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Effects of antioxidant enzyme inhibitors on free radical levels in mice and possible clinical applications for decreasing ROS damage in the cochlea

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EFFECTS OF ANTIOXIDANT ENZYME INHIBITORS ON FREE RADICAL LEVELS IN MICE AND POSSIBLE CLINICAL APPLICATIONS FOR DECREASING ROS DAMAGE IN THE COCHLEA

by

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Abstract

Reactive oxygen species (ROS) are involved in a number of neurological disease states as well as mediate cochlear injury due to ototoxic drugs and noise exposure. A study was done to measure cochlear ROS levels within various mice *in vivo* via cochlear perfusion. By perfusing the cochlea, we use a medium to capture 2,3-dihydrobenzoic acid (DHBA), a biological marker for ROS production in the presence of salicylate. Two perfusates were used, a control consisting of artificial perilymph and salicylate, and a perfusate containing mercaptosuccinate and aminotriazole, inhibitors of antioxidant protective enzymes glutathione peroxidase (GPx) and catalase. It was predicted that impairing the antioxidant protection would yield higher background levels of ROS, specifically ·OH. High performance liquid chromatography (HPLC) analysis for the 2,3-DHBA marker revealed inconclusive results. At this time experimental conditions are unable to produce results that support our predictions. Several variables merit additional examination, including pH, drug concentration, route of administration of salicylate, and flow rate. Additionally, cochlear temperature may play a large role. Additional research is needed to determine optimal conditions for measuring free radicals *in vivo*.

Introduction

Reactive oxygen species (ROS), and oxygen containing free radicals, have been linked to various injuries and disease states in the body. A free radical is an atom or molecule that contains one or more unpaired electrons. Free radicals are highly reactive, and overproduction can cause cell death and tissue damage. One of the most damaging radicals is the hydroxyl radical (·OH), which will react with most macromolecules close to its generation site. (Evans & Halliwell, 1999).

Chronically increased levels of ROS lead to oxidative stress throughout the body. This stress can impair or kill cells and initiate disease and degenerative processes such as aging, carcinogenesis, and immunodeficiencies. They are involved in a myriad of health problems including heart disease, rheumatoid arthritis, kidney disease, and certain brain pathologies amongst other conditions. (Lubec, 1996).

In addition to the aforementioned disease states that free radicals are involved in, the cochlea is also very susceptible to oxidative injury. This susceptibility is due to the fact that the inner ear is composed of highly metabolically active tissues; these tissues are very capable or making ROS and free radicals including the hydroxyl radical. (Kopke et al., 1999).

To understand what happens in conjunction with cochlear damage it is necessary to review the biochemistry of cellular injury, since it is the hair cells within the cochlea that are the vital link to our ability to process sound. A schematic of daily ROS production can be seen in Figure 1. Metabolism (construction) and catabolism (destruction) of macromolecules yields ROS. These ROS are usually removed by the body's antioxidant protectants, such as superoxide dismutase (SOD), glutathione

peroxidase (GPx), Catalase, Vitamin E, and others (Evans & Halliwell, 1999). Additionally, there are also heat shock proteins (HSP) that may play a role in either protecting or repairing cochlear structures under oxidative stress. (Kopke et al., 1999). When various cochlear insults are introduced such as noise exposure, ischemia, trauma, or ototoxic substances, there is an elevated production of reactive oxygen species (ROS). (Ohlemiller & Dugan, 1999). These reactive oxygen species include hydroxyl radicals, superoxide anions, and hydrogen peroxide.

A visual schematic of how different molecules relate and react with one another in the production and removal of ROS can be seen in Figure 2. In an oxidation-reduction reaction, oxygen is reduced to superoxide, which is further transformed by enzymes to hydrogen peroxide. This hydrogen peroxide can then be broken down via three separate pathways. Two pathways are facilitated by protective enzymes, catalase and glutathione peroxidase, to yield water, oxygen, and glutathione disulfide. A third route yields production of the free radical $OH^{\cdot}$ (hydroxyl radical). The protective role of GPx and catalase will be the major focus of this study. (Refer back to Figures 1 and 2 for a schematic of the place and role of protective enzymes in the production and removal of ROS.) There are many other enzymes involved in scavenging and breaking down ROS and free radicals.

There are two major types of hearing loss based on two different mechanisms. Conductive hearing loss results from the inability to get the signal to the inner ear, whereas sensorineural hearing loss is caused by damage to the inner ear (cochlea) and/or the eighth nerve. Although, conductive losses are usually treatable with medication or some form of surgery. Sensorineural loss is irreparable and permanent. Acute and

chronic oxidative stress may be the underlying cause of some sensorineural and age related hearing loss.

