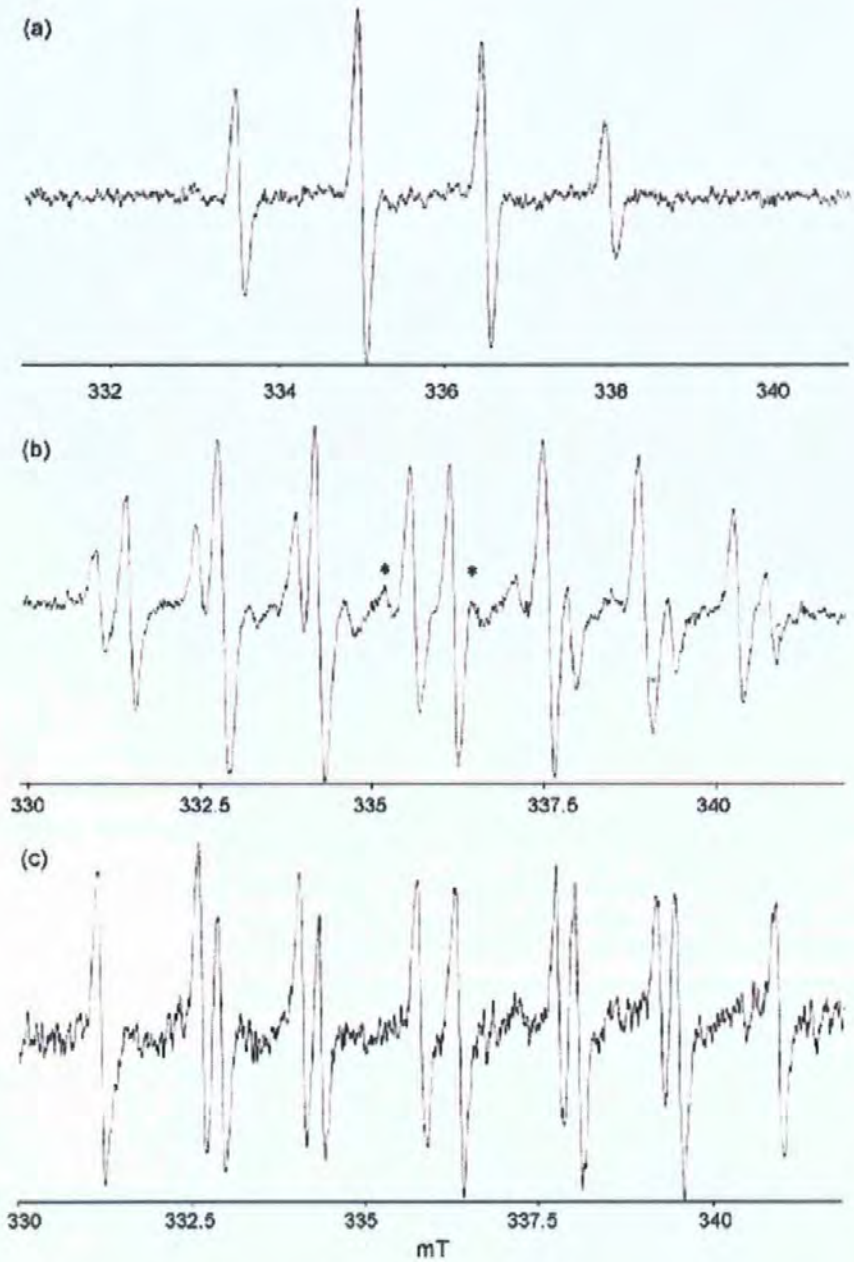

Fig. 5.5. ESR spectra of GFSk-S1 cells treated with 50 μg ml^{-1} TiO_2 for 24 hours and irradiated with UVA (a) in the presence of DMPO; (b) in the presence of DEPMPO, *, marks characteristic peaks of the DEPMPO–OOH spin adduct; (c) spectrum of unidentified adduct DEPMPO–X (*a*H = 1.72 mT, *a*N = 1.44 mT, *a*P = 5.16 mT), obtained by subtraction of the spectrum of DEPMPO–OH.

5.4 Discussion

As was mentioned earlier, there is conflicting evidence as to whether TiO_2 nanoparticles induce toxicity in the absence of UV irradiation. Results from this investigation seem to support both sides of the argument.

In terms of cytotoxicity, the neutral red retention assay showed that TiO_2 alone had a very weak capacity to affect lysosomal membrane integrity (cell death), even at very high concentrations (1000 $\mu g\ ml^{-1}$). This is in agreement with Zhang and Sun (2004) who had similar results over the same concentration range with human colon carcinoma cells (methyl tetrazolium cytotoxicity [MTT] assay). Linnainmaa *et al.* (1997) also reported no effect of 5 - 200 $\mu g\ cm^{-2}$ ultra-fine TiO_2 on viability of rat liver cells, using cell division (multi nuclei index) as a measure of cytotoxicity.

Contrastingly though, our cytotoxicity results do not fall in line with those of Uchino *et al.* (2002) who reported significant decreases in viability (MTT assay) of CHO cells to ~50% of controls when treated with 100 $\mu g\ ml^{-1}$ TiO_2. They also do not agree with Wang *et al.* (2007) who observed dose-dependent decreases in viability in human lymphoblastoid cells, also using the MTT assay. They recorded 61, 7 and 2% relative viability at 130 $\mu g\ ml^{-1}$ TiO_2 for 6, 24 and 48 hour exposures.

From the last two examples it appears as though the MTT assay may be a more sensitive and appropriate method of assessing cell viability in TiO_2-

treated cells than the NRR assay used in this investigation. However, the use of MTT assay for nanoparticle cytotoxicity evaluation has been called into question due to its apparent ability to yield false positive results, particularly in the case of carbon nanotubes (Wörle-knirsch *et al.*, 2006). On the other hand, Zhang and Sun (2004) also used the MTT assay, but found no cytotoxic effect of TiO_2, even at 1000 $\mu g\ ml^{-1}$. This suggests that differences in response between studies are not necessarily related to the assay method. Instead, it may be due to how the TiO_2 treatments differ in terms of the source of TiO_2, particle size, preparation method (sonication), degree of aggregation and incubation conditions.

A standard protocol for nanoparticle suspension preparation for *in vitro* studies would be useful for future investigations. Differential susceptibility between cell types could also introduce a great deal of variability in results. Differences in metabolic rate/capacity (temperature dependent), antioxidant enzyme machinery, and DNA repair capabilities may also explain the experimental outcome.

Results from the Comet assay indicate that TiO_2 nanoparticles are in fact genotoxic without UV irradiation, as all concentrations tested produced a significant increase in the level of Fpg-sensitive sites. This suggests that 8-hydroxyguanine (8-OH-G) was probably the most likely product of TiO_2-induced oxidative stress, although formamidopyrimidines (imidazole ring-opened purines) are also possible substrates for Fpg (Kielbassa *et al.*, 1997). 8-OH-G is important because it is relatively easily formed and is mutagenic

and carcinogenic (Valko *et al.*, 2006). Consequently it is considered a good biomarker of oxidative stress and a potential biomarker of carcinogenesis (Martinez *et al.*, 2003; Valko *et al.*, 2006). Singlet oxygen is known to react with the guanine moiety of cellular DNA to form 8-OH-G, as is the hydroxyl radical which reacts almost indifferently with all components of the DNA molecule damaging both purine and pyrimidine bases and also the deoxyribose backbone (Martinez *et al.*, 2003; Valko *et al.*, 2006). Therefore, from Comet assay results, it is not possible to conclude which species was responsible for the genotoxic effects of TiO_2, although as mentioned earlier, from ESR analysis it appears that it is most likely to be OH, as there is no evidence of $^1O_2$ production.

In common with other metals or organometalic compounds, in addition to the production of ROS, TiO_2 could also directly bind to DNA or repair enzymes leading to the generation of strand breaks (Jha *et al.*, 1992; Hartwig, 1998).

Our Comet assay results compare favourably with recent studies on mammalian cells which have investigated the genotoxic potential of TiO_2 nanoparticles at similar concentrations. Gurr *et al.* (2005) observed increased levels of oxidative DNA damage (Fpg-sensitive sites) when BEAS-2B cells, a human bronchial epithelial cell line, were treated with 10 $\mu g\ ml^{-1}$ anatase (10 nm) TiO_2 particles for 1 hour. Additionally, treatment of human lymphoblastoid cells with ultra-fine TiO_2 in the range 65–130 $\mu g\ ml^{-1}$ induced 2.5-fold increases in the frequency of micronuclei, 5-fold increases in DNA single strand breaks (Comet assay) and 2.5-fold increases in mutation frequency

(Wang *et al.*, 2007). Exposure to 1 μg cm^{-2} of TiO_2 (<20 nm) for 12–72 hours also caused a significant dose-dependent increase in the induction of micronuclei in Syrian hamster embryo cells (SHE) (Rahman *et al.*, 2002). However, as with the cytotoxicity results, there seems to be some evidence to the contrary. Nakagawa *et al.* (1997) reported no effect of ultra-fine (25 nm) TiO_2 on a series of genotoxicity parameters measured, including the Comet assay, in mouse lymphoma and Chinese hamster cells *in vitro*. TiO_2 nanoparticles also had no effect on micronuclei induction in cultured rat liver epithelial cells (Linnainmaa *et al.*, 1997).

As was discussed for the cytotoxicity results, disagreements in reported genotoxic potential of TiO_2 nanoparticles may be explained by a number of variables, including the TiO_2 treatment regime, the cell type used, the metabolic/antioxidant capacity of the cells, as well as DNA repair capabilities. It is particularly important to consider possible inherent differences between cells from different phylogenetic origin, namely fish (as used here) and mammalian cells. Indeed it has been previously shown that fish cells are generally more susceptible to toxic/oxidative injury than similarly treated mammalian cells (Raisuddin & Jha, 2004). Although this did not seem to be the case for the cytotoxicity studies with the NRR assay, it does seem evident that the goldfish cells show increased sensitivity to TiO_2-induced oxidative DNA damage, compared to mammalian cells used in otherstudies. A significant increase in the level of Fpg-sensitive sites (8-OH-G formation) was observed at only 1 μg ml^{-1} (24 hour treatment) in goldfish cells which seems to be the lowest reported in any of the literature. We also observed a significant increase in

base oxidation after just 2 hours of TiO_2 exposure and together with the fact that the longer incubation period (24 hours) was still revealing significant levels of base oxidation, this suggests that DNA repair mechanisms were unable to repair damage from the initial insult.

Fish are reported to have a much lower capacity for DNA excision repair than mammals (Baily *et al.*, 1996: Willet *et al.*, 2001) and this may help explain the apparent increased sensitivity of the fish cells towards TiO_2-induced oxidative DNA damage. In comparison, it has been clearly demonstrated that hydroxylated guanine bases are removed from cellular DNA via an efficient enzymatic repair process in mammalian cells (Demple & Harrison, 1994). The half life for removal of 8-OH-G from DNA in human lymphoblast cells at 37 °C has been reported to be only 55 min (Jaruga, 1996).

In line with mammalian studies, once TiO_2-treated cells had been co-exposed with UVA, a more pronounced toxic effect was found than in the absence of UV, both in terms of cytotoxicity and oxidative DNA damage (Nakagawa *et al.*, 1997; Wamer *et al.*, 1997; Uchino *et al*, 2002; Zhang and Sun, 2007; Dunford *et al.*, 2007). Although it may have been reported several times in mammalian cells, this is the first time that the photo-catalytic properties of TiO_2 nanoparticles have been shown in fish cells. From the NRR results it is clear that the photo-catalytic effects are dependent on both the concentration of TiO_2 and the dose of UVA administered.

Although it appears that increasing the concentration of TiO_2 increases the interactive effect with UVA, it has been previously shown that this may not be due to increased absorption of UVA by TiO_2. Using ESR analysis, Uchino *et al.* (2005) found that there was no relationship between OH radical production and UVA absorbance in all TiO_2 test samples, but they demonstrated a correlation between OH radical production and viability of CHO cells. ESR spin trapping with DMPO suggests that irradiation of an aqueous suspension of TiO_2 with UVA produces hydroxyl radicals. Whilst the absence of a detectable signal from DMPO–OOH cannot be taken as proof that superoxide is not produced, since DMPO–OOH decays rapidly to DMPO–OH, the absence of any effect of SOD on the observed ESR spectrum strongly suggests that there is no significant superoxide production. In contrast, the spin trap DEPMPO gives relatively stable adducts of both OH and $O_2^{\bullet-}$ but irradiation of TiO_2 produces only the OH adduct. Moreover, addition of mannitol, which reacts with hydroxyl radicals, but not superoxide radical anions, reduced the amount of DEPMPO–OH adduct observed and formed a DEPMPO–mannitol adduct.

Further evidence for the formation of hydroxyl radicals is provided by spin trapping with POBN. This is an efficient trap for C-centred radicals, but the POBN–OH spin adduct is very short lived. Consequently, UVA irradiation of TiO_2 outside the microwave cavity of the spectrometer produced no detectable radical adduct unless ethanol was present during irradiation, when the hydroxyethyl radical $\bullet CH(CH_3)OH$ was formed by H-abstraction by the hydroxyl radicals. In contrast, *in situ* UV-irradiation of aqueous TiO_2 in the presence of POBN was subsequently found to produce the POBN–OH adduct. The

absence of any ESR signal on irradiation of TiO_2 in the presence of 2,2,6,6-tetramethyl-4-piperidinol demonstrates that $^1O_2$ is not formed. Consequently it can be concluded that UVA-irradiation of aqueous TiO_2 produces only hydroxyl radicals.

ESR studies showed that UVA irradiation of control cells produced no detectable radicals. In contrast, irradiation of TiO_2- treated cells produced primarily OH radicals that could be trapped with DMPO or DEPMPO. Using the latter spin trap, a number of other radicals were detected, the most abundant of which is possibly due to peroxidation of lipid membranes by hydroxyl radicals, since a similar signal, together with that of DEPMPO–OH appears to be present in the spectrum of UV-irradiated vesicles of phosphatidylcholine containing TiO_2 (unpublished results). Moreover, the radical trapped by POBN on irradiation of TiO_2-treated cells is consistent with a lipid peroxyl radical (Barber *et al.*, 1993)). The nature of this radical and the source of the low concentration of superoxide radical anions that are produced require further investigation. However, it can be concluded that the primary cause of biological damage from TiO_2, combined with UVA is due to hydroxyl radicals which may react directly with DNA or indirectly through peroxidation of membrane lipids.

Our ESR spin trapping experiments indicate that TiO_2 can produce hydroxyl radicals in the absence of UVA, possibly due to ambient light, and these react with cells, although the concentration detected by spin trapping with DEPMPO

is reduced, due to the spin adducts being metabolised by the cells and by their inherently short lifetime when compared with exposure times.

In conclusion, we have shown for the first time that TiO_2 nanoparticles in the absence of photo-activation are potentially genotoxic to fish cells under *in vitro* conditions. This effect becomes more pronounced in the presence of UVA, along with cytotoxic effects which only occurred during combined expose of TiO_2 and UVA. ESR studies indicate that OH radicals are the predominant radical species generated both in aqueous solution as well as in the fish cells. These radicals are likely to play the major role in producing the genotoxic effects in terms of oxidative DNA damage. *In vivo* studies are now required to further establish how TiO_2 nanoparticles may affect various parameters of fish health during waterborne or dietary exposure of environmentally realistic concentrations.

CHAPTER 6: VALIDATION OF METHODS FOR EVALUATING ANTIOXIDANT STATUS, IMMUNE FUNCTION AND GENOMIC STABILITY IN HUSBANDRY STRESSED RAINBOW TROUT (*ONCHORYNCHUS MYKISS*)

6.1 Introduction

This work was done in collaboration with Sebastien Rider and forms part of a co-authored manuscript, to be duly submitted for publication. As such, the results presented here will also form part of Sebastien Rider's Doctoral thesis. In terms of the present thesis, the purpose of these experiments was the development and validation of novel assays for the evaluation of stress effects on fish. The aim being to apply these techniques to further studies on the potential protective effects of dietary micronutrient supplementation against husbandry induced stress in fish.

As outlined in chapter 1, husbandry stressors such as handling, weighing, netting and transportation may initiate a stress response leading to increased levels of stress hormones, modulations of the immune system, and suboptimal growth (Bly *et al.*, 1997; Iwama *et al.*, 1997). However, there appears to be a lack of knowledge on the effects of husbandry stress on the antioxidant system of fish and on oxidative stress induced genomic instability.

Therefore the objective of these experiments was to determine if chronic physical handling and confinement stress causes oxidative stress, genomic

instability and modulations in the immune system of trout. Total antioxidant capacity (TAC) of trout plasma and respiratory burst capacity of trout leukocytes were evaluated using test kits from Knight Scientific Ltd (Plymouth, UK). Neither of these assays has previously been used for the detection of TAC and respiratory burst in fish. Oxidative DNA damage was evaluated by using a modified Comet assay protocol using a lesions-specific bacterial repair enzyme (Fpg), which coverts oxidised purine bases to single strand breaks, greatly enhancing the specificity and sensitivity of the assay (Collins *et al.*, 1993; Dusinska and Collins, 1996). Only a couple of studies have utilised a modified Comet assay for the detection of oxidative DNA damage in fish using Fpg enzyme, but apparently not in relation to chronic handling and confinement stress. Akcha *et al.* (2003) used a modified Comet assay with Fpg to evaluate the effects of marine pollution on oxidative DNA damage in the dab, whereas Aniagu *et al.*, (2007) linked observed increases in oxidative DNA damage to exhaustive physical exercise in chub.

6.2. Material and methods

6.2.1. Materials

All materials were obtained from Sigma (Poole, UK) unless otherwise stated.

6.2.1. Fish and husbandry

Rainbow trout (*Oncorhynchus mykiss*), initially 105 ± 20 g, were obtained from Hatchlands Fisheries (Rattery, Devon, UK) and transferred to the University of Plymouth packaged in filtered water in clear polythene bags with pure oxygen. Fish were acclimated to the experimental system (System E; Plate 1) for four weeks before being separated into two 125 l 'control' and 'stressed' tanks three days prior to the study. All fish were fed twice daily a commercial fish feed (15-45 Grower Feed XS, Aller Aqua, Christiansfeld, Denmark) at 1.8% body weight per day during both the acclimation and experimental period. The following water quality parameters were monitored daily: pH was maintained at 7.5 ± 0.5, dissolved oxygen (DO) was ≥ 8.0 mgl^{-1} (90-95% saturation) and temperature was regulated automatically at 15 ± 0.5 °C. Nitrogenous waste levels were measured weekly using a Hach Lange Dr 2800 and test kits for ammonia (Lange LCK 304), nitrite (Lange LCK 341) and nitrate (Lange LCK 340). A 20 % water change was performed weekly to keep nitrate levels within acceptable limits, considered to be < 0.1 mgl^{-1}, < 0.1 mgl^{-1} and < 50 mgl^{-1} for ammonia (unionized), nitrite and nitrate, respectively. Photoperiod was set to 12 hours light: 12 hours dark using artificial light from fluorescent tubes.

Plate 6.1. Recirculation system (E) based in the experimental aquarium at the University of Plymouth, UK, where the experiment was carried out. The system comprised 20, 125 litre tanks with a flow rate through the tanks of ~ 5 $lmin^{-1}$.

6.2.2. Stress induction

Two different physical stressors were used in order to lessen the chance that fish may become desensitized to one particular kind of stress (Barnton *et al*, 1987). Netting/handling stress and low water confinement stress were applied on alternate days for 7 days. For netting stress all fish were netted from one tank at a time and collected in a large single net suspended over a bucket containing system water. This was followed by 10 second aerial emersion/ 10 second water immersion for 2 minutes (Salo *et al.*, 2000) after which fish were

immediately returned to their system tank. For low water confinement six tanks at a time were drained to a level which was just sufficient to cover the fish's dorsal fin (Fevolden *et al.*, 2003). The fish were further confined by dividing the tank into two with a plywood division. Fish were kept under these conditions for one hour after which divisions were removed and system water allowed to flow in at the normal rate until the water level returned to normal.

6.2.3. Anaesthetisation and sample collection

Fish were removed from their respective tanks and transferred into an anaesthetic bath containing Tricane Methane Sulphonate (MS222) [Farmaq, Fordingbridge, UK] at a concentration of 100 mgl^{-1} (Ross, 2001), dissolved in system water. Anaesthesia was monitored by loss of equilibrium in the fish which indicated it was suitably sedated for subsequent blood sampling. Blood was drawn from the caudal vein using a 1 ml syringe fitted with a 25 gauge needle. Following blood sampling, fish were allowed to recover in well aerated system water before being returned to their tanks. The blood samples were then dealt with according to the end point being assayed. Whole blood was sampled on day eight from 12 randomly selected individuals for the measurement of respiratory burst and TAC (stored in EDTA treated tubes and kept at room temperature). On day nine further blood samples were taken and stored on ice in heparin treated tubes the modified Comet assay.