Age is also a large factor in hearing loss. However, it can be theorized that it is not age per se, but rather a lifetime of continual free radical attack that leads to hearing loss. Presbycusis, high frequency hearing loss seen in about 1 out of every 3 or 4 persons above the age of 65, may be associated with chronic oxidative stress. The basal region of the cochlea appears more metabolically active, thus it is more easily damaged by oxidative stress. Combine that fact with years of cumulative effects of free radical damage, and it can be hypothesized that elevated high frequency thresholds seen in age-related hearing loss is due to oxidative stress. (Kopke et al., 1999; Evans & Halliwell, 1999).

Several studies have probed the specific damaging effects of ROS on the cochlea and cochlear function. It is already known that ROS-mediated damage can include initiation of DNA strand breaks, peroxidation of free fatty acids, and oxidation of proteins and carbohydrates. These cellular changes lead to alterations in cell surface morphology, membrane integrity, and ionic gradients. (Halliwell, 1992). An *in vitro* study was performed in guinea pigs to examine the effects of ROS in outer hair cells. (Clerici et al., 1995). The outer hair cell (OHC) is of importance because it is involved with the frequency tuning and sensitivity of the Organ of Corti. It is believed to be changes in OHC shape and stiffness during stimulation that biomechanically produces sharp frequency tuning. Any alterations to OHC morphology and/or viability could have severe implications. Clerici et al. found cell body shortening and death associated with ROS. Findings at the *in vitro* level are hard to extrapolate to *in vivo* (within a living

subject), however, elevated levels of ROS in the cochlea have been measured in mice after exposure to loud noise. (Ohlemiller et al., 1999a). When mice are exposed to high enough levels to cause a permanent threshold shift, as measured by ABR threshold shifts, ROS levels are higher as compared to controls. Thus, ROS elevation is seen in mice who suffer PTS following noise exposure. These data suggest that there is a relationship between noise exposure and oxidative stress induced hearing loss. (Ohlemiller et al, 1999a).

Ischemia is another cochlear insult believed to produce increases in cochlear ROS. Reduction of blood flow to the cochlea has been linked to elevated levels of ROS in the perilymph. In addition, these levels remained elevated for at least a few hours. (Ohlemiller & Dugan, 1999).

Studies that target protective enzymes such as SOD and GPx have shown increased susceptibility to hearing loss, and a link between genetic impairment of antioxidant defense mechanisms and susceptibility to cochlear injury (Ohlemiller et al, 1999a; Ohlemiller et al, submitted). Presently, scientists have the capability of genetically controlling aspects of mice through "knockout" techniques. Knockout techniques make it possible to create a model of chronic oxidative stress by simply impairing the genes that serve as antioxidant protectors. Knockout mice for *Sod1* and *GPx,* missing the gene that codes for *SOD* and *GPx,* showed increased susceptibility to noise-induced PTS.

Since links have been demonstrated between genetic impairment of antioxidant defenses and cochlear vulnerability; and between vulnerability and hearing loss, it will be

helpful to expand our understanding of the relation between cochlear antioxidant status and ROS production.

By measuring a molecular marker via high performance liquid chromatography techniques, one can indirectly measure the amount of $OH^{\cdot}$ produced (Lubec, 1999; Ohlemiller & Dugan, 1999). This study will attempt to measure increased levels of the 2,3-DHBA marker following a cochlear perfusion of a control perfusate (artificial perilymph with salicylate) and a perfusate containing enzyme inhibitors aminotriazole and mercaptosuccinate which inhibits catalase and GPx respectively. By introducing inhibitors to block catalase and GPx, most H_2O_2 should be converted to $\cdot OH$, leading to a larger quantity of 2,3-DHBA, and presumably an increase in oxidative stress. Our prediction is that HPLC analysis will yield greater amounts of 2,3-DHBA following perfusion of enzyme inhibition as compared to control perfusions.

Materials and Methods

Animals

This study included approximately 35 mice of the C57BL/6J and CBA strains. Due to a low success rate for both surgery and perfusion, far fewer mice were actually used in the data analysis. All procedures were approved by the Central Institute for the Deaf Animal Care and Use Committee.

High Performance Liquid Chromatography

Liquid chromatography is the separation of components of a solution following differential migration of the solutes in a liquid flowing through a column packed with particles of a specific size and hydropholicity (Lough & Wainer, 1996). Stated in simple terms, HPLC allows us to quantify small amounts of components. There are two processes that occur in series. The first process involves separation of compounds into their components (chromatography portion). In our study "reversed phase" chromatography was used, whereby different substances are retained based on their polarity. Those substances that are non-polar or hydrophobic, will be retained for shorter periods of time. Components are then separated by the amount of time they are retained (retention times), and by looking at the compound over time we can analyze the components separately.

Once the compounds are separated they are quantified electrochemically, whereby they are oxidized by electrodes set at specified voltages. The difference in electrochemical potentials will result in the removal of the hydrogen atoms. The HPLC machine quantifies substances based on the amount of hydrogen atoms being cleaved, characterized by the amount of current detected. A 'chromatogram', a plot of current

versus time, tracks the oxidation of compounds in sequence is drawn to show quantities of components in solution over time. When hydrogen atoms are cleaved they elicits a peak on the chromatogram. The number recorded for the quantity of substance is the area under this peak. Thus by a series of processes, the apparatus allows both separation and quantification of very small amounts

Raw chromatograms can be seen in figures 3 and 4. The chromatograms appear on a typical PC monitor and are split into two graphical representations, one for each detection. Channel A shows the peaks for the DHBA markers, and channel B shows the amount of salicylate. Below the graphs, the peaks are named with details of their retention times and their peak areas. Each chromatogram is only representative of the amount of compounds within one collected sample.

Acute Surgical Procedure and Cochlear Perfusion

Animals were anesthetized using sodium pentobarbital (60 mg/kg, i.p.) and placed ventral side up 10 minutes post anesthesia injection to keep the body temperature stable. If the mouse was still responsive to toe pinches, the mouse was given another 10 min. for full effects of the drug. Additional doses of anesthesia were given if the mouse was still responding after 20 min. A rectal temperature probe connected to a heating pad was inserted to maintain core temperature at 37.5 +/- 1° Celsius. The first incision was made vertically exposing the trachea. Intubation was then performed to insure against respiratory arrest. Following intubation, the mouse was secured in a custom head-holder. With the animal both secure and stable, surgery proceeded to expose and open the left bulla to gain access to the cochlea. An entrance hole was made in the round window with an insect pin, and an exit hole was made over the apex using a 90-degree-angle pick.

Typical surgery from time of dosing to start of perfusion was approximately 1 - 1.5 hours.

Perfusion was performed through PE 10 tubing flamed to a fine tip at the end, which was inserted into the round window opening, and sealed into place with dental cement. The perfusion apparatus consisted of about 60 mm of PE 10 tubing, which attached to a 5ml syringe containing the perfusate. A syringe pump operated at 4.2 micro liters/min pumped the perfusate into the cochlea. About another 60 mm of PE 10 tubing loosely placed into the anterior bulla to collect the emerging perfusate from the apical hole via a vacuum, and was dripped into 0.5 ml plastic tubes immersed in an ice water bath. Since 2,3-DHBA is degraded by heat, light, and metal cations, glass and metal were minimized in the flow path, and the experiment was conducted in a dim room with the operating microscope lights turned of once the perfusion had begun. A schematic of the cochlear perfusion can be seen in figure 5.

The artificial perilymph (AP) was made in milli Q water and consisted of 21.9mM sodium bicarbonate, 134.2 mM sodium chloride, 2.95mM potassium chloride, 3.3 mM calcium chloride, 3.95mM magnesium chloride, 3.7 mM dextrose, and 6.7 mM urea. The pH was adjusted to 7.4 before it was perfused via bubbling with $95\%O_2/5\%CO_2$.

Samples were collected every 20 minutes as long as the conditions remained viable. Additional pentobarbital was applied as needed at one-fourth the initial dose. A minimum of 5 data samples was needed for data analysis, (three control samples, one transition where the pumps were switched, and at least one trial). Therefore, the animal had to remain viable for close to 2 hours after perfusion began.

Each procedure was carried out under different experimental conditions as more contributing factors were considered. The setup on 1/17/2000 started out using artificial cerebrospinal fluid (ACSF) for which the chemical components are listed in the materials section. The pH of the solution was brought to 7.4 by bubbling with 5% CO_2 and 95%O_2 gas; 100uM of salicylate was put into this ACSF. The drug solution was composed of 67mM aminotriazole to inhibit catalase and 50 uM mercaptosuccinate to inhibit GPx, in ACSF. We perfused at a rate of 4.2 uL/min and collected samples every 20 minutes. Two control samples were collected with the control solution being perfused, and then subsequent samples were collected with the drug solution being perfused. Our results, as seen in aforementioned figures showed a decline in 2,3-DHBA.

The next procedure (1/28/2000) used a different buffering system (1mM phosphate) for the perfusate. Rather than using sodium bicarbonate we changed to 155mM NaCl. Additionally, we bubbled the pH to 7.0 and used a different water source. Carbon experiments had shown that the solution was becoming too alkaline.