6.2.5 ABEL (Analysis By Emitted Light) assays from Knight Scientific Ltd.

Total antioxidant capacity (TAC) of trout plasma and respiratory burst (cell activation) of trout whole blood were evaluated using ABEL (Analysis By Emitted Light) test kits with Pholasin® from Knight Scientific Ltd. (Plymouth, UK).

Pholasin® is the photoprotein of the marine, rock-boring bioluminescent mollusc, *Pholas dactylus*, the Common Piddock. Pholasin® does not glow on its own but is 'switched on' in the presence of free radicals and other oxidants and therefore is used in the assays as a chemiluminescent probe. It can be used to either detect the quenching or free radicals by antioxidants, such as in the Total Antioxidant Capacity (TAC) assay (Nourooz-Zadeh *et al.*, 2006), or to detect the production of free radicals by cells and/or enzymes, as in the cell activation (respiratory burst) assay (Roberts *et al.*, 1997).

6.2.5.1 Total antioxidant capacity (TAC)

Total antioxidant capacity (TAC) of trout plasma was assessed using an ABEL® 41M2 antioxidant test kit with Pholasin® using peroxynitrite (Knight Scientific Ltd, Plymouth, UK).

Peroxynitrite is formed in the assay by the reaction between superoxide and nitric oxide, released simultaneously and continually from SIN-1 (3-morpholino-sydnonimine HCl; $C_6H_{10}N_4O_2$. HCl).

$$O_2^{\bullet-}\text{ (superoxide)} + NO^{\bullet}\text{ (nitric oxide)} \rightarrow ONOO^{-}\text{ (peroxynitrite)}$$

When SIN-1 is injected into a 96 well microplate containing Pholasin®, light of gradually increasing intensity is detected, reaching a peak after a few minutes. If there are antioxidants in the sample capable of scavenging peroxynitrite then these will compete with the Pholasin® for the peroxynitrite. Any antioxidants in the sample will be gradually consumed, delaying the time at which the maximum peak of light is emitted, as well as, competing for the Pholasin®, resulting in a peak of lower intensity. The time at which peak luminescence occurs (peak time) after adding SIN-1, is expressed in vitamin E analogue equivalent units, VEA units, using 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (a water soluble vitamin E analogue). A standard curve is run as part of the assay; the formula for the linear regression can then be used to convert times-to-peak into VEA equivalent units.

The assay was performed according to the manufacturer's instructions. In brief, to 5 µl trout serum, 95 µl assay buffer, 50 µl Pholasin® and 50 µl SIN-1 was injected at time zero. Assays were performed at 25°C in a white 96 well microtitre plate using a BMG Lumistar Galaxy Luminometer. Mean VEA equivalent units ($\mu mol l^{-1}$) were determined for all individuals from duplicate assays.

6.2.5.2. Respiratory burst activity of leukocytes

Extracellular respiratory burst activity (cell activation) of trout leukocytes in whole blood was assessed using an ABEL® 04M antioxidant test kit with Pholasin® and Adjuvant K™ (Knight Scientific Ltd, Plymouth, UK).

Biocidal free radicals are released from activated leukocytes in response infection and disease and form a key part immune function. The cell activation assay with Pholasin® measures the release of such radicals after stimulation of cells with formyl-methionyl-leucyl-phenylanaline (fMLP) or phorbol myristoyl acetate (PMA). During activation, the NADPH oxidase forms a complex of proteins that assembles on the inner surface of the plasma membrane. There a constant stream of superoxide is release to the outside of the cell via electron transfer (from NADPH) through the membrane to oxygen. Degranulation may follow which involves fusion of granular membranes with the plasma membrane and the release of enzymes/proteins such as myloperoxidase (MPO) which are involved in the production of further oxidants such as hypochlorous acid.

Briefly, EDTA whole blood was diluted 1/100 with blood dilution buffer and 20 µl added to 90 µl assay buffer, 50 µl Pholasin® and 20 µl Adjuvant-K™ (enhances luminescence of Pholasin®). Leukocytes were activated by injecting 20 µl of PMA providing a final concentration of 0.5 µg PMA (final blood dilution 1/1000). The assay was run in a 96 well white microplate at room temperature (25°C) and emitted light read every 27 seconds for 90 minutes using a BMG

Lumistar Galaxy Luminometer. Mean of duplicates were used to calculate mean relative light units for individuals from stressed and control treatments.

6.2.6. Modified Comet assay

The modified Comet was performed as outlined in section 2.2.4.2 except that red blood cells diluted in PBS were used instead of trypsinised cultured cells.

6.2.8. *Statistical analysis*

Mann-Whitney *U* test was used to test for statistical significance between medium values in the Comet assay, whereas differences in means were analysed by the Student's t-test. Differences were considered significant when $P < 0.05$.

6.3 Results

No mortality occurred as a result of the applied stress throughout the study. The Total antioxidant capacity (TAC) of trout plasma, evaluated by the ability of serum antioxidants to scavenge peroxynitrite, was significantly lower in stressed fish compared to control fish ($P < 0.001$, Fig. 6.1). Husbandry stress also appeared to reduce the respiratory burst capacity of trout leukocytes, after stimulation with PMA (Fig.6.2). This difference was realized after ~50 minutes post PMA activation. Initial peaks of activity at ~9 minutes were recorded for both stressed and control fish, which were not significantly different between treatments. However, stressed fish showed a significantly lower secondary

peak (~4800 Relative Light Units [RLU] after ~33 minutes), compared to the secondary peak of control fish (6400 RLU measured after 90 minutes) ($P < 0.05$). The signal of the control fish may have continued to rise if the assay had been run for longer. Chronic husbandry stress also influenced genomic stability in trout erythrocytes as measured by the modified Comet assay (Fig 6.3). Direct single strand breaks (no Fpg digestion) did not increase to a significant level. However, oxidative DNA damage, measured by the modified Comet assay with Fpg digestion, significantly increased in fish subjected to stress ($P < 0.05$).

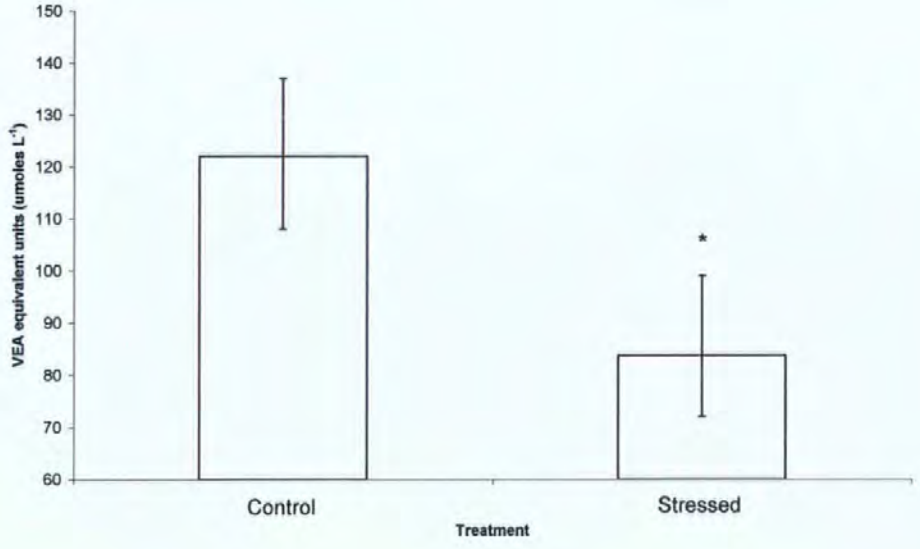

Fig. 6.1. Total antioxidant capacity (TAC) of plasma from 'stressed' and 'control' Rainbow trout (*O. mykiss*). Expressed as vitamin E equivalent (VEA) units ($\mu moll^{-1}$) ± S.E (n = 12). * denotes significant difference versus control.

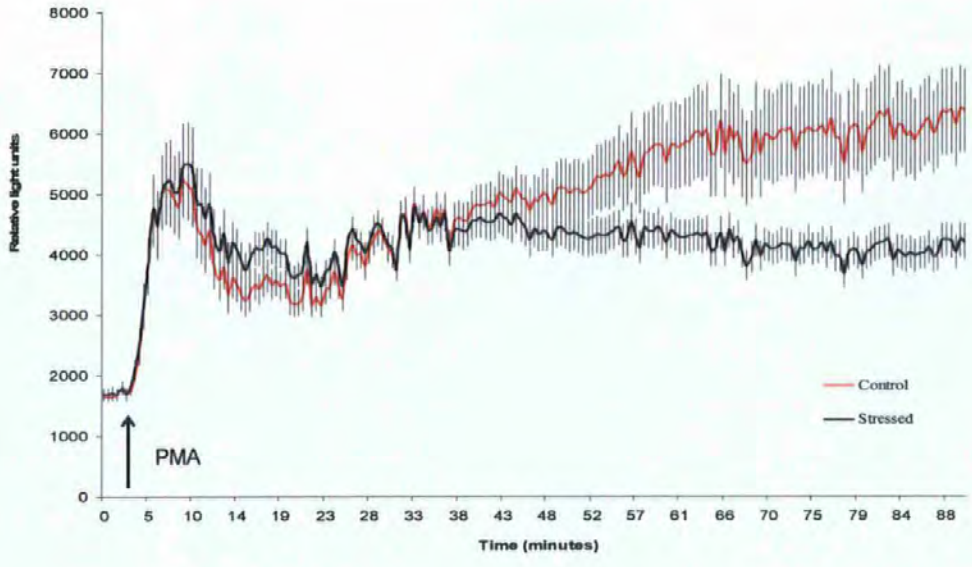

Figure 6.2 Respiratory burst capacity of leukocytes from 'stressed' and 'control' Rainbow trout (*O. mykiss*). Expressed as mean relative units ± standard error (n = 12). PMA injected after 3 minutes (indicated by arrow).

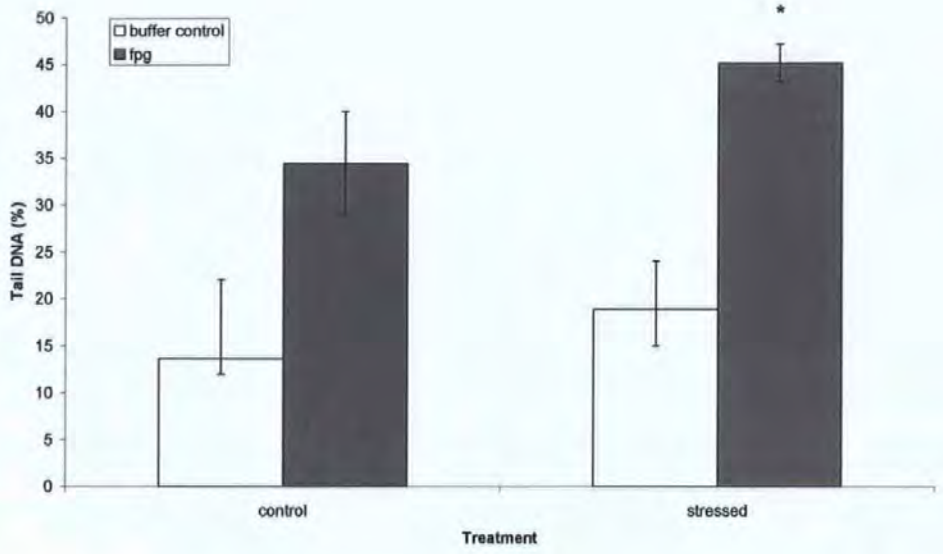

Figure 6.3. Oxidative DNA damage in erythrocytes of 'stressed' and 'control' Rainbow trout (*O. mykiss*). Expressed as median percentage tail DNA with interquartile range for single strand breaks and Fpg sensitive sites (n = 12). * denotes significant difference versus control.

6.4. Discussion

Overall the results from these experiments indicate that husbandry induced stress influences antioxidant status, immune status and genomic stability in rainbow trout. Total antioxidant capacity (TAC), as evaluated by the ability of plasma antioxidants to scavenge a continual production of peroxynitrite, was significantly reduced in response to husbandry stress. This particular method has not been adopted in studies on fish before. However, the TAC assay with Pholasin® has previously been validated in human studies and been shown to be a relatively simple, sensitive and reliable assay for the measurement of plasma TAC (Knight *et al.*, 2002; Nourooz-Zadeh *et al.*, 2006). The reductions

in TAC due to husbandry stress as seen this study agree with those of (Chirase *et al.*, 2004) who found reduced TAC in transport stressed cattle. Furthermore, in mammals, physical exercise may either increase TAC (Alipour *et al.*, 2006) or decrease TAC (Ficicilar *et al.*, 2003) depending on the nature of the exercise. However, exhaustive physical exercise has previously been shown to have no effect on the antioxidant status of chub (Aniagu *et al.*, 2006). In contrast to the present study though, they only measured the activity of superoxide dismutase (SOD) and reduced glutathione (GSH), which does not represent the *total* antioxidant capacity of the fish. As the measure of single antioxidants or antioxidant enzymes may not truly represent the overall antioxidant capacity of fish, these measurements should be accompanied by assays such as the TAC assay used in this study, which may be more biologically relevant.

The respiratory burst capacity of trout leukocytes after stimulation with PMA also appeared to be suppressed in fish subjected to husbandry stress. Initial responses to the stimulant PMA appeared to be similar as the peaks after ~ 9 minutes were not dissimilar between the two groups. This response was probably due to the release of superoxide from the inner membrane of blood leukocytes to the outside of the cell via electron transfer (from NADPH) through the membrane to oxygen via the NADPH oxidase system (Reichl *et al.*, 2001). For the control group a second peak was reached after 90 minutes which was possibly due to degranulation which involves fusion of granular membranes with the plasma membrane and the release of enzymes/proteins such as myloperoxidase (MPO) which are involved in the production of further

oxidants such as hypochlorous acid which react with Pholasin® (Reichl *et al.*, 2001). The fact that this did not happen in the stressed fish suggests that the degranulation and further release of oxidants as part of the respiratory burst process was suppressed due to stress.

Respiratory burst has not previously been measured in fish using the cell activation assay with Pholasin® as a chemiluminescent probe. Respiratory burst in fish is usually evaluated using assays such as the NBT (nitroblue tetrazolium) assay, which is based on a colour change due to the reduction of NBT by superoxide (Chung & Secombes, 1988). A chemiluminescent method enhanced with luminol has also been used to measure respiratory burst in fish (Jokinen *et al.*, 2000). However, the cell activation assay with Pholasin® appears to be superior to that of other chemiluminescent assays, such as those involving luminol, in measuring respiratory burst. Under optimal conditions Pholasin® produced a signal some 50–100 times that of luminol in the presence of activated human neutrophils, proving its greater sensitivity for the detection of free radicals (Roberts *et al.*, 1987). Furthermore, in addition to detection of superoxide released during the initial stages of the respiratory burst, Pholasin® may also detect the release of myloperoxidase (MPO) which produces hypochlorous acid during degranulation in activated leukocytes (Reichl *et al.*, 2001) in the latter part of the response to infection.

Husbandry stress has previously been shown to modulate the immune system of fish (Yin *et al.*, 1995; Tort *et al.*, 1996). Components of the respiratory burst

have been shown to be either stimulated (Pulsford *et al.*, 1994) or suppressed (Vazzana *et al.*, 2002; Montero *et al.*, 2001; Angelidis *et al.*, 1987).

Husbandry stress was also shown to significantly increase levels of oxidative DNA damage in trout erythrocytes, reflected by increases in Fpg-sensitive sites. Previously, the modification of the Comet assay protocol, which includes a digestion step with bacterial repair endonucleases to target oxidised DNA bases, has been shown to greatly enhance the sensitivity and specificity of the assay, compared to the standard protocol (Dusinska and Collins, 1996; Collins, 2004). In the present study, this is highlighted by the fact that the level of Fpg-sensitive sites (oxidised purines) increased to a greater degree than the level of direct SSB in stressed fish. The increase in Fpg-sensitive sites compared to direct SSB suggests that 8-hydroxyguanine (8-OH-G), the product of guanine oxidation, was probably the most likely adduct induced, although formamidopyrimidines (imidazole ring-opened purines) are also possible substrates for Fpg (Kielbassa *et al.*, 1997; Martinez *et al.*, 2003). Furthermore, 8-OH-G is considered one of the most important DNA lesions resulting from oxygen radical attack and a good biomarker of oxidative stress (Valavanidis *et al.*, 2006). Although the standard Comet assay has been used extensively for evaluating the genotoxic potential of contaminants in fish (see reviews Mitchelmore & Chipman, 1998; Cottelle & Ferard, 1999), there are only two other studies that have specifically evaluated oxidative DNA damage in fish cells using the modified Comet assay (Akcha *et al.*, 2003; Aniagu *et al.*, 2006).

Overall it appears as though the methods for detection of TAC, respiratory burst capacity and genomic stability used in this study are sensitive to the effects of stress that may be induced in fish during common husbandry techniques. Therefore, their application for further studies on the effects of husbandry stress on fish and how these may be modulated by micronutrient supplementation seems valid.

CHAPTER 7: EFFECTS OF HUSBANDRY STRESS AND MICRONUTRIENT SUPPLEMENTATION ON VARIOUS HEALTH PARAMETERS OF COMMON CARP (*CYPRINUS CARPIO*)

7.1. Introduction

As outlined in chapters 1 and 6, husbandry stressors such as handling, weighing, netting and transportation may initiate a stress response leading to increased levels of stress hormones, modulations of the immune system, and suboptimal growth in fish. In the last chapter assays for total antioxidant capacity (TAC), respiratory burst (cell activation) and oxidative DNA damage (measured by a modified Comet assay) were shown to be sensitive to a chronic episode of husbandry induced stress in trout. Therefore the same assays were used again in this study to observe effects of husbandry stress in carp.

Also, as outlined in chapter 1, selenium, zinc and vitamin E are all essential for optimal growth, antioxidant capacity and immune function in fish. Selenium and zinc especially are required at the active site of a number of enzymes, including the antioxidant enzymes glutathione peroxidase (Se dependent) and superoxide dismutase (Zn-dependent). Although the role of these micronutrients have been confirmed in fish kept under 'normal' conditions (stress free), there is a lack of literature available on how their requirement may change under conditions of stress such as those during common

husbandry practices. In other words, how husbandry induced stress effects may be modulated by dietary supplementation of micronutrients.

In light of the above, the aims of this study are two: (a) evaluate the effects of husbandry stress on antioxidant status, immune function and genomic instability in carp; (b) evaluate the effectiveness of selenium, zinc and vitamin E supplementation to modulate any stress induced changes. Antioxidant status was evaluated by measuring total antioxidant capacity (TAC), the activity of the antioxidant enzymes glutathione peroxidase and superoxide dismutase and as a measure of lipid peroxidation of cell membranes, the osmotic fragility of red blood cells. Assays for immune function included lysozyme, complement and respiratory burst. Oxidative DNA damage, as measure by the modified Comet assay, was also included.

7.2 Materials and methods

7.2.1 Materials

All chemicals were purchased from Sigma (Poole, UK) unless otherwise stated. Sources of other materials are cited in the relevant sections.

7.2.2 Fish and husbandry

Common carp (*Cyprinus carpio*), initially 9.8 ± 0.1 g, were obtained from Hampshire Carp Hatcheries (Winchester, UK) and transferred to the University

of Plymouth packaged in filtered water in clear polythene bags with pure oxygen. Fish were acclimated to the experimental system (Plate 7.1) for two weeks during which they were feed *ad lib* on the control diet. Also during this period, the water temperature was slowly raised (0.5°C day^{-1}) from ambient (~18°C) to 25°C, the optimal growth temperature for carp. Fish were then graded into 18 tanks in order to feed 6 experimental diets in triplicate. The following water quality parameters were monitored daily: pH was maintained at 7.5 ± 0.5, DO was ≥ 8.0 mgl^{-1} (90-95% saturation) and temperature was regulated automatically at 25 ± 0.5 °C. Nitrogenous waste levels were measured weekly and maintained within acceptable limits as described in section 6.2.1. Photoperiod was set to12 hours light: 12 hours dark using artificial light from fluorescent tubes. During the trial fish were fed 3 % of their body weight per day ($BW.d^{-1}$) interspersed between 2 feeds. Fish were weighed fortnightly and feed amounts adjusted accordingly.

7.2.3 Experimental diets

7.2.3.1. Diet preparation

All diets were made at the University of Plymouth using raw materials supplied by Waltham Centre for Pet Nutrition (Melton Mowbray, UK). The basal diet followed the formulation of Aquarian® goldfish pellets (Waltham) and met the minimum nutrient requirements for carp, as set by the National Research Council (NRC) (1999). Six experimental diets were made with the addition of micronutrient supplements as outlined in Table 7.1, which were considerably

above the minimum requirements, yet not considered to have any adverse toxic effect (NRC, 1999).

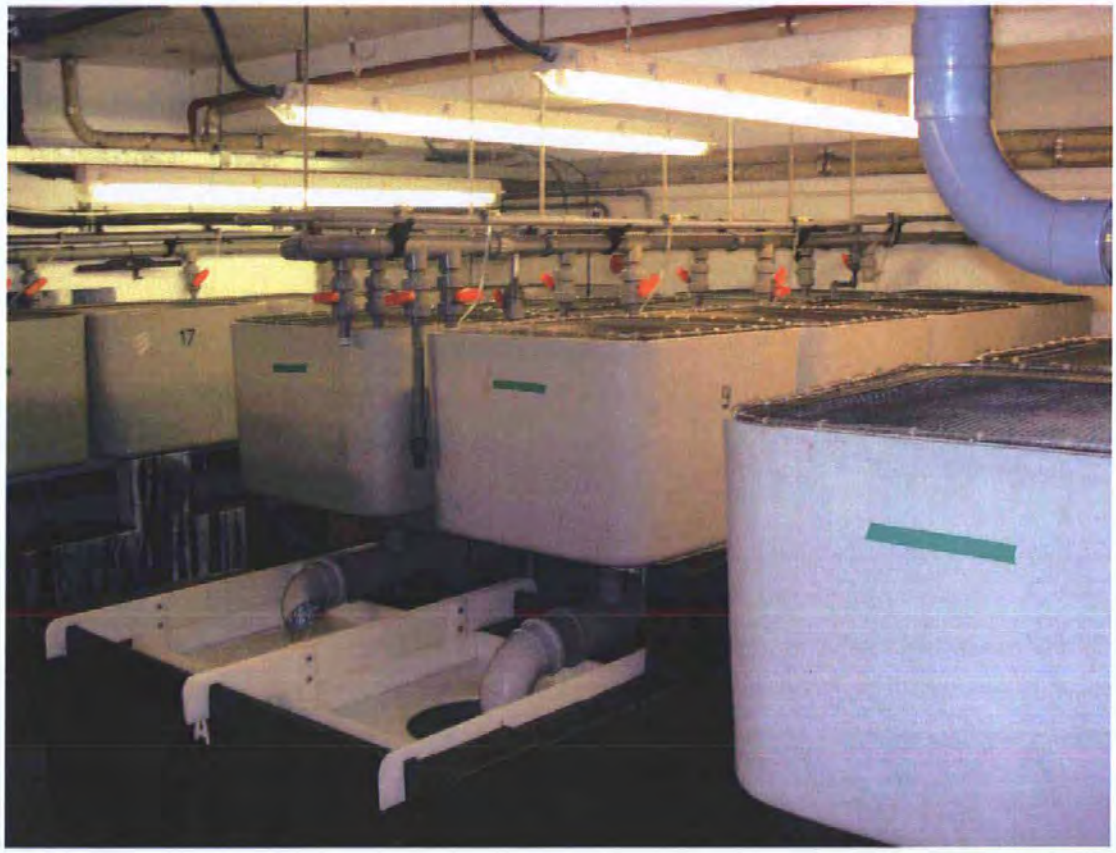

Plate 7.1 Recirculation system (A) based in the experimental aquarium at the University of Plymouth, UK, where the trial was run. The system comprised 18, 170 litre tanks with a flow rate through the tanks of ~ 10 $lmin^{-1}$.

Where necessary, supplement premixes were added to the dry diet mix at 5% w/w, using corn flower as a carrier, to give the desired inclusion level (Table 7.1). Sodium selenite and zinc sulphate heptahydrate were first dissolved in water, mixed with cornflower, dried overnight and ground to a fine powder before addition to the final diet mix. Other supplements were added in dry as a fine powder. To prepare the experimental diets, all the dry ingredients were

thoroughly mixed until homogenous using a Hobart food mixer. Oil was added and mixed in well, before water was added at ~ 30% v/w. The semi-moist mixture was transferred to a Jackson extruder and pressure-pelleted using a 2-mm die and oven-dried over 2 days at 45°C, until moisture was < 10%.

Table 7.1. Source and supplementatetion level of micronutrients supplemented in 6 experimental diets fed to Common Carp (*C. carpio*) over 10 weeks.

Diet no.	Supplement	Source	Targeted level
1	**Control (none)**	-	-
2	**Vitamin E** (*α-tocopheral acetate*)	Waltham	1000 IU kg^{-1}
3	**Inorganic zinc** (*Zinc sulphate heptahydrate,* $ZnSO_4 \cdot 7H_2O$)	Sigma	300 mg kg^{-1}
4	**Organic zinc** (*Zinc Biolpex*)	Alltech	300 mg kg^{-1}
5	**Inorganic selenium** (*Sodium selenite,* Na_2SeO_3)	Sigma	2.5 mg kg^{-1}
6	**Organic selenium** (*Selplex*)	Alltech	2.5 mg kg^{-1}

7.2.3.2 Proximate analysis

As all diets were made at the University of Plymouth; proximate analysis was carried out to ensure they accurately resembled Waltham Goldfish pellets, according to AOAC (1995) methods. Proximate compositions of the diets are reported in Table 7.2, which do not differ significantly between diets and match extremely well to Aquarian® goldfish pellets (Waltham Centre for Pet Nutrition,

Melton Mowbray, UK). Specifics of the formulation of Aquarian pellets are required to remain confidential.

Moisture

The gravimetric measurement of moisture in the diets was expressed as a percentage of the initial sample weight (W1). A representative sample of ~ 10 g (weighed out in triplicate) was dried to constant weight in a fan assisted oven (Genlab Ltd, UK) maintained at 110°C (~ 36-48 hours). The sample was removed from the oven, cooled in a dessicator, and reweighed (W2). Moisture was calculated according to the formula:

$$\text{Moisture (\%)} = [(W1 - W2) / W1] \times 100$$

Ash

Ash content was determined as total inorganic matter by incineration of the sample at 550° C in a muffle furnace (Carbolite, UK). Remaining inorganic materials are reduced to their most stable form, oxides or sulphates and are considered 'ash'. Samples of diet (0.5 g) were weighed out in triplicate, and then reweighed after 16 hours incineration. Ash was calculated as:

$$\text{Ash (\%)} = \{[W1 - (W1 - W2)] / W1\} \times 100$$

Lipid

Crude lipid in the diets was determined by solvent extraction using a Soxtech extraction unit (Tecator Soxtech HT 1043 Extraction unit, Sweden). The method depends upon the heating of a solvent (petroleum ether), which is allowed to pass through the sample to extract the lipid. The extract is collected in a cup and, when the process is completed, the solvent is evaporated and can be recovered. The remaining crude lipid in the cup is dried and weighed. Feed (2.5 g) was weighed out in triplicate into extraction thimbles (W1) and covered with absorbent cotton while 40 ml of solvent was added to a pre-weighed collection cup. Subsequently, samples were subjected to boiling (115° C) in solvent for one hour followed by rinsing for 50 minutes. Solvent was then evaporated from the cup to the condensing column. Extracted lipid in the cup was placed in an oven at 110° C for 1 hour and after cooling was weighed (W2) and % crude lipid calculated thus:

Crude lipid (%) = (W2 / W1) x 100

Protein

Crude protein was determined by the Kjeldahl method which is based on sample digestion with concentrated sulphuric acid and catalysts, so that the organic compounds are oxidized and the nitrogen is converted to ammonium sulphate. Upon making the reaction mixture alkaline, ammonia is liberated, removed by steam distillation, collected and titrated.

Diet samples (150 mg) were weighed out in triplicate and transferred into Kjeldahl digestion tubes along with a catalyst tablet (3 g K_2SO_4 ,105 mg $CuSO_4.5H_2O$ 105 mg TiO_2, BDH Ltd, UK) and 10 ml of H_2SO_4 (Sp.Gr. 1.84, BDH Ltd, UK). Digestion was performed using a Gerhardt Kjeldatherm digestion machine (Gerhardt Instruments, Germany) and comprised three steps:

1. 100 °C for 30 min.
2. 220 °C for 60 min.
3. 380 °C for 45 min.

After digestion, NaOH was added to produce ammonia which reacts with boric acid. A back-titration was carried out using 0.1M H_2SO_4. The protein percentage was calculated thus:

Protein (%) = [(ST x 2) – (BT x 2)] x 0.1 x 14 x 6.25 x (100 / SW)

Where, ST = sample titre (litres)

BT = blank titre (litres)

SW = sample weight (g)

0.1 is the acid strength in moles

14 in the molecular weight of nitrogen

6.25 is a constant (protein is assumed to be 16% nitrogen)

Table 7.2. Proximate analysis of control and experimental diets. Data represented as percentages (± SE) from triplicate samples.

%	Control	+ Vit. E	+ In. Zn	+ Org. Zn	+ In. Se	+ Org. Se
Protein	**34.4** (0.46)	**35.7** (1.07)	**35.4** (0.07)	**34.9** (0.54)	**35.5** (0.03)	**35.1** (0.20)
Lipid	**7.9** (0.04)	**7.7** (0.00)	**8.0** (0.08)	**7.8** (0.03)	**7.8** (0.61)	**8.0** (0.1)
Ash	**7.9** (0.11)	**8.3** (0.09)	**8.2** (0.12)	**7.9** (0.17)	**8.1** (0.16)	**7.7** (0.18)
Moisture	**9.0** (0.12)	**8.3** (0.03)	**8.0** (0.07)	**8.1** (0.10)	**7.7** (0.18)	**8.1** (0.03)

7.2.3.3. Vitamin and mineral analysis

Actual selenium and vitamin E levels in experimental diets are yet to be determined. Zinc levels were determined by flame atomic absorption spectroscopy (FAAS) at the University of Plymouth and are reported in Table 7.3.

Table 7.3. Zinc analysis of control and experimental diets. Mean levels from triplicate samples (± SE).

Diet	Control	+ Vit. E	+ In. Zn	+ Org. Zn	+ In. Se	+ Org. Se
Zinc $\mu g\ g^{-1}$	**63.5** *(2.5)*	**64.9** *(2.9)*	**280.7** *(10.1)*	**403.3** *(20.3)*	**70.8** *(7.0)*	**72.4** *(1.6)*

7.2.4. Experimental design and stressors

The trial was run for 12 weeks with fish being weighed fortnightly in order to adjust feed input and to chart growth performance. At the end of the trial whole

blood and serum samples were collected in order to evaluate dietary effects on the various health parameters measured. Following a 1 week recovery period, fish were stressed using two different physical stressors in order to lessen the chance that fish may become desensitized to one particular kind of stress (Barnton *et al*, 1987). Netting/handling stress and low water confinement stress were applied on alternate days for 7 days. For netting stress all fish were netted from one tank at a time and collected in a large single net suspended over a bucket containing system water. This was followed by 10 second aerial emersion/ 10 second water immersion for 2 minutes (Salo *et al.*, 2000) after which fish were immediately returned to their system tank. For low water confinement six tanks at a time were drained to a level which was just sufficient to cover the fish's dorsal fin (Fevolden *et al.*, 2003). The fish were further confined by dividing the tank into two with a plywood division. Fish were kept under these conditions for one hour after which divisions were removed and system water allowed to flow in at the normal rate until the water level returned to normal. Further blood samples were taken after the 7 day stress period in order to investigate the interaction between dietary status and stress.

7.2.5 Growth parameters

Growth indices calculated include: live weight gain (LWG), specific growth rate (SGR), thermal-unit growth co-efficient (TGC) and feed conversion efficiency (FRC).

LWG (g) = FBW – IBW

Where, FBW = final wet body weight (g)

IBW = initial wet body weight (g)

SGR (%BW.D^{-1}) = [(ln FBW – ln IBW) / t] x 100

Where, % BW.D^{-1} = % body weight gain (g) per day

ln FBW = natural log of FBW

ln IBW = natural log of IBW

t = time (days)

TGC = (FBW$^{1/3}$ – IBW$^{1/3}$) / (T x t x 100)

Where, T = average system water temperature (°C).

FCR = (feed input (g) / LWG)

7.2.6. Anaesthetisation and sample collection

Fish were removed from their respective tanks and transferred into an anaesthetic bath containing Tricane Methane Sulphonate (MS222) [Farmaq, Fordingbridge, UK] at a concentration of 100 mgl^{-1} (Ross, 2001), dissolved in system water. Anaesthesia was monitored by loss of equilibrium in the fish which indicated it was suitably sedated for subsequent blood sampling. Blood

was drawn from the caudal vein using a 1 ml syringe fitted with a 25 gauge needle. Following blood sampling, fish were allowed to recover in well aerated system water before being returned to their tanks. The blood samples were then dealt with according to the end point being assayed. The enzyme assays, complement assay and TAC assay required the preparation of serum. For these samples, collected blood was transferred to microcentrifuge tubes (without anti-coagulant) and left to clot at room temperature for 3-4 hours before centrifugation at 9000 x g for 5 minutes. The resulting serum was stored at -80°C and assayed within 1 month. For the Comet assay, blood was immediately transferred to heparin-coated tubes. Then, 10 µl of heparin treated blood was mixed with 990 µl of freezing medium (RPMI 1640 medium containing 25% FBS and 20% DMSO) to give a 1/100 dilution. This was flash frozen in liquid nitrogen, stored at -80°C and assayed within 1 month as recommended (Belpaeme *et al.*, 1998). Respiratory burst assays were carried out at Knight Scientific (Plymouth, UK). Whole blood samples were immediately transferred to their laboratory in anticoagulant (EDTA) treated tubes. At each sampling point 3 fish were sampled from each tank to give at least 9 fish per dietary treatment for each assay.

7.2.7. Analytical techniques

7.2.7.1. Glutathione peroxidase (GSHPx) activity

Glutathione peroxidase (GSHPx) is a selenium-dependent enzyme which catalyzes the reduction of hydrogen peroxide (H_2O_2) and organic

hydroperoxides (ROOH) to water and the corresponding stable alcohol. GSHPx uses glutathione as the ultimate electron donor to regenerate the reduced form of the selenocysteine at the active site.

Glutathione peroxidase activity in carp serum was indirectly measured by utilising a coupled reaction with glutathione reductase (GR) (Tran *et al.*, 2007). Oxidized glutathione (GSSG), produced upon reduction of an organic hydroperoxide by GSHPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A_{340} is directly proportional to the GSHPx activity in the sample.

Carp serum (5 µl) was added to a 96 well plate and mixed with 30 µl of co-substrate solution (0.2 mM NADPH, 2mM reduced glutathione, 1 U ml^{-1} glutathione reductase) and 255 µl of Tris-HCl buffer (0.1 M Tris, 0.5 mM EDTA, 2.5 mM sodium azide, pH 8.0). The reaction mixture was incubated at 25°C for five minutes. The assay was initiated by the addition of 10 µl of 3 µM H_2O_2 and the absorbance at 340 nm continually recorded for 5 minutes using an Optimax microplate reader with Kinetic software SOFTmax® (Molecular Devices, USA) at 25°C. One unit (U/L) of GSHPx activity was expressed as the formation of 1 µmol $NADP^+$ from NADPH per min at pH 8.0 at 25°C calculated as follows:

U/L = ΔA340/minute x (TV/SV) X 10^3 / 6.22, where

TV = Total Volume in ml

SV = Sample Volume in ml

10^3 = converts ml to l

6.22 = millimolar absorbance coefficient of NADPH

7.2.7.2. Alkaline phosphatase (ALP) activity

Alkaline phosphatase (ALP) is a hydrolase enzyme which is most effective in an alkaline environment. It is responsible for removing phosphate groups in the 5- and 3- positions of many molecules, including nucleotides, proteins, alkaloids and the artificial molecule *p*-nitrophenyl phosphate, which is the substrate used in this assay. The process of removing a phosphate group is known as dephosphorolation.

This assay uses *p*-nitrophenyl phosphate as a substrate to measure the activity of ALP in a sample by the amount of *p*-nitrophenol liberated in 30 minutes (Walter and Schutt, 1974). *p*-nitrophenol is a yellow salt so it can be measured colourmetrically at 405 nm. It is important the substrate, *p*-nitrophenyl phosphate is in excess otherwise it will be limiting.

For each sample 2 parallel tubes were assayed containing 'active' enzyme and 'inactivated' enzyme. To both, 2 ml of assay buffer/substrate solution (1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) was added. To the active enzyme tube only, 50 µl of serum was also added. Both tubes were subsequently

incubated for 30 minutes at 25°C before the reaction was stopped by the addition of 10 ml of 0.05 M NaOH. Finally, 50 µl of serum was added to the inactivated enzyme tube. The absorbance of both tubes was read at 405 nm and the absorbance of the inactivated enzyme tube subtracted from that of the activated enzyme tube. The following equation was used to calculate ALP activity which is expressed as mM of p-nitrophenol liberated per min per litre of serum (U/l):

$$\frac{\text{Absorbance x Total reaction volume (12.05) x 1000 ml}}{\text{Reaction time (30min) x Ext coefficient (18.5*) x sample volume (0.05)}}$$

** The extinction coefficient of 4-nitrophenol in alkaline solution is 18.5 cm^2 / µM at 405nm*

7.2.7.3. Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD), which catalyzes the dismutation of superoxide anion ($O_2^{\cdot-}$) into hydrogen peroxide and molecular oxygen. Cytosolic SOD has a greenish colour and consists of two subunits: one subunit contains copper and the other zinc (Cu/Zn-SOD). Mitochondrial SOD has a reddish-purple colour and contains manganese (Mn-SOD).

SOD activity in pooled carp serum was measured by using an SOD Assay Kit (Dojindo Molecular Techniques Inc., Maryland, USA). The kit utilises a highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-

(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with superoxide anion which is continually produced by the xanthine/xanthine oxidase (XO) enzyme system. Since the absorbance of the formazan dye at 440 nm is proportional to the amount of superoxide anion produced, the inhibition activity of SOD or SOD mimetics in a sample can be quantified by measuring the decrease in the colour development at 440 nm.

The assay was performed according to the manufacturer's specifications, with slight modifications. The assay was carried out at 25°C which is optimal for carp instead of 37°C which is used for mammalian work. The protocol was also modified to allow the measurement of Cu/Zn-SOD and Mn-SOD independently as the standard protocol will measure total SOD only. To measure Mn-SOD activity, the activity of Cu/Zn-SOD was completely blocked by using 1mM potassium cyanide (KCN). To measure Cu/Zn-SOD activity total SOD activity was measured and then the activity of Mn-SOD subtracted from this.

7.2.7.4 ABEL (Analysis By Emitted Light) assays from Knight Scientific Ltd.

Total antioxidant capacity (TAC)

Total antioxidant capacity (TAC) of carp serum was assessed using an ABEL® 41M2 antioxidant test kit with Pholasin® using peroxynitrite (Knight Scientific Ltd, Plymouth, UK), as outlined in the previous chapter (6).

Respiratory burst (Cell Activation)

Extracellular respiratory burst of carp leukocytes was assessed using an ABEL 04M2 Cell Activation whole blood test kit with Pholasin® and Adjuvant K™ (Knight Scientific Ltd, Plymouth, UK), as outlined in the previous chapter (6).

7.2.7.5. Complement (alternative pathway)

Haemolytic activity driven by the alternative complement pathway was measured using rabbit red blood cells (RBC) as target cells in a Gelatin Veronal Buffer (GVB) (0.01M EGTA [blocks the classical pathway], 0.01M MgCl, pH 7.5) as described by Tort *et al.* (1996). Serial doubling dilutions of Carp serum were made in GVB in 1.7 ml microcentrifuge tubes to give the following concentrations in a total volume of 100 µl : 100%, 50%, 25%, 12.5%, 6.25% and 3.125% (v/v). Rabbit RBC in Alsever's solution (TCS Biosciences Ltd, Buckingham, UK) were washed three times in GVB and the concentration adjusted to 2 x 10^8 cells ml^{-1}. To each serum dilution tube, 100 µl of the washed rabbit RBC suspension was added and tubes incubated at 20°C for 90 min with manual shaking. After incubation, the volume in each tube was made up to 1 ml using GVB. Tubes were then centrifuged at 1600 x *g* for 5 minutes. The upper supernatant (200 µl) from each tube was transferred to a 96-well plate. The extent of haemolysis was determined by measuring the optical density of the supernatant at 414 nm using an Optimax microplate reader with Kinetic software SOFTmax® (Molecular Devices, USA). Complete haemolysis

(100%) was determined by adding 100 µl of the washed rabbit RBC suspension to 900 µl of distilled water. Zero haemolysis was determined by adding 100 µl of the washed rabbit RBC suspension 900 µl GVB buffer. The alternative complement pathway haemolytic activity was reported as the reciprocal of the serum dilution causing 50% lysis of rabbit RBC (ACH50).