In subsequent experiments (2/18/2000), two additional changes were made to the experimental design. In order to ensure that we were using enough drugs to see a marked change we doubled the dosage, putting 100 uM mercaptosuccinate and 134 mM aminotriazole. In addition the salicylate was applied systemically, rather than putting it into the ACSF 3 mg (1mg/kg body weight). The salicylate was injected subcutaneously before the surgery.

Although salicylate injections seemed to cause a bit more bleeding during the surgical procedure (since salicylate is a blood thinner), we decided to continue with that condition for the next few mice, including the one analyzed on 3/27/2000. Every

condition remained the same as before, except the amount of drug was increased again such that the trial perfusate contained 200 uM mercaptosuccinate and 268 mM aminotriazole.

HPLC Analysis of Samples

Samples were analyzed immediately using high liquid chromatography (HPLC) to quantify salicylate and its oxidation products, 2,3- and 2,5-DHBA. Using a C15 column and a flow rate of 0.5ml/min, the stationary phase was 5 micron, C15, 3.2mm x 25cm, and the mobile phase consisted of 50mM Sodium Acetate, 50mM Citric Acid, 25% Methanol, 5% Isopropyl Alcohol, with phosphoric acid and a pH of 2.5. The electrochemical portion consisted of two separate coulometric electrodes in series with each other. The first cell was set to a potential of 250 mV, the second was set to 750mV. (Since both 2,3 and 2,5 DHBA are completely oxidized at the first voltage and salicylate is completely oxidized at the latter.) It should be noted that since the 2,5-DHBA isomer can be generated from enzymatic oxidation of salicylate by P450 enzymes, analyses focused mainly on the level of 2,3-DHBA. (Halliwell et al., 1991 as cited in Ohlemiller et al., 1999a; Ohlemiller & Dugan, 1999). Chromatograms were obtained for both control and drug trials.

If it was not feasible to analyze the samples immediately, they were temporarily stored on ice or frozen.

Results

Approximately 35 animals were used through the study, 10% of the surgeries were not successfully completed, 25% of the animals did not survive long enough to collect through the first perfusion, 50% did not remain viable through enough perfusions

for analyzable data. Data collected from the five of the most successful surgeries and perfusions (remaining15%) were analyzed. Since there was no continuity on the amount of samples run between animals, all data was analyzed by taking the mean of the control samples and the mean of the trials.

Results showed that under several different experimental conditions, the amount of 2,3-DHBA marker did not increase during the drug trials as compared to the controls as measured by peak areas on the HPLC chromatograms. Instead, the trend shows a decreasing amount of 2,3 DHBA on average for drug trials. (Figure 6). In fact, the highest percentage of 2,3 DHBA found in trials as compared to control was 80.37% on 1/28/2000. (Figure 6). In addition to DHBA, amount of salicylate was also measured. The same trend occurred over time for salicylate, as there was a decrease in all subjects except for the final surgery (Figure 7). When analyzing control vs. trial for amounts of DHBA and salicylate, it can be seen that although both are smaller, there are higher percentages of salicylate than 2,3-DHBA in the drug trials compared to the control trials (Figure 8). The importance of this finding is that it shows that salicylate is still getting to the cochlea, and is not depleted by the rate of perfusion, or travelling to other areas.

Several conditions are necessary for the appropriate reactions to occur and be measured efficiently. We examined a number of experimental parameters including pH via CO_2 bubbling, perfusion rate, route of salicylate delivery to the cochlea, and cochlear temperature. As a consequence, however, few experiments were performed for any single set of conditions. Further research needs to be done to look at iron content in the collected samples, as iron plays a key factor in the free radical reactions; it is possible that cochlear iron content was depleted over time. Additionally, during the final surgery

cochlear temperature was measured and found to be around 30-32°C, much lower than >37°C expected. Thus depressed cochlear temperature may have played a role.

Discussion

Data were inconclusive and did not support our hypothesis. Measurements did not show that the impairment of antioxidant protective agents results in elevated ROS levels. Specifically, the introduction of aminotriazole and mercaptosuccinate to impair catalase and GPx did not show increases in our 2,3-DHBA marker for ·OH levels in the cochlea.

There are several possible reasons why these experiments did not appear to work. The first possibility is perfusion flow rate. The experimental procedure assumes that DHBA is steadily being produced in the cochlea, and by flushing with an artificial perilymph, we provide a medium to capture this DHBA. It is possible that the perfusate was either diluting the DHBA too much, or perhaps even exhausting the cochlea's ability to produce it. In an effort to control for this, the last surgery was perfused at a slower rate (2.0uL/min). (With a slower perfusion rate samples were collected every 30 minutes. This longer sample time results in the necessity for the animal to remain viable for a minimum of 2.5 hours after the start of perfusion.