7.2.7.6. Lysozyme activity

Lysozyme activity was measured using the turbidometric method (Stolen *et al.*, 1993) in 96 well micro-titre plates. To each sample well, 15 µl of serum was mixed with 285 ul of buffer/substrate solution (0.06M Na_2HPO_4, pH 6.2 containing 0.09% NaCl and 0.2 mg ml^{-1} *Micrococcus lysodeikticus*). After 30 seconds of incubation, the absorbance at 530 nm was followed for 5 minutes using an Optimax microplate reader with Kinetic software SOFTmax® (Molecular Devices, USA). After the blank absorbance had been subtracted, lysozyme activity was calculated as the amount of sample that caused a decrease in absorbance of 0.001 units min^{-1}. Hen egg white lysozyme was used as a positive control.

7.2.7.7 Erythrocyte haemolysis / osmotic fragility assay

This assay serves as a method of evaluating membrane stability in circulating erythrocytes (red blood cells), but could also bee deemed as an indirect method of assessing lipid peroxidation. The method, adapted from Freitas *et al.* (2007), evaluates the resistance of erythrocytes to osmotic shock from exposure to increasingly hypertonic solutions.

A series of NaCl dilutions were made up in dH_2O between 0.1 and 0.9 % (w/v) (~ 0.85 % being iso-osmotic). Solutions were not buffered, as it had previously been shown that pH values between 3 and 10 do not effect haemolysis of human erythrocytes (Freitas *et al.*, 2007). 1 ml of each concentration was added to an Eppendorf tube to which 20 µl of fresh whole blood was added. Tubes were gently homogenised by inversion and incubated at room temperature for 30 minutes. Subsequently, tubes were centrifuged at 1300 x *g* for 10 minutes and the absorbance of the supernatant read spectrophotometrically at 540 nm using 1 ml disposable plastic cuvettes.

7.2.7.8 Modified Comet assay

Comet assay was performed as described in the previous chapter (5), with the exception of cells which were flash frozen and stored at -80°C instead of cells from fresh blood. This was due to the larger number of samples taken in this trial.

7.2.8. Statistical analysis

Mann-Whitney *U* test was used to test for statistical significance between medium values, whereas differences in means were analysed by the student's t-test. Differences were considered significant when $P < 0.05$.

7.3. Results

7.3.1. Growth

After the 10 week feeding trial fish in all tanks had more than quadrupled their initial starting weight (9.8 g) attaining a mean individual weight of 45.5 – 47.5 g (Fig. 7.1.). However, there were no significant differences between any of the dietary groups in any of the growth parameters calculated ($P > 0.05$, Table 7.4.).

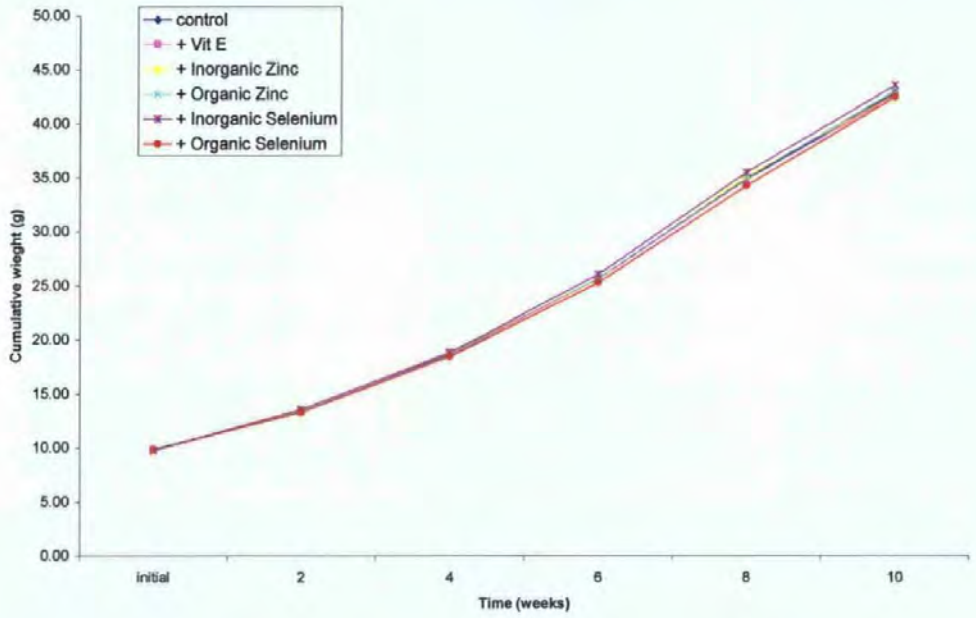

Fig. 7.1. Cumulative wet weight gain of Common Carp (*Cyprinus carpio*) fed 6 experimental diets for 10 weeks.

Table 7.4. Live weight gain (LWG), feed conversion ratio (FCR), specific growth rate (SGR), thermal growth co-efficient (TGC) and survival rate in Common Carp (*Cyprinus carpio*) fed 6 experimental diets for 10 weeks. Values are means (± S.E.) from triplicate groups of fish.

Diet	Supplement	LWG (g) *(s.e)*	FCR *(s.e)*	SGR *(s.e)*	TGC *(s.e)*	Survival (%)
1	Control	**33.01** *(0.77)*	**1.31** *(0.01)*	**2.10** *(0.02)*	**0.220** *(0.02)*	**100**
2	Vitamin E	**32.86** *(0.57)*	**1.31** *(0.01)*	**2.10** *(0.02)*	**0.220** *(0.03)*	**100**
3	Inorganic Zn	**32.90** *(1.22)*	**1.32** *(0.02)*	**2.10** *(0.04)*	**0.220** *(0.05)*	**100**
4	Organic Zn	**33.36** *(1.32)*	**1.30** *(0.02)*	**2.13** *(0.05)*	**0.223** *(0.06)*	**100**
5	Inorganic Se	**33.72** *(1.54)*	**1.30** *(0.03)*	**2.13** *(0.05)*	**0.224** *(0.07)*	**100**
6	Organic Se	**32.64** *(0.75)*	**1.30** *(0.02)*	**2.09** *(0.02)*	**0.219** *(0.03)*	**100**

7.3.2 Glutathione peroxidase (GSHPx) activity

Prior to stress induction there were no significant differences in GSHPx activity in fish fed supplemented diets compared to the control diet. However, post stress levels of GSHPx activity were significantly elevated (compared to the control diet) in fish fed diets supplemented with selenium, with the organically bound Selplex being more effective than the inorganic source, sodium selenite ($P < 0.001$ and $P < 0.05$, respectively, Fig. 7.2).

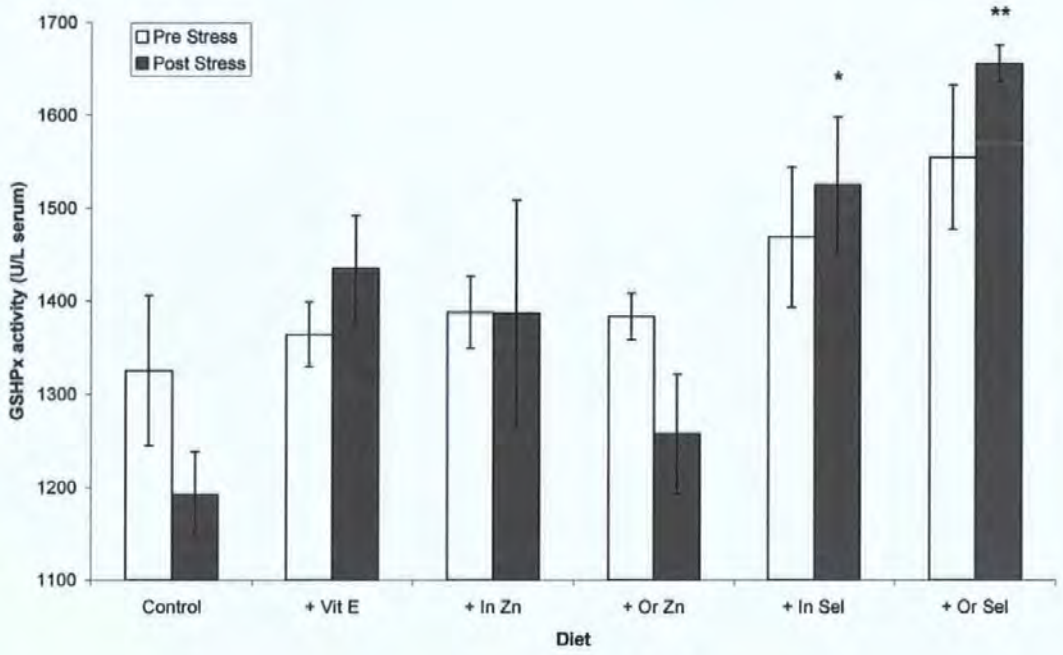

Fig. 7.2 Serum glutathione peroxidase (GSHPx) activity of Common Carp (*C. Carpio*) feed six experimental diets for 10 weeks. Activity measured both pre and post stress at end of trial and expressed as μmol NADPH oxidised per min per litre serum (U/L). Data are represented as mean activity ± s.e (n = 9). Significant differences vs control are denoted as * $P < 0.05$ and ** $P < 0.001$.

7.3.3. Alkaline phosphatase activity

There were no significant differences in ALP activity in fish fed any of the supplemented diets compared to fish fed the control diet, both pre and post stress ($P > 0.05$, Fig 7.3.). However, regardless of the dietary treatment ALP activity seemed to decrease due to husbandry stress although not to a significant level.

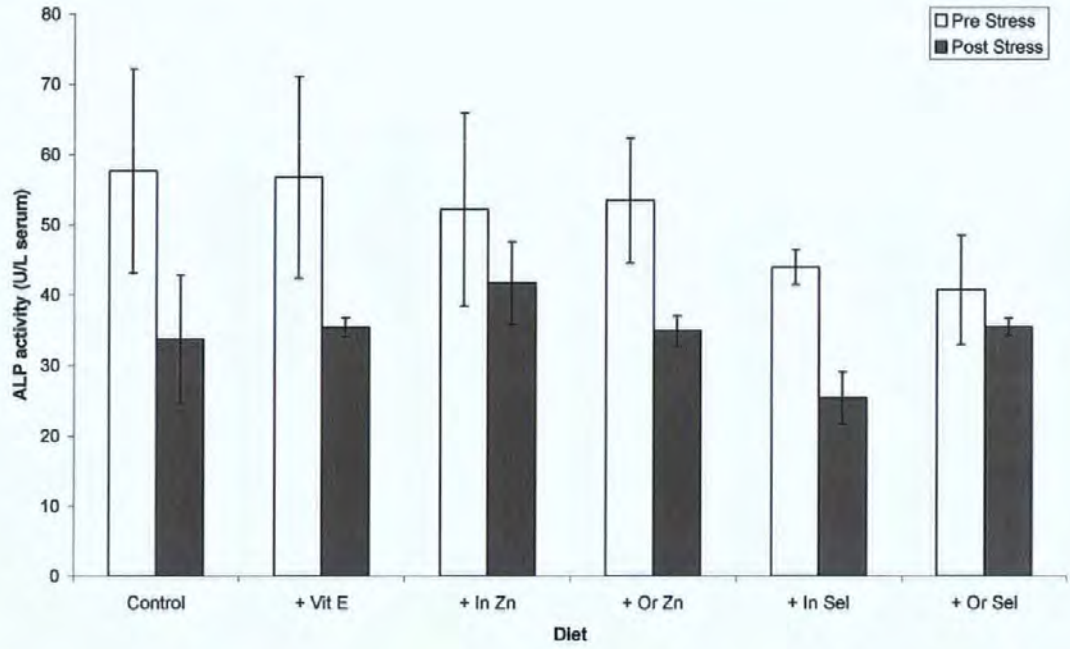

Fig. 7.3. Serum alkaline phosphatase (ALP) activity of Common Carp (*C. Carpio*) feed six experimental diets for 10 weeks. Activity measured both pre and post stress at end of trial and expressed as mmoles of *p*-nitrophenol liberated per min per litre of serum (U/L). Data are represented as mean activity ± s.e (n = 9).

7.3.4. Superoxide dismutase (SOD) activity

SOD activity was measured as Mn-SOD, Cu/Zn-SOD and total SOD. Of the total SOD activity, approximately 70% was due to Cu/Zn-SOD activity. There were no significant differences in activity of any form of SOD either due to stress induction or due to dietary supplementation (Fig. 7.4., $P > 0.05$).

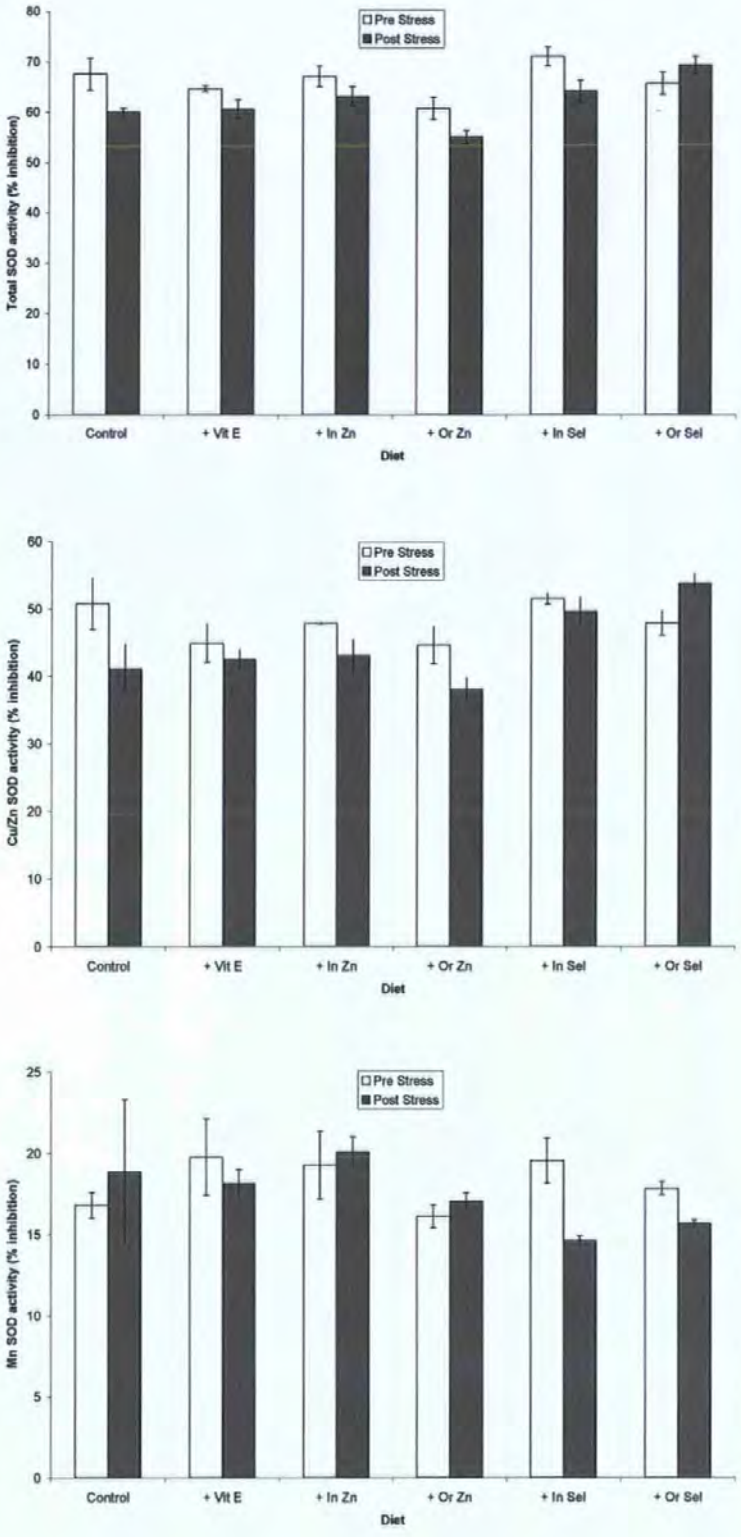

Fig. 7.4 Serum total (a), Zn/Cu dependent (b) and Mn dependent (c) superoxide dismutase (SOD) activity of Common Carp (*C. Carpio*) feed six experimental diets for 10 weeks. Activity measured both pre and post stress at end of trial and expressed as percentage inhibition of superoxide production. Data are represented as mean activity ± s.e (n = 9).

7.3.5. Total Antioxidant Capacity (TAC)

Husbandry induced stress caused a significant decrease in total antioxidant capacity (TAC) of carp serum compared to pre stress levels ($P < 0.001$; Fig. 7.5). However, there was no significant difference in TAC values in fish fed micronutrient supplemented diets compared to the control diet, either pre-stress or post stress ($P > 0.05$).

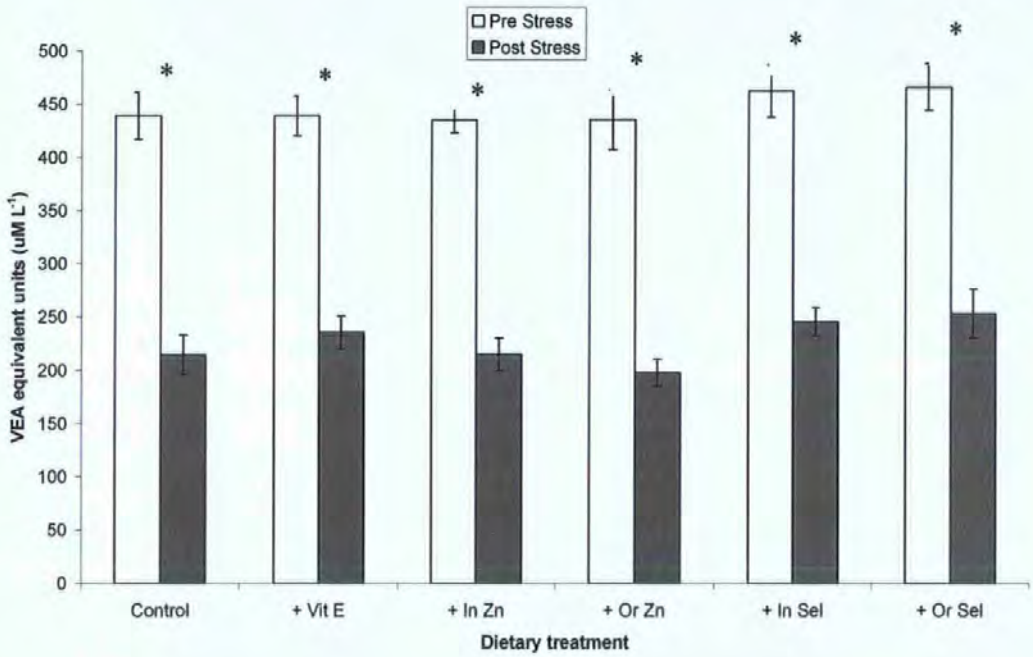

Fig. 7.5. TAC of Common Carp (*C. Carpio*) feed six experimental diets for 10 weeks. Activity measured both pre and post stress at end of trial and expressed as VEA equivalent units ($\mu M\ L^{-1}$). Data are represented as mean activity ± standard error from triplicate groups of fish (n = 9). * denotes significant difference between stressed and control fish ($P < 0.05$).

7.3.6 Respiratory burst

Husbandry stress appeared to enhance the respiratory burst activation of carp leukocytes when activated with PMA (Fig 7.6). This effect was realised after ~15 minutes where mean relative light units increased significantly from ~580 RLU to ~800 RLU in stressed fish post PMA stimulation ($P < 0.05$). This was in contrast to control fish where the pre PMA stimulation max of 480 RLU was only marginally increased to 510 RLU post stimulation.

The dietary treatment appeared to have no significant effect on the respiratory burst capacity of PMA stimulated leukocytes either pre husbandry stress (Fig 7.7) or post stress (Fig. 7.8).

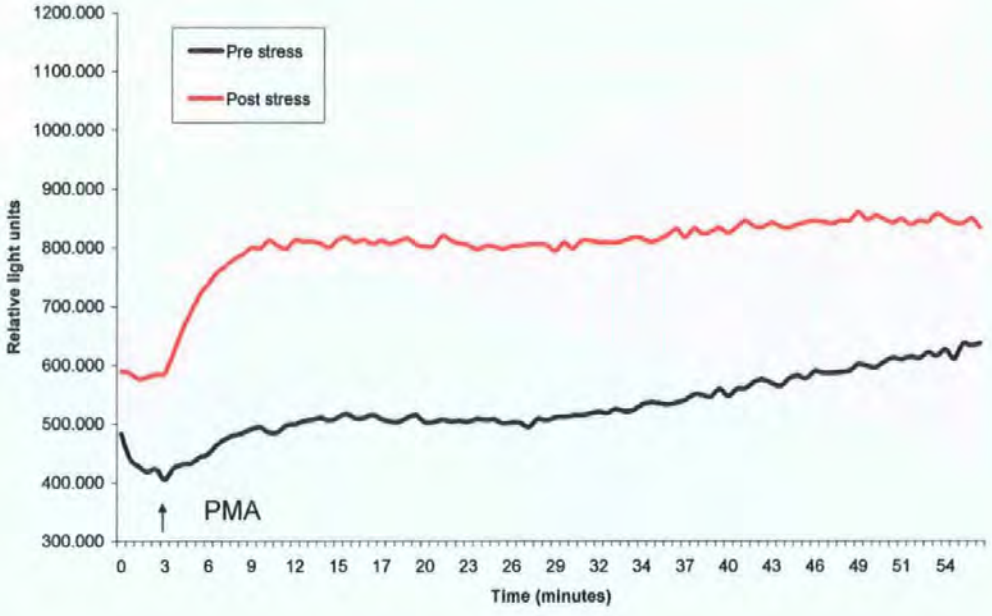

Fig. 7.6 Respiratory burst capacity of Common Carp (*Cyprinus carpio*) before and after husbandry stress induction. Represented as mean relative light units ± SE bars. Results pooled from dietary treatments to give a sample size of 54 fish. PMA activation at time 3 minutes (indicated by arrow).

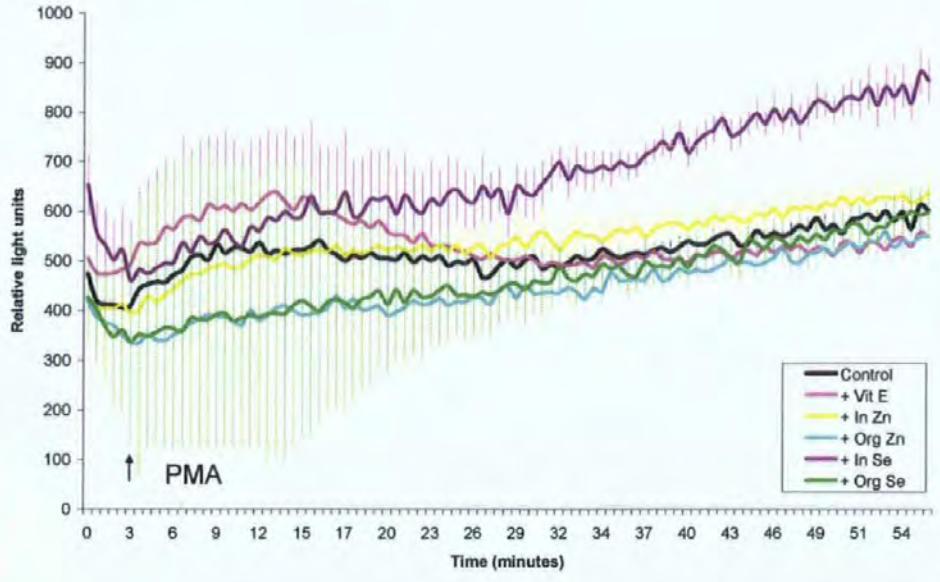

Fig. 7.7 Respiratory burst capacity of Common Carp (*Cyprinus carpio*) fed 6 experimental diets for 10 weeks pre stress induction. Represented as mean relative light units ± SE bars. PMA activation at time 3mins (indicated by arrow).

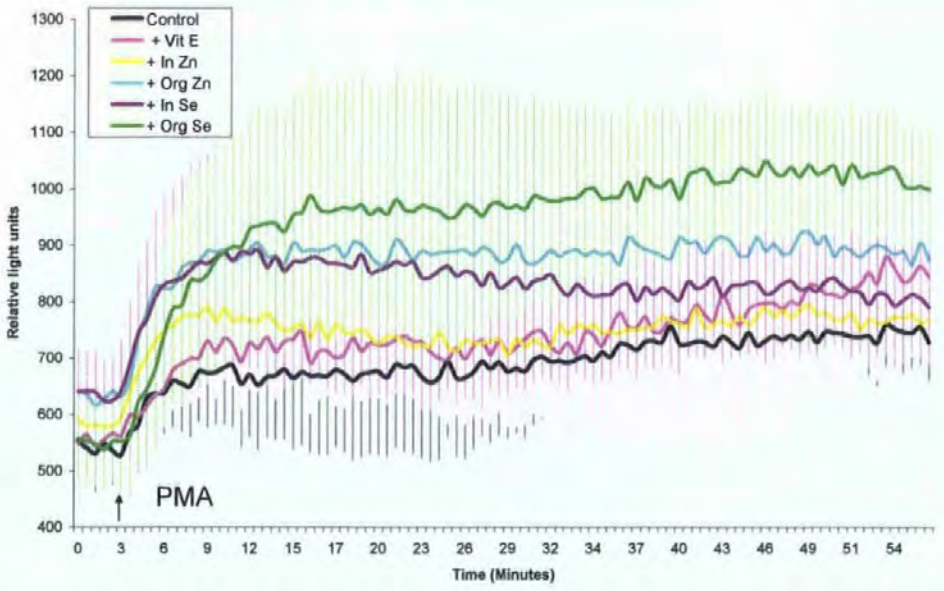

Figure 7.8 Respiratory burst capacity of Common Carp (*Cyprinus carpio*) fed 6 experimental diets for 10 weeks post stress induction. Represented as mean relative light units ± SE bars. PMA activation at time 3mins (indicated by arrow).

7.3.7 Complement assay

There were no significant differences in alternative complement activity (ACH50) either between dietary treatments or due to stress induction (Fig 7.9.).

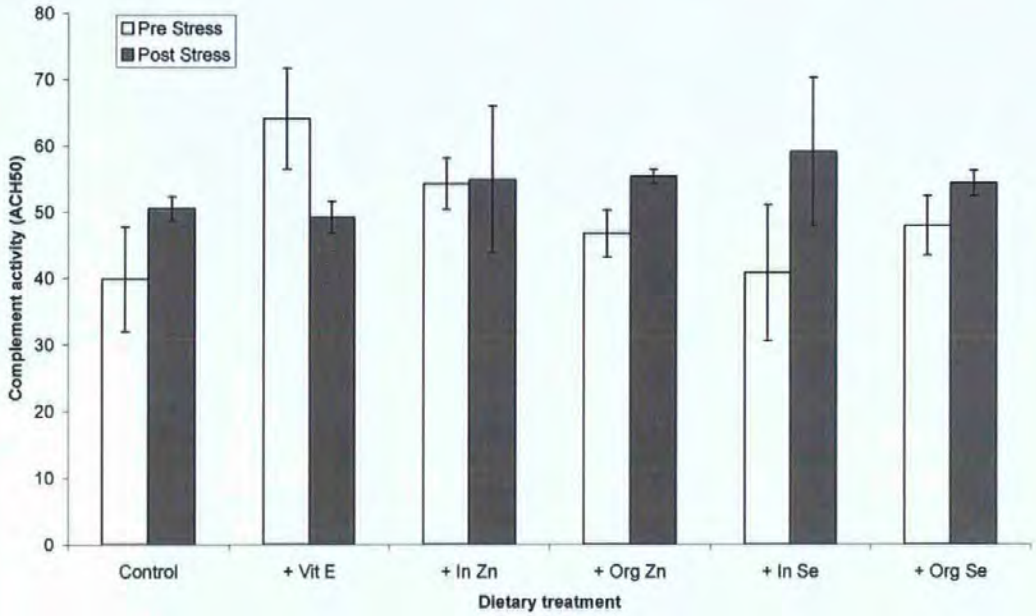

Fig. 7.9. Alternative complement activity in serum from Common Carp (*Cyprinus carpio*) fed 6 experimental diets for 10 weeks. Activity measured both pre and post stress at end of trial and expressed as ACH50 units. Values are means ± standard error from triplicate groups of fish (n =9).

7.3.8 Lysozyme assay

Lysozyme activity was assayed in 9 individual fish per dietary group, both for pre and post stress treatments. However, no activity was detectable in any of

the carp serum samples tested. In order to confirm the validity of the assay several reference samples of trout serum (obtained from the nutrition aquarium at the University of Plymouth, UK) were tested for lysozyme activity using an identical protocol. Trout serum was collected and frozen in exactly the same way as carp serum collected at the end of the trial (see section 7.2.6). Lysozyme activity of trout serum was detected at 524 U/ml, confirming the validity of the assay. Attempts were made to optimise the assay for carp serum by increasing the sample size, changing the buffer and varying the pH of the buffer, and varying the concentration of *Micrococcus lysodeikticus*. However this was unsuccessful.

7.3.9 Red Blood Cell (RBC) Osmotic fragility assay

Carp red blood cells were exposed to a series of increasingly hypotonic solutions to establish their osmotic fragility. There were no significant differences in the ability of carp red blood cells to resist osmotic shock either between dietary treatments or due to stress induction (Fig 7.10.).

7.3.10 Modified Comet assay

Carp red blood cells that were processed for the analysis of oxidative DNA damage by the modified Comet assay were not able to be effectively scored by image analysis because of the extremely high levels of DNA damage observed in the stained slides. Instead of the usual appearance of fluorescently stained nucleoids (that have been subjected to some form of stress) as 'Comets' with a

distinctive head and tail (see figure 2.1, section 2.1.3), carp blood cells exhibited extremely high levels of DNA strand breakage and separation, with DNA fragments dispersed throughout the gel. The result of which was that no reliable data could be collected on the effect of stress and micronutrient supplementation on oxidative DNA damage. Attempts were made to optimise the assay for carp red blood cells by reducing the incubation time during the unwinding and electrophoresis stages of the assay; however, this was unsuccessful.

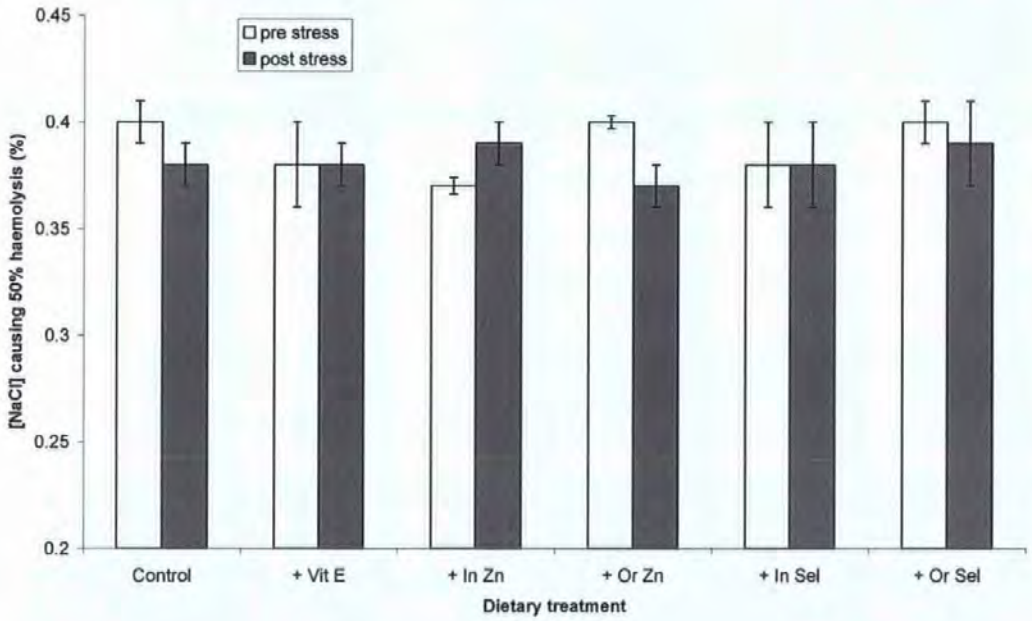

Fig. 7.10. Red blood cell 50% haemolysis values of from Common Carp (*Cyprinus carpio*) fed 6 experimental diets for 10 weeks. Activity measured both pre and post stress at end of trial and expressed the concentration of NaCl causing 50% haemolysis of red blood cells. Values are means ± standard error from triplicate groups of fish (n =9).

7.4 Discussion

There were no differences in any of the growth parameters measured between dietary treatments, and no mortalities were observed, throughout the 10 week feeding trial. However, this was not unexpected as the basal diet used (before supplementation) adequately supplied the minimum recommended levels of selenium (0.35 $\mu g\ g^{-1}$), zinc (30 $\mu g\ g^{-1}$) and vitamin E (100 $\mu g\ g^{-1}$) (NRC, 1999). In any case, the objective of this study was to assess the impacts of high levels of micronutrient supplementation on more subtle markers of health, and to assess how stress may effect their requirement. The fact that no mortalities were observed and growth was not affected indicates that the high supplementation levels used for each micronutrient are not grossly toxic and did not cause any adverse effects. For zinc, carp have been shown to tolerate up to 1700 $\mu g\ g^{-1}$ without adverse effects on growth or survival (Ogino & Yang, 1979) which is well below the 400 $\mu g\ g^{-1}$ used in this study. For vitamin E a high dose of 1000 $\mu g\ g^{-1}$ as α tocopherol acetate, as used in this study, was not detrimental to carp health but actually increased immunocompetence (Sahoo & Mukherjee, 2002). Also the selenium level used in this study (2.5 $\mu g\ g^{-1}$) is well below the reported level to cause chronic dietary selenium toxicity (13 $\mu g\ g^{-1}$) (Hilton *et al.*, 1980). As stress was only administered from 7 days effects on growth could not be accurately evaluated. However, chronic husbandry stress over 2 weeks has been shown to reduce growth and survival of sea bream fed vitamin E deficient diets (Montero *et al.*, 2001).

Prior to stress induction there were no significant differences in selenium dependent GSHPx activity in fish fed supplemented diets compared to the control diet. This is probably because the selenium level in the basal diet (~0.65 μg g^{-1}) was adequate to achieve maximal GSHPx activity and therefore supplementing extra selenium (as in diets 5 and 6) would have no beneficial effect. This in agreement with previous studies in fish that have shown that increasing the level of selenium above the recommended minimum dose (~0.35 μg g^{-1}) (and often the average selenium concentration in commercial diets) shows no concurrent increases in GSHPx activity in the plasma of rainbow trout and the serum of Atlantic salmon (Hilton *et al.*, 1980; Julshamn *et al.*, 1990). However, this is in stark contrast to the findings of Felton *et al.* (1996) who found that hepatic GSHPx activity was significantly increased in Coho salmon when dietary selenium was increased from 1.1 to 8.6 μg g^{-1}, without any signs of toxicity. Lorentzen *et al.* (1994) also found serum GSHPx activity to significantly increase in Atlantic salmon serum when the dietary level was raised from 1.5 μg g^{-1} to 2.1-3.4 μg g^{-1}. These differences between studies indicate that the selenium requirement for optimal GSHPx activity may need more attention.

Post stress levels of GSHPx activity were significantly elevated (compared to the control diet) in fish fed diets supplemented with selenium, with the organically bound Selplex being more effective than the inorganic source. Stress had no effect on the GSHPx activity in fish feed the other 4 diets including the control. This indicates that the burden of husbandry stress may increase the requirement of selenium in carp. However, this is the reverse of

the effect found by Felton *et al.* (1996) who found that transport stress actually increased levels of GSHPx in the control diet, but decreased the level in selenium supplemented diets – they could not explain this effect. Except the aforementioned study there appears to be no information on how stress may change the requirement for selenium with respect to GSHPx activity. Therefore, this is definitely an area warranting more research. However, Roche and Boge (1996) found that GSHPX activity was increased due to thermal stress, although they did not relate enzyme activity to the dietary content of selenium.

With respect to the dietary source of selenium, the organically bound form of selenium (Selplex) elevated the level of post stress GPX activity, above the control diet level, to a higher degree than that of the inorganic form (sodium selenite). This is in agreement with previous studies and may been due to the greater bioavailability of the organic sources of selenium supplements (Jovanovic *et al.*, 1997; Wang and Lovell, 1997).

The enzymes, alkaline phosphatase and superoxide dismutase were measured due to their dependence on zinc in the active site for functionality. Pre stress levels of ALP showed no significant differenced between dietary treatments. This suggests that the level of zinc in the basal diet (~ 60-70 $\mu g\ g^{-1}$) was sufficient for the optimal activity of the enzyme. This is in agreement with other studies on fish that indicate that the level of zinc for optimal ALP activity (30 $\mu g\ g^{-1}$) is below the basal level used in the control diet of this study (Ogino and Yang, 1979; Gatlin and Wilson, 1983). Post stress ALP levels also

showed no significant differenced between dietary treatments. Interestingly though, regardless of the dietary treatment ALP activity seemed to decrease due to husbandry stress although not to a significant level. This indicates that supplementing extra zinc to the diet above recommended levels may not be beneficial in reducing any husbandry stress induced reductions is ALP activity. Therefore there may be other factors influenced by husbandry stress, apart from the requirement of zinc, which may affect ALP activity. Humans studies indicate that ALP activity is reduced during episodes of ethanol induced oxidative stress in the brain and may be related to ascorbate levels (Das *et al.*, 2007). There appears to be no literature available on whether dietary zinc supplements can modulate stress effects on ALP activity in fish.

There was no significant difference in superoxide dismutase (SOD) activity, between the dietary regimes pre or post husbandry stress. This was evident for total SOD, Mn-dependent SOD, and Cu/Zn-dependent SOD. Again this suggest that the levels of zinc (as well as other micronutrients necessary for the active site of SOD, manganese and copper) in the basal diet were sufficient for the optimal activity of the enzyme even during episodes of stress, or that stress has no real effect on SOD levels. Aniagu *et al.* (2006) found no significant difference in SOD activity after husbandry stress induced acute exhaustive exercise in chub. The modulation of SOD levels through zinc supplementation has received no attention to this author's knowledge. However, trout fed Zn-deficient diets appeared to lack Cu/Zn SOD isozymes in the liver, compared to control fish, as confirmed by western blotting analysis. Surprisingly one study by Jovanovic *et al.*, (1997) found that SOD activity

increased due to selenium supplementation in carp (not the case in this study). It was postulated that this could have been due to the overall increase in metabolism, growth and health of Se-supplemented fish (53% increases in growth). It may have also been due to the fact that selenium rich yeast was the source of the dietary supplement. Other micronutrients present in yeast such as copper, zinc and manganese may have contributed in the rise in SOD activity.

Although there appeared to be no differences in the activity of Zn-dependent enzymes between diets either pre or post stress, organically bound dietary sources of zinc have previously been shown to be better utilized in fish, particularly in relation to ALP activity (Apines *et al.*, 2008).

No significant differences were observed in total antioxidant capacity (TAC) between dietary treatments, either pre stress or post stress, indicating that TAC may not necessarily be related the levels of selenium, zinc and vitamin E in the diet. However, across all dietary regimes husbandry stress reduced TAC scores by ~50%. Reductions in TAC in fish due to husbandry stress have not been evaluated previously. However, reductions in TAC from stress due to transportation have been measured in cattle using the chemiluminescence of isoluminol (Chirase *et al.*, 2004).

The total antioxidant capacity of fish in relation to dietary supplements is a fairly novel concept, as usually the efficacy of dietary supplements is evaluated by measuring the activity of *particular* antioxidants or antioxidant enzymes

such as GSHPx and SOD. The measure of single antioxidants or antioxidant enzymes may not truly represent the overall or total antioxidant capacity of fish as is highlighted in the present study. Although GSHPx is known to reduce peroxynitrite (Sies and Arteel, 2000), the oxidant used to challenge carp serum in the ABEL® TAC assay, increases in GSHPx activity after the induction of husbandry stress in selenium supplemented diets had no effect on the TAC score of the same fish. Also, although TAC reduced due to stress in this study, SOD activities remained unchanged suggesting that the measurement of SOD is not a good indicator of antioxidant status. Therefore, assays for measuring TAC, such as the one us in the current study, should be considered in future studies examining the effects of diet and stress on the antioxidant system of fish.