Another factor could have to do with the type and amount of inhibitor. Did we use the "right" inhibitors at sufficiently high concentrations? It may just be that in order to truly measure a noticeable increase in ROS production, that inhibition of protective agents for background ROS levels is not enough. It is possible that a cochlear insult needs to be introduced.

Another confound could be the possibility of cerebrospinal fluid (CSF) contamination. In the normal cochlea, spaces containing perilymph and CSF connect via the cochlear aqueduct. When the round window is opened during the surgery, perilymphatic spaces predominantly contain CSF, because pressure within the cranial cavity forces CSF into the cochlea. Due to the small size of mice, it is not currently feasible to block the cochlear aqueduct. We believed that the flow rate of artificial perilymph was fast enough to minimize the concentration of CSF. If a contamination had occurred, the result would be a diluted sample and decreased amounts of both salicylate and 2,3-DHBA. Since we did see a decrease in both salicylate and 2,3-DHBA over time it is possible that there was some CSF contaminating our samples. However, sample volumes were as predicted based on pump flow rate suggesting that any additional CSF volume was minimal.

Temperature measurements indicated that the cochlea was well below normal core temperature. The previous mice had been stabilized to core body temperatures usually not exceeding 37 degrees C, since that had seemed to keep them viable longer. However, to boost the cochlear temperature high enough in the final perfusion, the mouse's core temperature at times became elevated, at times in excess of 38.5 °C. If cochlear temp was low with a mouse that was being kept with a core temp close to 2 °C warmer than previous subjects were, it is quite possible that temperature played a role in previous results. All biochemical reactions are very temperature dependent. If the temperature is cooler than necessary, it is possible that the cooling would result in a reduced production of ROS.

Despite this experiment's inability to show increased levels of free radical formation from enzyme inhibiting drugs, it is well documented in the literature that they can be harmful in conjunction with cochlear insult, and lead to cochlear damage. Ford et al. (1997,) showed that cisplatin rapidly produced ototoxicity in the chinchilla, presumably via the generation of ROS. Additionally, hair cell destruction was noted at the basal end of the cochlea, and it is thought that cisplatin affects the antioxidant defense system, which would point to probable oxidative damage. Therefore, antioxidants could be a key player in the development of better defenses against ototoxic agents.

Had the data supported our predictions, it may have followed one of three theorized patterns. Perhaps, the drugs would have resulted in a steady increase in ROS production over time. Another possibility is that the ROS levels would have increased to a point of saturation, or even after a certain amount of time started to show a slight decrease due to depletion of iron in the cochlea. A graphical representation of theorized 2,3-DHBA levels over time according to our theories can be seen in figure 9. A bar graph of what the mean control vs. trial levels would look like for these three theories can be seen in figure 10. The differences between this graph based on theoretical data, and our actual numbers (Figure 6) are quite obvious.

It has already been shown that depletion of antioxidant enzymes in the cochlea via ototoxic drugs such as cisplatin can cause ABR threshold shifts. However, these negative effects can be blocked by a series of antioxidant compounds given in combination with cisplatin. It is theorized that protection of the cochlea from ototoxic injury can focus on several major mechanisms including prevention of ROS formation. (Rybak et al., 1999).

Several scientists have suggested use of gene therapy or free-radical scavengers to increase the amount of antioxidant enzymes in the cochlea. Perhaps, protective agents can be injected intratympanically to additionally protect against apoptosis and cell death. (Rybak et al., 1999). Additionally, administration of iron chelators could help to inhibit the Fenton Reaction, and render cochlear iron stores less reactive with H_2O_2 (Kopke et al., 1999). The future use of antioxidants and other agents to protect against currently incurable sensorineural hearing losses related to oxidative stress is quite promising.

Acknowledgements

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Figure Legends

Figure 1. Schematic drawing of ROS production, removal, and damage in the body. Shows the relationship between antioxidant protectants and cochlear insults in increasing and decreasing possibility for ROS damage.

Figure 2. Diagram explaining the biochemistry behind ROS and hydroxyl radical formation in an oxidation reduction reaction.

Figure 3. Raw chromatogram representing results of a control perfusion. Chromatogram compares peak area and retention times for 2,3-DHBA, 2,5-DHBA, and salicylate.

Figure 4. Raw chromatogram representing results from a trial perfusion. Chromatogram compares peak area and retention times for 2,3-DHBA, 2,5-DHBA, and salicylate. It is clear that the 2,3-DHBA peak is smaller for the trial than the control in figure 3.