There were no significant differences in the resistance of carp red blood cells to osmotic shock, between the dietary regimes pre or post husbandry stress. This indicates that the levels of selenium, zinc and vitamin E in all diets were sufficient to maintain the integrity of red blood cell membranes, even during episodes of husbandry induced stress. Red blood cell fragility has previously been shown to increase in fish fed diets depleted in selenium and vitamin E (Bell *et al.*, 1987; Diminov, 1998; Montero *et al.*, 2001) and in rats deficient in zinc (Johanning and O'Dell, 1989). Supplements of vitamin E (280 $\mu g\ g^{-1}$) above the basal levels have been shown to increase resistance of red blood cells to lipid peroxidation although dietary selenium supplements (0.8 $\mu g\ g^{-1}$) had no effect in channel catfish (Wise *et al.*, 1993). The role of zinc in the maintenance of red blood cell membrane integrity under normal and stressful

conditions is yet to be determined. It appears as though there is no evidence to suggest that husbandry stress may affect red blood cell fragility. Red blood cell fragility was unaffected by 15 weeks chronic crowding stress and repetitive chasing as an acute stressor in sea bream (Montero *et al.*, 2001).

Respiratory burst of leukocytes, lysozyme activity and alternative complement activity were measured in order to establish the relationship between micronutrient supplementation, husbandry stress immune function. When respiratory burst capacity of leukocytes was averaged across diets husbandry stress appeared to have a stimulatory effect on the production of free radicals. This is in agreement with previous studies that have shown husbandry stress to increase respiratory burst in fish (Pulsford *et al.*, 1994; Ruane *et al.*, 2002). The observed increase may be due to increased migration of activated leukocytes from the head kidney to the blood during stress (Narnaware & Baker, 1996; Mesegeur, 2001). However, other studies have shown husbandry stress to suppress respiratory burst capacity (Vazzana *et al.*, 2002, Montero *et al.*, 2001; Angelidis *et al.,* 1987). It is not clear whether theses modulations of the immune system may be beneficial or damaging to health. With regard to dietary supplements, adding selenium, zinc, or vitamin E to the diets above normal levels did not have significant effects on respiratory burst capacity either pre or post stress. This indicates that sufficient levels of these micronutrients were present in the diet to cause any deficiency dependent modulations of the respiratory burst capacity, and that supplementing high levels would have no extra beneficial effect. In terms of vitamin E this is contrary to findings of Saho & Mukerjee (2001) who observed increased in

respiratory burst capacity of carp when feed high levels of α-tocopherol (1000 μg g^{-1}). Respiratory burst was also shown to significantly increase in channel catfish fed four times the recommended dose of selenium (Wise *et al.*, 1993). In the present study, supplementation of these micronutrients also had no effect on the stress induced changes in respiratory burst. There appears to be no information in the literature on how stress induced changes in respiratory burst capacity of fish may be modulated by dietary enhancement of the nutrients.

Levels of complement activity in this study were not affected by dietary treatment either pre or post stress. Previously though, complement activity has been shown to be dependent on Vitamin E (Montero, 2001). The dependency of complement on selenium and zinc requires further investigation in fish as there appears to be no available literature on this area. Husbandry stress has previously been shown to suppress complement activity in fish (Tort *et al.*, 1996), although this was not the case in this study.

Despite efforts to optimise the assay, in the present study no detectable levels of lysozyme were found in carp serum from any dietary treatment either pre or post stress. Reasons for this are unknown; especially as using exactly the same protocol high levels of lysozyme activity were easily detected in reference samples of trout serum. Although not detected in this study, levels of fish lysozyme have been shown to be effected by stress (Demers and Bayne, 1997). Lysozyme activity is also dependent on selenium (Jaramillo & Gatlin 2004) and vitamin E (Montero, 2001) in the diet.

Unfortunately in this study it was not possible to measure oxidative DNA damage evaluation in response to dietary treatment and stress because of the extremely high levels of background damage observed. Previously, one study reported that husbandry stress increased oxidative DNA in chub, as measured by a modified Comet assay (Aniagu *et al.*, 2006). However, there are no studies that have evaluated the role of dietary micronutrients on genomic stability in fish. This is surprising when considering the amount of research in this are in human nutrition, especially for selenium, due to the role of oxidative DNA damage in carcinogenesis (El-Bayoumy, 2001)

Overall the novel aspect of this study was the attempt to provide a link between such husbandry induced stress effects as oxidative stress and immune function, and the dietary requirement of micronutrients. Previous studies in fish have usually focused on one of these aspects – that is, the effects of stress on fish health *or* the effects of diet on fish health. Results from the present study indicate that husbandry stress has effects on GSHPx activity (in terms of selenium requirement), total antioxidant capacity, ALP activity and respiratory burst capacity of leukocytes. Stress had no effect on SOD activity, complement activity and red blood cell fragility. The only effects of dietary supplementation was the selenium dependent maintenance of GSHPx activity in fish subjected to stress, which is the first time this has been reported in fish.

CHAPTER 8: CONCLUDING REMARKS AND FURTHER WORK

The overall theme of this thesis has been the study of the effects of well known and potentially novel stressors on fish health and how some of these may be modulated by dietary supplements of micronutrients, using both *in vitro* and *in vivo* analysis.

In vitro studies were carried out with cultured fish cells (EPC-A1, GFSk-S1) to evaluate the potential benefits of niacin and selenium supplementation against ultraviolet radiation (UVR) induced cell death and DNA damage. UVR is known to cause detrimental health effects in fish (Zagarese & Williamson, 2001), whereas niacin and selenium are essential micronutrients (Lovell, 1998). However, there is a lack evidence to suggest whether niacin and selenium may modulate UVR induced stress effects, such as DNA damage.

In the present study, niacin (NAM) supplementation in EPC-A1 cells significantly reduced UVB induced DNA single strand breaks as measured by the Comet assay. This is a novel observation in fish nutrition. The exact mechanism of protection is not known. It could possibly be due to increased DNA repair as, in humans, niacin is required for the activity of the DNA repair enzyme PARP-1 (Hageman and Stierum, 2001). However, this is unlikely as cells were not afforded a recovery period that would be necessary for repair mechanism to kick in. Furthermore, fish are known to have a low capacity for DNA excision repair (Willet, *et al.*, 2001). A more likely explanation is the increase in antioxidant capacity of the cells due to NAM supplementation

which could quench free radicals produced by UVB - niacin is reported to have a greater antioxidant capacity than ascorbic acid (Kamat & Devasagayam, 1996). The possible role of niacin-dependent PARP-1 and DNA repair in protecting fish from genomic instability remains to be defined, and warrants further investigation.

Selenium supplementation in GFSk-S1 cells significantly reduced UVA and UVB induced oxidative DNA damage and cytotoxicity, as measured by a modified Comet assay and the NRR assay, respectively. This also is a novel observation in fish nutrition. Possible reasons for selenium dependent protection against UVR may relate to increases in the expression/activity of important selenoenzymes. Glutathione peroxidase (GSHPx) and thioredoxin reductase (TR) reduce harmful hydroperoxides and peroxynitrites which are produced in the skin during UVR exposure (Rotruck *et al.*, 1973; Ebert *et al.* 2006). Interestingly, although GSHPx activity has been shown to be dependent on dietary selenium in fish, measurements of TR in fish appear absent, and therefore require investigation. The activity of these enzymes was not measured during *in vitro* experiments in this study because of the lack of cells that could be cultured in order to measure a response. However, further studies that evaluate oxidative DNA damage and cytotoxicity in fish cells in relation to selenium supplements must endeavour to concurrently measure how the levels GSHPx and TR may also be modulated. Other possible mechanisms of seleno-protection may related to metal ion co-ordination - the tendency of selenium to bind to metals, such as copper, thus reducing their

availability for Fenton-type reactions which produce the highly reactive hydroxyl radical from hydrogen peroxide (Battin *et al.*, 2006).

The final part of the *in vitro* work for this thesis was the evaluation of nanoparticulate titanium dioxide as a novel source of stress for fish. Despite growing concern over the potential risk of manufactured nanoparticles to the environment (Owen & Depledge, 2005; Moore, 2006), there is a paucity of information on their potential toxic effects on natural biota, including fish. This is in contrast to mammalian studies that have reported oxidative stress effects including DNA damage in cultured cells. The results of the current study are the first to confirm in fish what has been documented in mammalian models. Exposure of GFSk-S1 TiO_2 nanoparticles alone did not cause any significant cytotoxic effect although when cells were co-exposed to TiO_2 and UVA a cytotoxic response was realised. The exposure of TiO_2 in the absence of UVA was enough to induce increases in oxidative DNA damage, an effect which was exacerbated by a combined exposure with UVA. The exact free radical species responsible for TiO_2 induced cytotoxicity and DNA damage appeared to be hydroxyl radical (OH), as evaluated by electron spin resonance (ESR) and spin trapping. ESR is the only general approach that can provide direct evidence for the presence of a free radical. In addition, analysis of the ESR spectrum generally enables determination of the identity of the free radical (Halliwell and Gutteridge, 1997). This appears to be the first time that ESR has been used in a study with cultured fish cells, although the technique has been used to measure free radical production in toxicant exposed goldfish *in vivo* (Luo *et al.*, 2006). It would be an interesting technique to utilise in future

studies on dietary supplements in fish *in vivo*, particularly those involving micronutrients involved in antioxidant defence.

Generally speaking, in relation to selenium and niacin, and other micronutrients, further work must be done to establish if dietary supplements in feeding trials may help protect fish from environmental stress factors, such as UVR, which may result in genomic instability *in vivo*. This is even more prevalent as cell culture based studies are often criticised for their lack of biological relevance and applicability (Knasmuller *et al.*, 2008). For this reason the effects of TiO_2 exposure observed in cultured cells must also be confirmed *in vivo*.

In the second part of this thesis, two experiments were carried out to evaluate the potential health effects of husbandry induced stress (netting and confinement stress) in trout and carp. Previously, husbandry stress has been shown to increase the production of stress hormones and modulate the immune system of fish leading to reduced disease resistance and growth (Bly *et al.*, 1997; Iwama *et al.*, 1997). However husbandry stress induced changes in antioxidant status and genomic stability are lacking.

In the second experiment on carp, a 10 week feeding trial was also conducted prior to stress induction to evaluate the potential modulatory effects of selenium, zinc and vitamin E on any husbandry induced stress effects observed. Health parameters were also evaluated pre stress in order to elicit any effects of micronutrient supplementation on health parameters in

unstressed fish. Selenium, zinc and vitamin E are all essential in fish for optimal growth, immune function and antioxidant defences (Lovell, 1998), although how there requirement may change under stressful conditions requires further study.

In both studies on trout and carp, certain health parameters were shown to be sensitive to husbandry stress, and may be useful biomarkers of stress in future studies. Total antioxidant capacity (TAC) was reduced in both carp and trout after stress. Although this is the first report of this effect in fish, TAC has previously been shown to decrease in transport-stressed cattle (Chirase *et al.*, 2004). Respiratory burst capacity of blood leukocytes was also affected by stress but differently in carp than in trout. In carp, husbandry stress stimulated the production free radicals by leukocytes whereas in trout it was suppressed. Stimulatory (Pulsford *et al.*, 1994) or suppressive (Vazzana *et al.*, 2002; Montero *et al.*, 2001; Angelidis *et al.*, 1987) effects on the respiratory burst have previously been reported in fish.

The measurement of oxidative DNA damage was attempted in both trout and carp in response to stress. In trout, stress was shown to increase oxidative DNA damage, as measured by the Comet assay. This finding aggress with Aniagu *et al.*, (2006) who found that exhaustive physical exercise resulted in increased levels of oxidative DNA damage in chub. Unfortunately oxidadative DNA damage could not be effectively measured in carp either pre or post stress due to the apparent high background levels of damage. This could have been due to the method of sample preparation as damage could have

occurred to DNA during the freezing process in the preparation of carp blood cells for the Comet assay. This process was necessary due to the high number of samples taken for analysis. In comparison for the study on trout, fresh blood was used for Comet analysis, within a couple of hours of sampling. It may be the case that methods for Comet analysis on frozen blood cells needs optimising as previous studies have shown that frozen blood cells may be used for analysis of DNA damage by the Comet assay (Belpaeme *et al.*, 1998). Therefore, further work must be done on this.

Health parameters that appeared not to be significantly affected by husbandry stress in this study include SOD activity, ALP activity, red blood cell fragility and complement (for carp only). Therefore these assays may not necessarily be suited as markers of husbandry stress in fish. However, some such as complement have previously been shown to be effected by stress (Tort *et al.*, 1996).

In the study in carp the only beneficial effect of micronutrient supplementation on husbandry induced stress was the maintenance of post stress GSHPx activity through selenium supplementation. This indicates that the burden of husbandry stress may increase the requirement of selenium in carp. However, this is the reverse of the effect found by Felton *et al.* (1996) who found that transport stress actually increased levels of GSHPx in the control diet, but decreased the level in selenium supplemented diets – they could not explain this effect. Except the aforementioned study there appears to be no information on how stress may change the requirement for selenium with

respect to GSHPx activity. Therefore, this is definitely an area warranting more research. However, Roche and Boge (1996) found that GSHPX activity was increased due to thermal stress, although they did not relate enzyme activity to the dietary content of selenium.

No other differences were observed between dietary treatments for any parameter measured either pre stress or post stress, indicating that levels of selenium, zinc and vitamin E were probably sufficient in the control diet.

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APPENDIX

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Mutation Research 640 (2008) 113–122
Fundamental and Molecular Mechanisms of Mutagenesis
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Hydroxyl radicals (•OH) are associated with titanium dioxide (TiO_2) nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells

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Received 5 July 2007; received in revised form 4 December 2007; accepted 19 December 2007
Available online 31 December 2007

Abstract

TiO_2 nanoparticles (<100 nm diameter) have been reported to cause oxidative stress related effects, including inflammation, cytotoxicity and genomic instability, either alone or in the presence of UVA irradiation in mammalian studies. Despite the fact that the aquatic environment is often the ultimate recipient of all contaminants there is a paucity of data pertaining to the potential detrimental effects of nanoparticles on aquatic organisms. Therefore, these investigations aimed to evaluate the potential cytotoxic and genotoxic effects of TiO_2 nanoparticles on goldfish skin cells (GFSk-S1), either alone or in combination with UVA. Whilst neutral red retention (NRR) assay (a measure of lysosomal membrane integrity) was used to evaluate cell viability, a modified Comet assay using bacterial lesion-specific repair endonucleases (Endo-III, Fpg) was employed to specifically target oxidative DNA damage. Additionally, electron spin resonance (ESR) studies with different spin traps were carried out for qualitative analysis of free radical generation. For cell viability, TiO_2 alone (0.1–1000 $\mu g\,ml^{-1}$) had little effect whereas co-exposure with UVA (0.5–2.0 $kJ\,m^{-2}$) caused a significant dose-dependent decrease which was dependent on both the concentration of TiO_2 and the dose of UVA administered. For the Comet assay, doses of 1, 10 and 100 $\mu g\,ml^{-1}$ in the absence of UVA caused elevated levels of Fpg-sensitive sites, indicating the oxidation of purine DNA bases (i.e. guanine) by TiO_2. UVA irradiation of TiO_2-treated cells caused further increases in DNA damage. ESR studies revealed that the observed toxic effects of nanoparticulate TiO_2 were most likely due to hydroxyl radical (•OH) formation.

Keywords: Titanium dioxide; Nanoparticles; UVA; Oxidative stress; DNA damage; ESR

1. Introduction

Nanotechnology concerns the development and use of substances which have a particle size <100 nm, the aim being to significantly increase the surface area:mass ratio, thereby greatly enhancing chemical/catalytic reactivity (amongst other properties), compared to normal-sized particles of the same substance. Although this provides many benefits for industry, there is increasing concern that substances previously considered biologically inert may indeed become toxic in a nanoparticulate state due to their increased reactivity and possible easier penetration of cells. In this context, normal-sized (>100 nm) TiO_2 has been considered to be biologically inert to animals and humans [1,2] and it is widely used as an ingredient in white paint, food colourant, sunscreen and cosmetic products [3]. TiO_2 nanoparticles, on the other hand, are incorporated into cellular membranes and cytoplasm of mammalian cells in culture [4,5], although studies on the toxic effects of TiO_2 nanoparticles provide conflicting evidence. Zhang and Sun [6] found human colon carcinoma cells were still 90% viable even after a 24 h exposure to 1000 $\mu g\,ml^{-1}$ TiO_2 nanoparticles. Nakagawa et al. [7] reported no effect of ultra-fine (25 nm) TiO_2 on a series of genotoxicity parameters measured in mouse lymphoma and Chinese hamster cells, including the Comet assay, microbial and cell mutation assays and chromosomal aberrations. TiO_2 nanoparticles also had no cytotoxic (cell growth) or genotoxic (micronuclei induction) effect on cultured rat liver epithelial cells [8]. Conversely, exposure of Syrian hamster embryo cells (SHE) to 1 $\mu g\,cm^{-2}$ TiO_2 (<20 nm) for 12–72 h caused a significant dose-dependent increase in the induction of micronuclei and apoptosis [9]. Additionally, anatase (10 and 20 nm) TiO_2 particles, in the absence of photo-activation, induced oxidative DNA damage, lipid peroxidation, micronuclei formation, and increased hydrogen peroxide and nitric oxide production in a

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doi:10.1016/j.mrfmmm.2007.12.010

human bronchial epithelial cell line [3]. Wang et al. [10] also found that TiO_2 nanoparticles are cytotoxic (MTT assay), genotoxic (Comet and Mn assay) and mutagenic (HPRT mutation assay) towards cultured WIL2-NS human lymphoblastoid cells whilst Long et al. [11] found that TiO_2 causes oxidative stress in brain microglia cells under *in vitro* conditions. *In vivo* toxicity studies have also demonstrated that inhalation of TiO_2 nanoparticles causes pulmonary inflammation in rats and mice [12]. This was characterised by increased numbers of macrophages and neutrophils and increased concentrations of soluble markers in bronchoalveolar lavage fluid (BALF).

Many studies have documented the phototoxic and photogenotoxic effects of TiO_2 (both normal and nano-sized) [5–7,13,14] and consequently its properties as a photo-catalytic compound have been applied to waste water disinfection [15] and photodynamic therapy of certain cancers [16]. Whilst, there is ample evidence of the formation of reactive oxygen species (ROS) when TiO_2 is exposed to UV light [14,17–19], there is disagreement as to the exact nature of the species produced and their involvement in cell death. Possible ROS that could be formed are hydroxyl radicals ($^{\bullet}OH$), superoxide radical anions (O_2^-), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). These species can be identified by means of electron spin resonance (ESR), using the technique of spin trapping.

Despite growing concern over the potential risk of manufactured nanoparticles to the environment [20,21], there is a paucity of information on their potential toxic effects on natural biota. In particular, except for a 48 h acute toxicity test on the fresh water flea, *Daphnia magna* [22], there is no information available in the literature on potential detrimental effects of TiO_2 nanoparticles on aquatic organisms. The input of engineered nanoparticles in the aquatic environment is likely to be due to industrial (i.e. initial and downstream manufacturers) or non-industrial sources. Non-industrial sources include consumer products (sunscreens and cosmetics) from both direct (e.g. bathing) and indirect (sewer) sources, leaching from landfill or soil-applied sewage sludge and atmospheric sources from waste combustion [20]. Given that nanotechnology industries plan large scale production, it is inevitable that these products and their by-products will accumulate in the aquatic environment [21,23], and their potential genotoxic effects could have short and long term consequences for the biota [24]. Furthermore, although only a small percentage of solar ultraviolet radiations (UVR) reaches the earth's surface, they may penetrate up to >20 m in the water column [25,26] and such radiation has been suggested to have deleterious effects on aquatic organisms, including fish [27,28]. It is also worth remembering that properties of the target cell influence the toxic responses of contaminants [29] and in order to protect human and ecosystem health, it is necessary to develop sensitive assays and to identify responsive cells and species.

In light of the above information, the aims of the present study were: (a) to evaluate whether TiO_2 nanoparticles are capable of inducing cytotoxicity and genotoxicity in a primary fish cell line, either alone or in combination with UVA radiation; (b) to observe any link between observed cytotoxic and genotoxic effects; (c) to evaluate what types of generated free radicals, if any, could be responsible for the observed toxic effects. While cytotoxic effects were determined using neutral red retention (NRR) assay, a modified Single Cell Gel Electrophoresis or Comet assay, using lesions-specific bacterial repair enzymes (Fpg and Endo-III), was used to determine oxidative DNA damage. Electron Spin Resonance (ESR) technique using a range of spin trap was used to qualitatively determine the generation of free radicals.