Figure 5. Schematic drawing and explanation of the perfusion mechanism showing where how the perfusate was pumped into the cochlea and then collected for analysis.

Figure 6. Mean 2,3-DHBA measured in control runs vs. mean 2,3-DHBA (peak area) measured in trials for each of the five animals analyzed. There was no significant increase in the trials as compared to the controls, in fact there was a decrease.

Figure 7. Mean salicylate measured in control runs vs. mean salicylate (peak area) measured in trials for each of the five animals analyzed. Trends show a larger amount of salicylate in the controls for 4 out of 5 animals, though not significant.

Figure 8. Percent of 2,3-DHBA and salicylate in trials as compared to control perfusions. Percentages for both were smaller for trials with the exception of the % salicylate being greater for the trial than for the control in the last animal.

Figure 9. Hypothetical graph of the amount of 2,3-DHBA measured over time. Shows speculated trends for increased levels of 2,3-DHBA (either a steady increase, an increase with eventual saturation, or an increase followed by a plateau and eventual decrease.)

Figure 10. Mean 2,3-DHBA measured in control runs vs. mean 2,3-DHBA (peak area) measured in trials for each of the three theorized groups of data. An overall increase in 2,3-DHBA for all three theories is obviously different than the trends seen from our actual data in (Figure 6).

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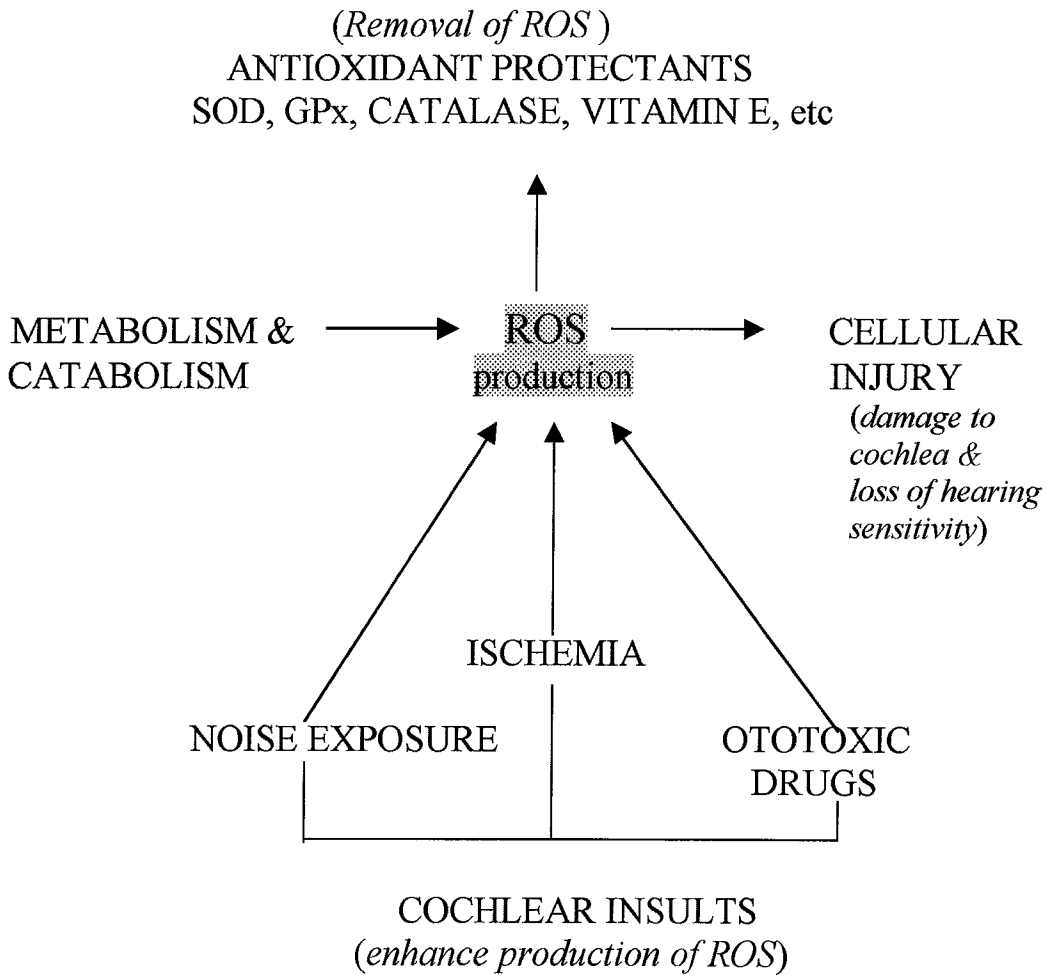
Figure 1