2. Materials and methods

2.1. Chemicals and cell culture

All chemicals and reagents were obtained from Sigma Chemical Co. (Poole, UK) unless otherwise stated. Cell culture media, sera and reagents were obtained from Invitrogen (Paisley, UK). DEPMPO was from Axxora Ltd. (Nottingham, UK). Fpg and Endo-III were supplied by Professor Andrew Collins (University of Oslo, Norway). GFSk-S1 cells (obtained from Professor Lucy Lee, Wilfred Laurier University, Canada) are a primary cell line developed from the skin of goldfish (*Carassius auratus*) and possess mainly a fibroblast-like morphology [30,31]. Cells were cultured in Leibovitz's L-15 medium containing 10% foetal bovine serum (FBS) at 20 ± 1 °C and typically seeded at 1×10^5 ml^{-1}.

2.2. Validation of NRR and Comet assay

Prior to experimentation, the NRR assay and Comet assay were both validated using a range of doses of H_2O_2 and UVA. H_2O_2 concentrations used were 1–5 mM for 24 h for the NRR assay [32] and 1–20 μM for 5 min for the Comet assay [29]. UVA doses used for validation were 0.25–10 kJ m^{-2} for NRR and 2.5–10 kJ m^{-2} for the Comet assay, these being based on previous work with UVA-irradiated carp and goldfish cells [33,34].

2.2.1. NRR assay

The NRR assay was used as a measure of cytotoxicity due to its recommended use for regulatory cytotoxicity and in particular phototoxicity evaluation of chemicals by OECD and NIH, USA [35]. The assay protocol was adapted from the methods of Babich et al. [36]. After treatment, old medium was removed and cells washed twice with PBS. This was followed by a 3 h incubation in neutral red dye-containing (70 μg ml^{-1}) L-15 medium at 20 ± 1 °C. Post-incubation, neutral red was removed and cells were washed three times with PBS, followed by addition of 200 μl acetyl ethanol (1% acetic acid, 50% ethanol in dH_2O). Plates were briefly shaken and optical density (OD) of lysates recorded at 540 nm using an Optimax microplate reader with Kinetic software SOFTmax® (Molecular Devices, USA). Percentage viability was calculated by dividing the OD of the treated wells by the OD of the control wells, with controls representing 100%. Results were obtained from two independent experiments. For each experiment, 6 replicate wells were used for each treatment.

2.2.2. Comet (single cell gel electrophoresis) assay

Comet assay was performed as described previously [29,37]. Briefly, frosted end microscope slides were coated with 1.5% normal melting point (NMP) agarose and allowed to air dry. Trypsinised cells (approximately 5×10^4) were centrifuged and resuspended in 170 μl molten 0.75% low melting point (LMP) agarose. This was then applied to the pre-coated slides as 2 drops of 85 μl. Coverslips were placed over each drop and gels were allowed to set at 4 °C for 1 h. When gels had solidified coverslips were gently removed and slides were immersed in cold (4 °C) lysing solution (1% Triton X-100, 10% DMSO, 2.5 mM NaCl, 100 mM Na_2EDTA, 100 mM Tris, 10% sodium lauroyl sarcosinate, pH 10) for at least 1 h. After the lysing period, an enzyme digestion step was performed in order to specifically target oxidised bases [38,39]. Formamidopyrimidine DNA glycosylase (Fpg) and Endonuclease III (Endo-III) were used to convert oxidised purines and pyrimidines, respectively, into DNA single strand breaks (SSB). Comet scores were compared to reference slides (buffer incubation with no enzyme). Slides removed from lysis were washed 3 times with enzyme reaction buffer (0.1 M KCl, 0.5 mM Na_2EDTA, 40 mM HEPES, 0.2 mg ml^{-1} bovine serum albumin, pH 8) at 4 °C. Then, 50 μl of either buffer alone (reference slides) or 1.5 μg ml^{-1} of Fpg or Endo-III was applied to the centre of each gel, along with a coverslip, prior to incubation at 37 °C for 45 min in a

humidified atmosphere. Immediately after enzyme digestion, slides were placed in a horizontal electrophoresis unit containing freshly prepared electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH, pH > 13). The DNA was allowed to unwind for 20 min before electrophoresis was performed at 25 V, 300 mA for 20 min. Afterwards, the slides were gently immersed in neutralization buffer (0.4 M Tris–HCl, pH 7.5) for 10 min, before a final 10 min wash in distilled H_2O. To visualise Comets, 40 μl of ethidium bromide stain was applied to each gel. Scoring was achieved using a fluorescence microscope (Leica DMR) and Komet 5.0 image analysis software (Kinetic Imaging Ltd., Liverpool, UK). 50 cells were scored per gel (100/slide). Duplicate slides were prepared for each treatment and were independently coded and scored without knowledge of the code. Results were obtained from two independent experiments. Out of the possible 34 parameters measured, %tail DNA was chosen as it is considered to be the most meaningful [40], although others parameters, including Olive tail moment, showed similar patterns. It is also recommended for regulatory studies [41].

2.3. TiO_2 and UVA treatments

TiO_2 in the anatase form, with an average particle size of 5 nm, was purchased from Sigma (Poole, UK). For low concentrations (0.1–100 μg/ml), TiO_2 nanoparticles were suspended in sterilized phosphate buffered saline (PBS), as recommended [3], to an initial working concentration of 1000 μg ml^{-1}. Final dilutions were made in complete growth medium to desired concentrations. For concentrations >100 μg ml^{-1} initial dilutions were made in complete growth medium. For cytotoxicity studies, cells were treated with 0.1–1000 μg ml^{-1} (0.06–625 μg cm^{-2}) TiO_2 for 24 h (no UV) or 2 h (+UV) [6]. NRR assay protocol was essentially as outlined above for validation (Section 2.2.1). For genotoxicity studies, cells were incubated with TiO_2 for 2 or 24 h at concentrations ranging from 1 to 100 μg ml^{-1} (0.2–20.8 μg cm^{-2}) [3,10], and the Comet assay performed as described above (Section 2.2.2). For ESR studies, cells were treated with TiO_2 (10–500 μg ml^{-1} [2.6–133 μg cm^{-2}]) for 24 h [14] (protocol below, Section 2.4). Although, while light was excluded during incubation, there was some unavoidable exposure to light during handling as the experiments involving addition of accurate concentrations of the particles to the cells could not be carried out in darkness. Therefore, the possibility of some damage due to exposure to ambient light, compared to concurrent controls, cannot be totally excluded. For experiments requiring UVA treatment, medium was removed and cells were washed twice with PBS. The monolayer was then covered with PBS, to prevent desiccation, and cells were irradiated on ice with the plate lids removed. Control cells were sham irradiated, i.e. were placed under the lamp for the same time as exposed cells, but were completely covered with aluminium foil to keep them in the dark. UVA was delivered from a bench lamp containing two 40 W bulbs (Spectroline XX-40, USA) with an output of 340–420 nm (peak 370). Radiation intensity was quantified using a spectroradiometer (Macam Photometrics, Scotland, model SR9910) sensitive to wavelengths 240–800 nm. Exposure time was adjusted based on radiation intensity readings to yield the doses required.

2.4. Electron spin resonance (ESR)

Spectra were obtained at room temperature, using a Bruker ECS 106, X-band spectrometer. Typical spectrometer settings were: magnetic field centre 335.9 mT, field scan 10–12 mT, microwave frequency 9.43 GHz, microwave power 10 mW, modulation frequency 100 kHz, modulation amplitude 0.1 mT, time constant 0.3 s, sweep time 168 s. Briefly, trypsinised cells were washed three times in PBS and cell pellets, containing approximately 2×10^6 cells were resuspended in 25 μl of spin trap in PBS, which was drawn into a 3 cm length of thin-walled 0.8 mm i.d. Teflon tubing (Zeus Industrial Products Inc., Orangeburg SC, USA). The Teflon tubing was folded in half and placed inside a 4 mm i.d quartz tube which allowed air to circulate and prevented the cells becoming anoxic. Spectra were recorded before and after exposure to UVA. Cell samples were exposed to UVA for 3 min, outside the spectrometer, to a dose of 0.25 kJ m^{-2} and then spectra were recorded, starting 1 min after the end of irradiation. The spin traps used were 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), alone or in combination with superoxide dismutase (SOD) [42]; α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN); 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) and, for detection of singlet oxygen [43], 2,2,6,6-tetramethyl-4-piperidinol. Any spin traps that showed free radical signals before treatment were shaken with activated charcoal which was then removed by centrifugation.

2.5. Statistical analysis

For NRR assay data, differences between means were analysed using the Student's *t*-test. Mann–Whitney *U*-test was performed on Comet data to compare medium %tail DNA values. Regression analysis was performed on NRR and Comet data to confirm dose response relationships where appropriate. Analyses were performed using Minitab V.13.

3. Results

3.1. Validation of NRR and Comet assay

Validation experiments for the NRR assay showed clear dose–response relationships for both H_2O_2 and UVA exposure (regression analysis, $P \leq 0.001$) (Fig. 1). Concentrations of $H_2O_2 \geq 3$ mM produced significant decreases in cell viability (*t*-test, $P < 0.001$, $LD_{50} = 3.3$ mM) as did UVA doses ≥ 2.5 kJ m^{-2} ($P < 0.001$, $LD_{50} = 7.0$ kJ m^{-2}). From these results it was decided that during irradiation experiments with TiO_2, UVA doses used would be 0.5 and 2.0 kJ m^{-2}, as at these doses cell viability was >90%. A modified Comet assay was used to detect oxidation of DNA bases in TiO_2-treated gold fish cells. Again, Comet assay validation showed a clear dose–response relationship for H_2O_2 exposure for all classes of damage (regression analysis, $P < 0.05$) (Fig. 2a). Concentrations of $H_2O_2 \geq 5$ μM produced significant increases in all 3 classes of DNA damage (Mann–Whitney *U*, $P < 0.001$). The order of magnitude being, Fpg-sensitive sites > Endo-III-sensitive sites > direct single strand breaks. For UVA, significant increases were also observed in Fpg-sensitive sites (≥ 2.5 kJ m^{-2}), Endo-III-sensitive sites (≥ 7.5 kJ m^{-2}) and direct SSB (10 kJ m^{-2}) (Mann–Whitney *U*, $P < 0.001$) (Fig. 2b). Significant dose–response relationships were only observed for enzyme-sensitive sites (regression analysis, $P < 0.05$). No such

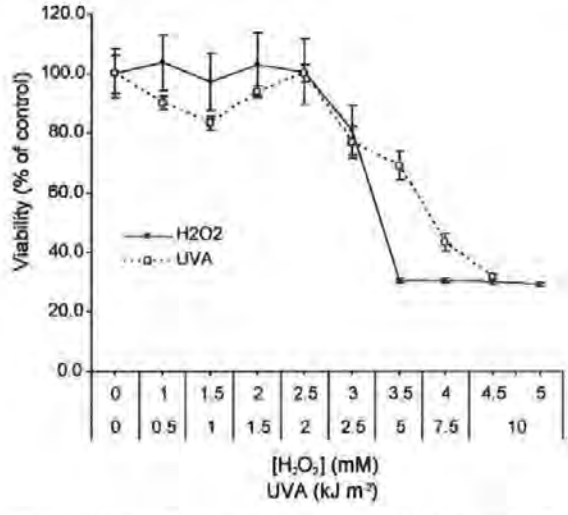

Fig. 1. Validation of the NRR assay with a range of concentrations of H_2O_2 (24 h exposure) and doses of UVA. Data represented as means ± 1 standard error.

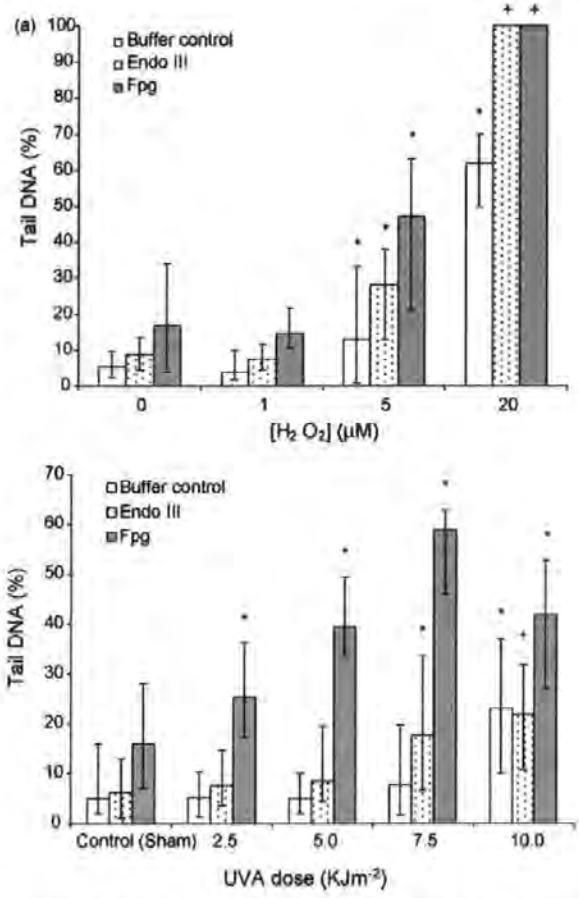

Fig. 2. Validation of Comet assay with (a) H_2O_2 and (b) UVA. Data points represented as medium values. Interquartile ranges give a measure of data spread. Symbol (*) denotes significant verses control + denotes unable to score – complete separation of head and tail.

dose–response relationship was evident for nucleoids not treated with lesions-specific enzymes. From these results 5 μM H_2O_2 was chosen as a positive control for further experiments with Comet. For combined exposures with UVA, 2.5 kJ m^{-2} was considered optimal as this level of expose caused only a weak response during validation experiments.

3.2. Cytotoxicity of TiO_2 (NRR assay)

Exposure of goldfish cells to TiO_2 nanoparticles for 24 h, in the absence of UVA, caused only a slight decrease in cell viability as measured by the NRR assay. Even at the highest concentration (1000 μg ml^{-1}), viability was still ~80% of control, although the decrease was statistically significant vs. the negative control (*t*-test, $P<0.05$) (Fig. 3). However, when TiO_2-treated cells were co-exposed with UVA (2.0 kJ m^{-2}), a significant dose-dependent decrease in cell viability was observed (regression analysis, $P<0.01$) (Fig. 3). At the highest concentration of TiO_2 (1000 μg ml^{-1}) cell viability dropped 2-fold (compared to non-irradiated cells) to ~40% of control. No such photosensitisation was observed at a lower dose of UVA (0.5 kJ m^{-2}), therefore, the effect was dependent on both the concentration of TiO_2 and the dose of UVA administered.

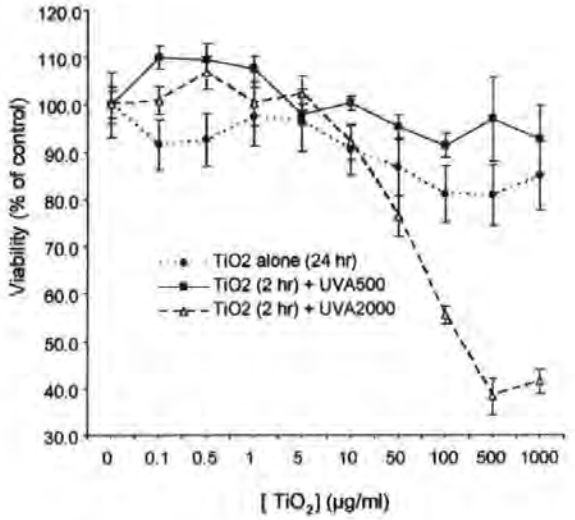

Fig. 3. Effect of TiO_2 concentration and UVA exposure on viability of GFSk-S1 cells, as evaluated by NRR assay. Cells were treated with TiO_2 for either 24 h (no UVR) or 2 h (+UVR) at concentrations ranging from 0.1 to 1000 μg ml^{-1}. UVA was administered at two levels—500 and 2000 J m^{-2}. Data represented as means ± S.E.

3.3. Genotoxicity of TiO_2 (Comet assay)

Cells were treated with 1, 10 and 100 μg ml^{-1} TiO_2 nanoparticles for 24 h in the absence of UV. All dose levels caused significant increases in oxidative DNA damage, as represented by increased levels of Fpg-sensitive sites (Man–Whitney *U*, $P<0.001$) (Fig. 4). Regression analysis showed that there was not a dose-dependent trend (Mann–Whitney *U*, $P>0.05$). Levels of Endo-III-sensitive sites remained unchanged, and

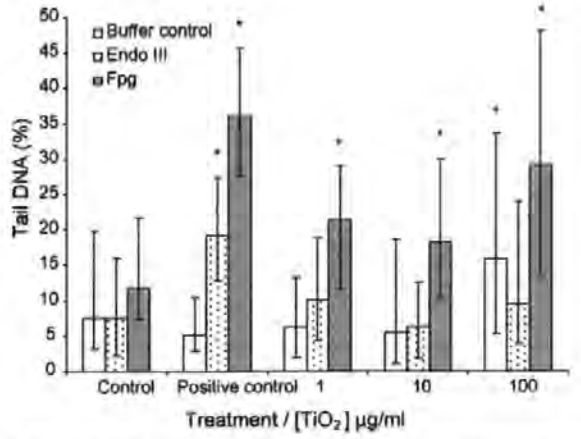

Fig. 4. Effect of TiO_2 nanoparticle exposure (in the absence of UVA) on oxidative DNA damage as measured by the Comet assay. For TiO_2 treatment cells were exposed at concentrations of 1, 10 and 100 μg ml^{-1} for 24 h. The positive control was 5 μM H_2O_2 for 10 min. Data points represented as medium values. Interquartile ranges give a measure of data spread. Symbol (*) denotes significant verses control.

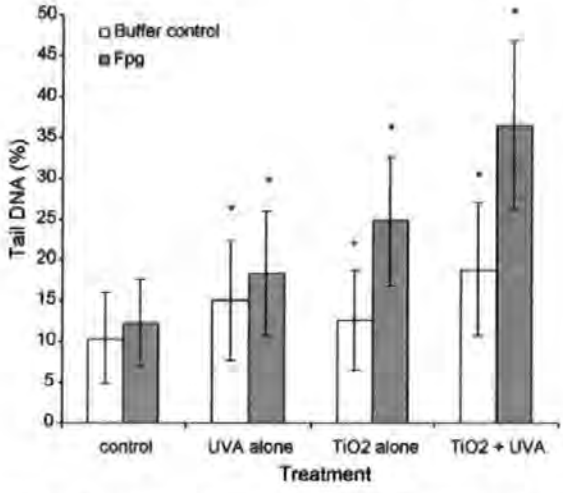

Fig. 5. Effect of UVA irradiation of TiO_2 nanoparticle-treated GFSk-S1 cells on oxidative DNA damage as measured by the Comet assay. UVA alone was a single UVA dose of 2.5 kJ m^{-2}. TiO_2 alone was 10 μg ml^{-1} for 2 h. The combined exposure (TiO_2 + UVA) was 10 μg ml^{-1} TiO_2 for 2 h followed by a UVA dose of 2.5 kJ m^{-2}. Data points represent as median values. Interquartile ranges give a measure of data spread. Symbol (*) denotes significant versus control.

therefore Endo-III incubation was not carried out in subsequent experiments. A significant increase in background SSB was only observed at the highest concentration (100 μg ml^{-1}) (Mann–Whitney U, $P < 0.001$). Oxidative DNA damage was also detected in cells incubated with 10 μg ml^{-1} TiO_2 for a shorter incubation period (2 h) (data not shown). When TiO_2-treated cells were irradiated with UVA, a further increase in oxidative DNA damage was evident, compared to TiO_2 or UV alone (Mann–Whitney U, $P < 0.001$) (Fig. 5).

3.4. ESR

UVA irradiation of an aqueous suspension of TiO_2 in the presence of DMPO produced the DMPO–OH adduct (Fig. 6a), which was unaffected by the prior addition of SOD. Using POBN as the spin trap, no adduct was observed after UVA irradiation unless a trace of ethanol was added before irradiation, when the POBN–$CH(OH)CH_3$ adduct was formed on irradiation (Fig. 6b). The spin trap DEPMPO showed the formation of the DEPMPO–OH adduct after UV irradiation of aqueous TiO_2, but in the presence of 100 mM mannitol, the OH adduct was reduced and another spectrum, thought to be a DEPMPO–mannitol radical adduct was also formed (Fig. 6c and d). No signals were observed when 2,2,6,6-tetramethyl-4-piperidinol was used as the trap, although an intense 3-line spectrum of the 4-hydroxy-2,2,6,6-piperidine-1-oxyl radical was observed when tetrasodium-meso-tetraphenylporphine sulphonate (TPPS) and white light was used as a positive control for this trap. Storage of an aerated solution of TiO_2 and DEPMPO in PBS for 3–4 h produced a well-defined DEPMPO–OH adduct, in the absence of UVA. However, subsequent experiments have shown that DEPMPO–OH is also formed on exposure of aqueous TiO_2 to white light.