Explanation:

1. Construction (Metabolism) and Destruction (Catabolism) within cells is constantly happening within all systems in the body

2. ROS are a product of Metabolism and Catabolism

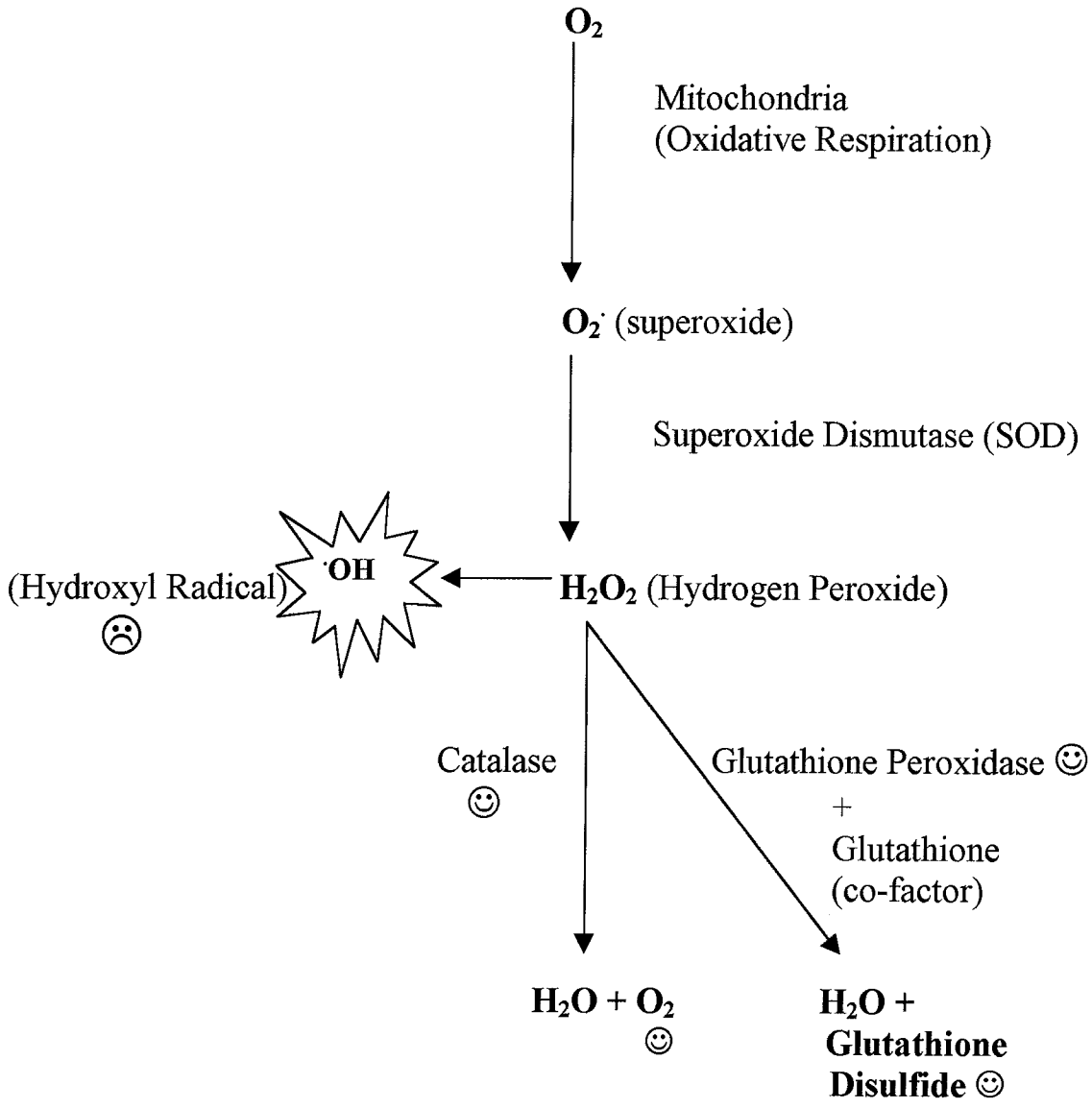
3. The body uses antioxidant protective measures to eliminate ROS, these protective agents include SOD, GPx, Catalase, Vitamin E, and others

4. Cochlear injury via noise exposure, ischemia, and ototoxic drug exposure can increase levels of ROS

5. If ROS are not eliminated, they can go on to cause cell injury and death leading to disease. When ROS are allowed to react in the cochlea, hearing sensitivity will decline.