UVA irradiation of TiO_2-treated cells, in the presence of DMPO, produced the DMPO–OH adduct (Fig. 7a), whilst control cells showed no such formation of radicals. Addition of SOD to the TiO_2-treated cells appeared to have no effect on the formation of the DMPO–OH adduct. UVA irradiation of TiO_2-treated cells in the presence of the spin trap POBN produced a spin adduct with a 6-line spectrum, characterised by $a_N = 1.56$ mT and $a_H = 0.33$ mT. Using DEPMPO as the spin trap, the ESR spectra of TiO_2-treated cells, observed after UVA irradiation (Fig. 7b), showed three different species, DEPMPO–OH, an unidentified adduct, DEPMPO–X (Fig. 7c) and a very small contribution from DEPMPO–OOH. The presence of the latter component was confirmed by addition of SOD, prior to UV irradiation, which suppressed two small peaks near the centre of the spectrum, whilst having a negligible effect on the rest of the spectrum. Addition of 500 U ml^{-1} catalase prior to irradiation had no detectable effect on the resulting spectrum. The ratio of DEPMPO–OH:DEPMPO–X was found to be dependent on the concentration of TiO_2. Cells treated with 10 or 50 μg ml^{-1} showed DEPMPO–OH as the major component, while with 100 or 500 μg ml^{-1} DEPMPO–X was equal to or greater than DEPMPO–OH. The overall radical concentration increased by an order of magnitude between 10 and 500 μg ml^{-1} TiO_2. Storage of TiO_2-treated cells in the presence of DEPMPO for several hours showed the formation of a low concentration of the mixed DEPMPO adducts, in the absence of UVA.

4. Discussion

Our validation experiments clearly showed, for the first time, that both the NRR assay and the Comet assay are sensitive assays for detecting oxidative stress-induced cytotoxicity and genotoxicity, respectively, in the GFSk-S1 cell. Previously, the modified Comet assay protocol, which includes a digestion step with bacterial repair endonucleases to target oxidised DNA bases, has been shown to greatly enhance the sensitivity and specificity of the assay, compared to the standard protocol [38]. In the present study, this is highlighted in a fish cell line by the fact that the level of Fpg-sensitive sites (oxidised purines) increased to a greater degree than the level of direct SSB in all treatments (H_2O_2, UVA, TiO_2, TiO_2/UVA). Although Endo-III (which targets oxidised pyrimidines) did not show any response to TiO_2 treatments, it did enhance the sensitivity of the Comet assay when cells were treated with H_2O_2 and UVA during validation experiments, and therefore may be important in future oxidative stress related studies. Although the standard Comet assay has been used extensively for evaluating the genotoxic potential of contaminants in fish (see reviews [44,45]), there are only two other studies that have specifically evaluated oxidative DNA damage in fish cells using the modified Comet assay [46,47]. Furthermore, this is the only study using fish cells to have validated the modified Comet assay protocol with reference genotoxins.

As was mentioned earlier, there is conflicting evidence as to whether TiO_2 nanoparticles induce toxicity in the absence of UV irradiation. Results from this investigation seem to support both sides of the argument. In terms of cytotoxicity, the neutral red retention assay showed that TiO_2 alone had a very weak capac-

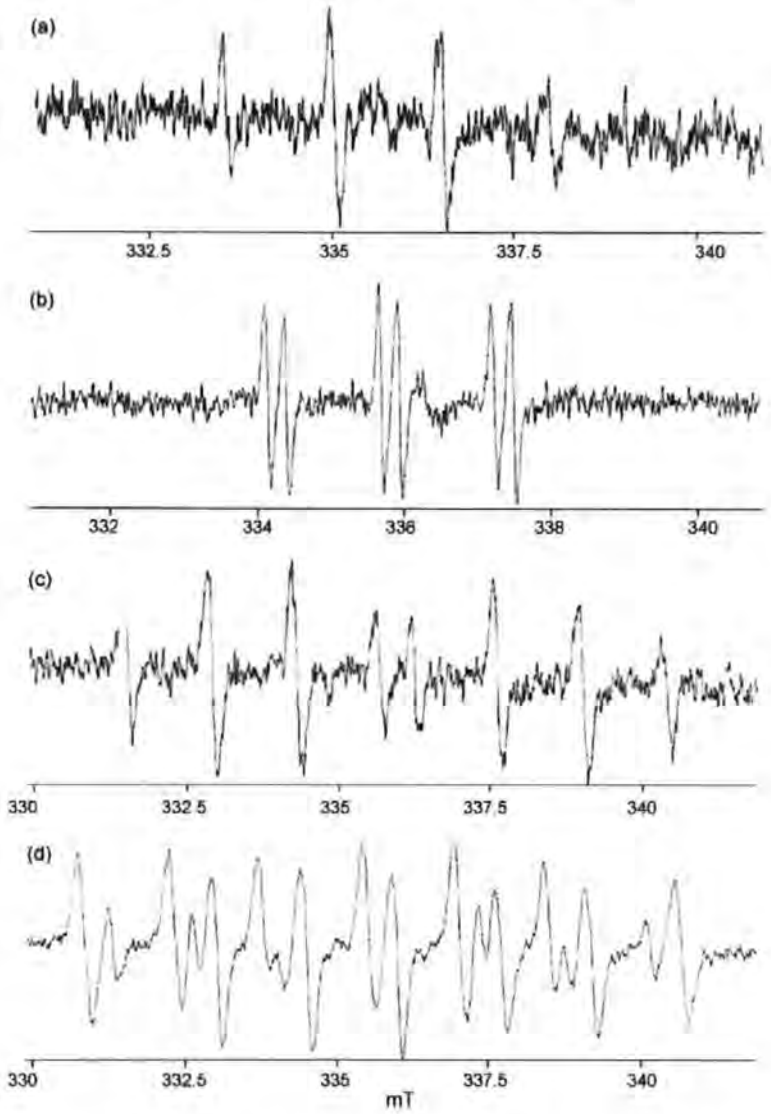

Fig. 6. ESR spectra of an aqueous suspension of TiO_2 after irradiation with UVA (a) in the presence of DMPO ($a_N = a^{\beta}_H = 1.49$ mT); (b) in the presence of POBN and ethanol ($a_N = 1.57$ mT, $a_H = 0.24$ mT); (c) in the presence of DEPMPO ($a_H = a_N = 1.37$ mT, $a_P = 4.71$ mT); (d) in the presence of DEPMPO and mannitol (additional signal from a mannitol radical: $a_H = 2.14$ mT, $a_N = 1.48$ mT, $a_P = 4.68$ mT).

ity to affect lysosomal membrane integrity (cell death), even at very high concentrations (1000 $\mu g\,ml^{-1}$). This is in agreement with Zhang and Sun [6] who had similar results over the same concentration range with human colon carcinoma cells (methyl tetrazolium cytotoxicity [MTT] assay). Linnainmaa et al. [8] also reported no effect of 5–200 $\mu g\,cm^{-2}$ ultra-fine TiO_2 on viability of rat liver cells, using cell division (multi nuclei index) as a measure of cytotoxicity. Contrastingly though, our cytotoxicity results do not fall in line with those of Uchino et al. [14] who reported a significant decreases in viability (MTT assay) of CHO cells to ~50% of control when treated with 100 $\mu g\,ml^{-1}$ TiO_2. Nor do they agree with Wang et al. [10] who observed dose-dependent decreases in viability in human lymphoblastoid cells, also using the MTT assay. They recorded 61, 7 and 2% relative viability at 130 $\mu g\,ml^{-1}$ TiO_2 for 6, 24 and 48 h exposures. From the last two examples it appears as though the MTT assay may be a more sensitive and appropriate method of assessing cell viability in TiO_2-treated cells than the NRR assay used in this investigation. However, the use of MTT assay for nanoparticle cytotoxicity evaluation has been called into question due to its apparent ability to yield false positive results, particularly in the case of carbon nanotubes [48]. On the other hand, Zhang and Sun [6] also used the MTT assay, but found no cytotoxic effect of TiO_2, even at 1000 $\mu g\,ml^{-1}$. This suggests that differences in response between studies are not necessarily related to the assay method. Instead, it may be due to how the TiO_2 treatments differ in terms of the source of TiO_2, particle size, preparation method (sonication), degree of aggregation and incubation conditions. A standard protocol for nanoparticle solution preparation for *in vitro* studies would be useful for future investigations. Differential susceptibility between cell types could also introduce a great deal of variability in results.

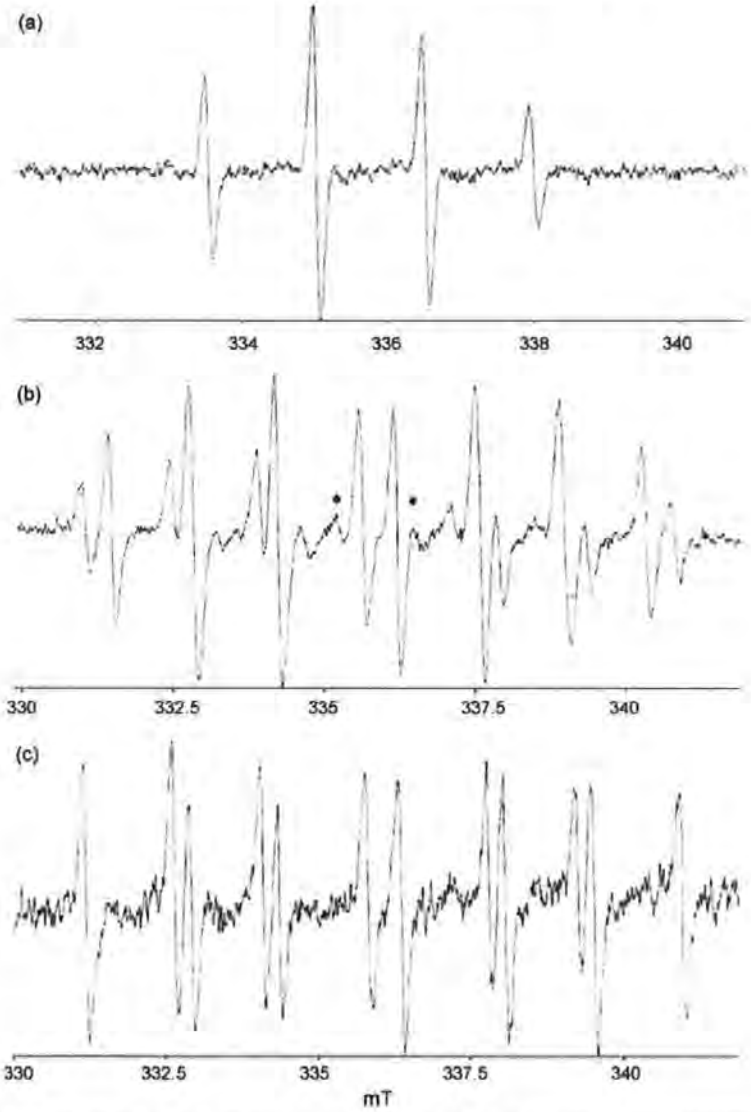

Fig. 7. ESR spectra of GFSk-S1 cells treated with 50 μl ml^{-1} TiO_2 for 24 h and irradiated with UVA (a) in the presence of DMPO; (b) in the presence of DEPMPO, *, marks characteristic peaks of the DEPMPO–OOH spin adduct; (c) spectrum of unidentified adduct DEPMPO–X (a_H = 1.72 mT, a_N = 1.44 mT, a_P = 5.16 mT), obtained by subtraction of the spectrum of DEPMPO–OH.

Differences in metabolic rate/capacity (temperature dependent), antioxidant enzyme machinery, and DNA repair capabilities may also explain the experimental outcome.

Results from the Comet assay indicate that TiO_2 nanoparticles are in fact genotoxic without UV irradiation, as all concentrations tested produced a significant increase in the level of Fpg-sensitive sites. Together with the fact that levels of Endo-III-sensitive sites remained unchanged, the increase in Fpg-sensitive sites suggests that 8-hydroxyguanine (8-OH-G) was probably the most likely product of TiO_2-induced oxidative stress, although formamidopyrimidines (imidazole ring-opened purines) are also possible substrates for Fpg [49]. 8-OH-G is important because it is both relatively easily formed and is mutagenic and carcinogenic [50]. Consequently it is considered a good biomarker of oxidative stress and a potential biomarker of carcinogenesis [50,51]. Singlet oxygen is known to react with the guanine moiety of cellular DNA to form 8-OH-G, as is the hydroxyl radical which reacts almost indifferently with all components of the DNA molecule damaging both purine and pyrimidine bases and also the deoxyribose backbone [50,51]. Therefore, from Comet assay results, it is not possible to conclude which species was responsible for the genotoxic effects of TiO_2, although as mentioned earlier, from ESR analysis it appears that it is most likely to be $^{\bullet}OH$, as there is no evidence of $^1O_2$ production. In common with other metals or organometalic compounds however, in addition to the production of ROS, TiO_2 could also directly bind to DNA or repair enzymes leading to the generation of strand breaks [52,53].

Our Comet assay results compare favourably with recent studies on mammalian cells which have investigated the genotoxic potential of TiO_2 nanoparticles at similar concentrations. Gurr et al. [3] observed increased levels of oxidative DNA

damage (Fpg-sensitive sites) when BEAS-2B cells, a human bronchial epithelial cell line, were treated with 10 $\mu g\,ml^{-1}$ anatase (10 nm) TiO_2 particles for 1 h. Additionally, treatment of human lymphoblastoid cells with ultra-fine TiO_2 in the range 65–130 $\mu g\,ml^{-1}$ induced 2.5-fold increases in the frequency of micronuclei, 5-fold increases in DNA single strand breaks (Comet assay) and 2.5-fold increases in mutation frequency [10]. Exposure to 1 $\mu g\,cm^{-2}$ of TiO_2 (<20 nm) for 12–72 h also caused a significant dose-dependent increase in the induction of micronuclei in Syrian hamster embryo cells (SHE) [9]. However, as with the cytotoxicity results, there seems to be some evidence to the contrary. Nakagawa et al. [7] reported no effect of ultra-fine (25 nm) TiO_2 on a series of genotoxicity parameters measured, including the Comet assay, in mouse lymphoma and Chinese hamster cells *in vitro*. TiO_2 nanoparticles also had no effect on micronuclei induction in cultured rat liver epithelial cells [8]. As was discussed for the cytotoxicity results, disagreements in reported genotoxic potential of TiO_2 nanoparticles may be explained by a number of variables, including the TiO_2 treatment regime, the cell type used, the metabolic/antioxidant capacity of the cells, as well as DNA repair capabilities. It is particularly important to consider possible inherent differences between cells from different phylogenetic origin, namely fish (as used here) and mammalian cells. Indeed it has been previously shown that fish cells are generally more susceptible to toxic/oxidative injury than similarly treated mammalian cells [29,54]. Although this did not seem to be the case for the cytotoxicity studies with the NRR assay, it does seem evident that the goldfish cells show increased sensitivity to TiO_2-induced oxidative DNA damage, compared to mammalian cells used in other studies. A significant increase in the level of Fpg-sensitive sites (8-OH-G formation) was observed at only 1 $\mu g\,ml^{-1}$ (24 h treatment) in goldfish cells which seems to be the lowest reported in any of the literature. We also observed a significant increase in base oxidation after just 2 h of TiO_2 exposure and together with the fact that the longer incubation period (24 h) was still revealing significant levels of base oxidation, this suggests that DNA repair mechanisms were unable to repair damage from the initial insult. Fish are reported to have a much lower capacity for DNA excision repair than mammals [55,56] and this may help explain the apparent increased sensitivity of the fish cells towards TiO_2-induced oxidative DNA damage. In comparison, it has been clearly demonstrated that hydroxylated guanine bases are removed from cellular DNA via an efficient enzymatic repair process in mammalian cells [57]. The half life for removal of 8-OH-G from DNA in human lymphoblast cells at 37 °C has been reported to be only 55 min [58].

In line with mammalian studies, once TiO_2-treated cells had been co-exposed with UVA, a more pronounced toxic effect was found than in the absence of UV, both in terms of cytotoxicity and oxidative DNA damage [5–7,14]. Although it may have been reported several times in mammalian cells, this is the first time that the photo-catalytic properties of TiO_2 nanoparticles have been shown in fish cells. From the NRR results it is clear that the photo-catalytic effects are dependent on both the concentration of TiO_2 and the dose of UVA administered. Although it appears that increasing the concentration of TiO_2 increases the interactive effect with UVA, it has been previously shown that this may not be due to increased absorption of UVA by TiO_2. Using ESR analysis, Uchino et al. [14] found that there was no relationship between •OH radical production and UVA absorbance in all TiO_2 test samples, but they demonstrated a correlation between •OH radical production and viability of CHO cells.

ESR spin trapping with DMPO suggests that irradiation of an aqueous suspension of TiO_2 with UVA produces hydroxyl radicals. Whilst the absence of a detectable signal from DMPO–OOH cannot be taken as proof that superoxide is not produced, since DMPO–OOH decays rapidly to DMPO–OH, the absence of any effect of SOD on the observed ESR spectrum strongly suggests that there is no significant superoxide production. In contrast, the spin trap DEPMPO gives relatively stable adducts of both •OH and O_2^- but irradiation of TiO_2 produces only the •OH adduct. Moreover, addition of mannitol, which reacts with hydroxyl radicals, but not superoxide radical anions, reduced the amount of DEPMPO–OH adduct observed and formed a DEPMPO–mannitol adduct. Further evidence for the formation of hydroxyl radicals is provided by spin trapping with POBN. This is an efficient trap for C-centred radicals, but the POBN–OH spin adduct is very short lived. Consequently, UVA irradiation of TiO_2 outside the microwave cavity of the spectrometer produced no detectable radical adduct unless ethanol was present during irradiation, when the α-hydroxyethyl radical $•CH(CH_3)OH$ was formed by H-abstraction by the hydroxyl radicals. In contrast, *in situ* UV-irradiation of aqueous TiO_2 in the presence of POBN was subsequently found to produce the POBN–OH adduct. The absence of any ESR signal on irradiation of TiO_2 in the presence of 2,2,6,6-tetramethyl-4-piperidinol demonstrates that $^1O_2$ is not formed. Consequently it can be concluded that UVA-irradiation of aqueous TiO_2 produces only hydroxyl radicals.

ESR studies showed that UVA irradiation of control cells produced no detectable radicals. In contrast, irradiation of TiO_2-treated cells produced primarily •OH radicals that could be trapped with DMPO or DEPMPO. Using the latter spin trap, a number of other radicals were detected, the most abundant of which is possibly due to peroxidation of lipid membranes by hydroxyl radicals, since a similar signal, together with that of DEPMPO–OH appears to be present in the spectrum of UV-irradiated vesicles of phosphatidylcholine containing TiO_2 (unpublished results). Moreover, the radical trapped by POBN on irradiation of TiO_2-treated cells is consistent with a lipid peroxyl radical [59]. The nature of this radical and the source of the low concentration of superoxide radical anions that are produced require further investigation. However, it can be concluded that the primary cause of biological damage from TiO_2, combined with UVA is due to hydroxyl radicals which may react directly with DNA or indirectly through peroxidation of membrane lipids. Our ESR spin trapping experiments indicate that TiO_2 can produce hydroxyl radicals in the absence of UVA, possibly due to ambient light, and these react with cells, although the concentration detected by spin trapping with DEPMPO is reduced, due to the spin adducts being metabolised by the cells

and by their inherently short lifetime when compared with exposure times.

In conclusion, we have shown for the first time that TiO_2 nanoparticles in the absence of photo-activation are potentially genotoxic to fish cells under *in vitro* conditions. This effect becomes more pronounced in the presence of UVA, along with cytotoxic effects which only occurred during combined expose of TiO_2 and UVA. ESR studies indicate that $^{\bullet}OH$ radicals are the predominant radical species generated both in aqueous solution as well as in the fish cells. These radicals are likely to play the major role in producing the genotoxic effects in terms of oxidative DNA damage. *In vivo* studies are now required to further establish how TiO_2 nanoparticles may affect various parameters of fish health during waterborne or dietary exposure of environmentally realistic concentrations.

Acknowledgements

The authors would like to thank Professor Lucy Lee (Wilfred Laurier University, Canada) for kindly providing GFSk-S1 cells and Professor Andrew Collins (University of Oslo, Norway) for supplying the material and relevant training for the use of Endo-III and Fpg in the Comet assay. We would also like to thank Zeus Industrial Products Inc., Orangeburg SC, USA, for kindly donating Teflon tubing for ESR analysis.

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