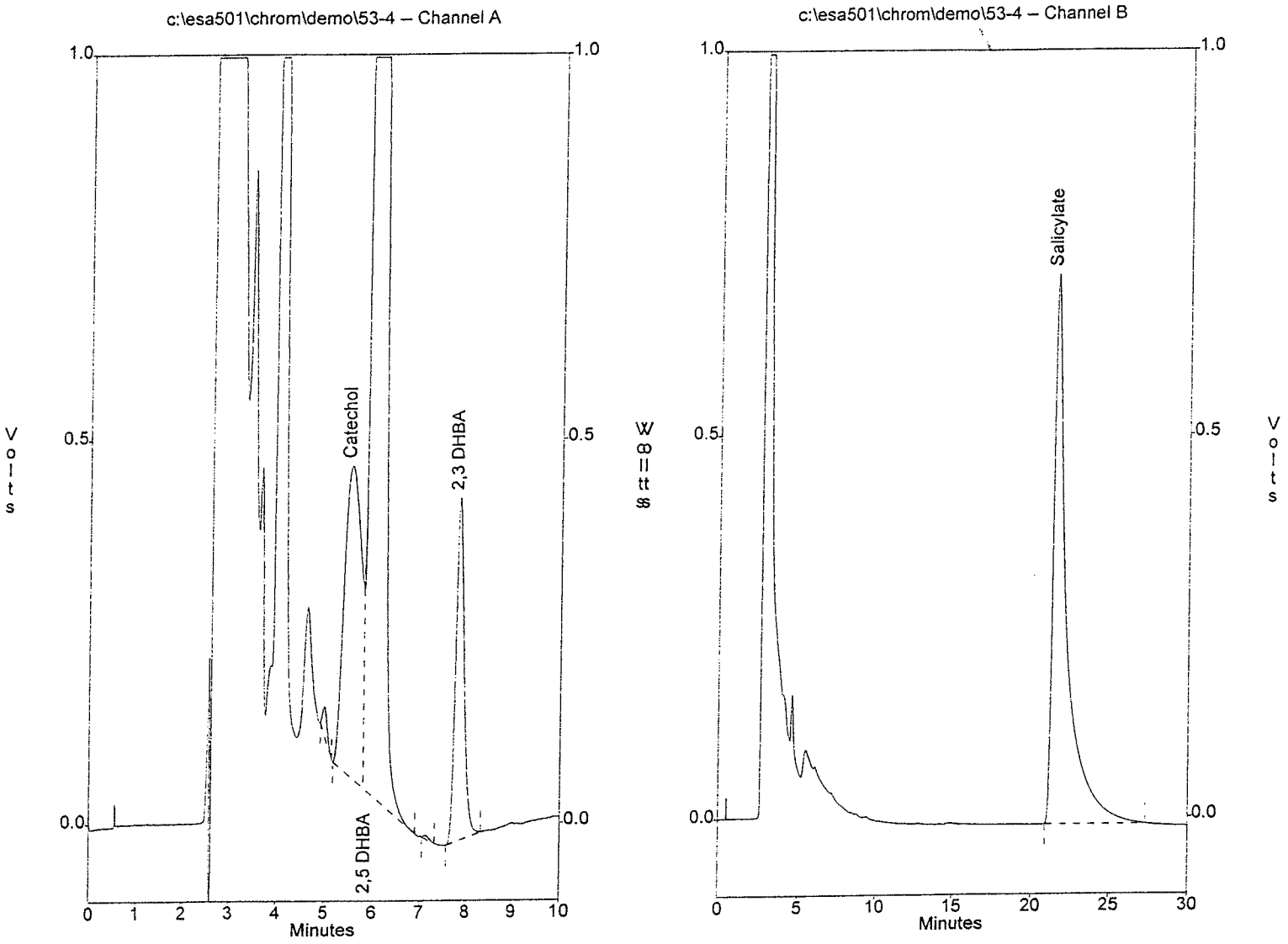
Figure 2

THE BIOCHEMISTRY OF FREE RADICAL FORMATION AND REDUCTION



CONTROL Figure 3

E1=250mV, R1=200nA; E2=750mV, R2=20uA
MP- 50mM NaAcetate, 50mM Citric Acid, 25% MeOH, 5% IsopropylOH (pH 2.5)
Flow 0.5 mL/min, 20uL injections
Sample ID :
Acquired : Feb 18, 2000 13:12:26



Channel A Results

#	Peak Name	Time	Peak Area	Area Percent	Peak Height
2	Catechol	5.55	9800730	23.9	405735
3	2,5 DHBA	5.91	24818962	60.5	954802
5	2,3 DHBA	7.84	5785044	14.1	446304

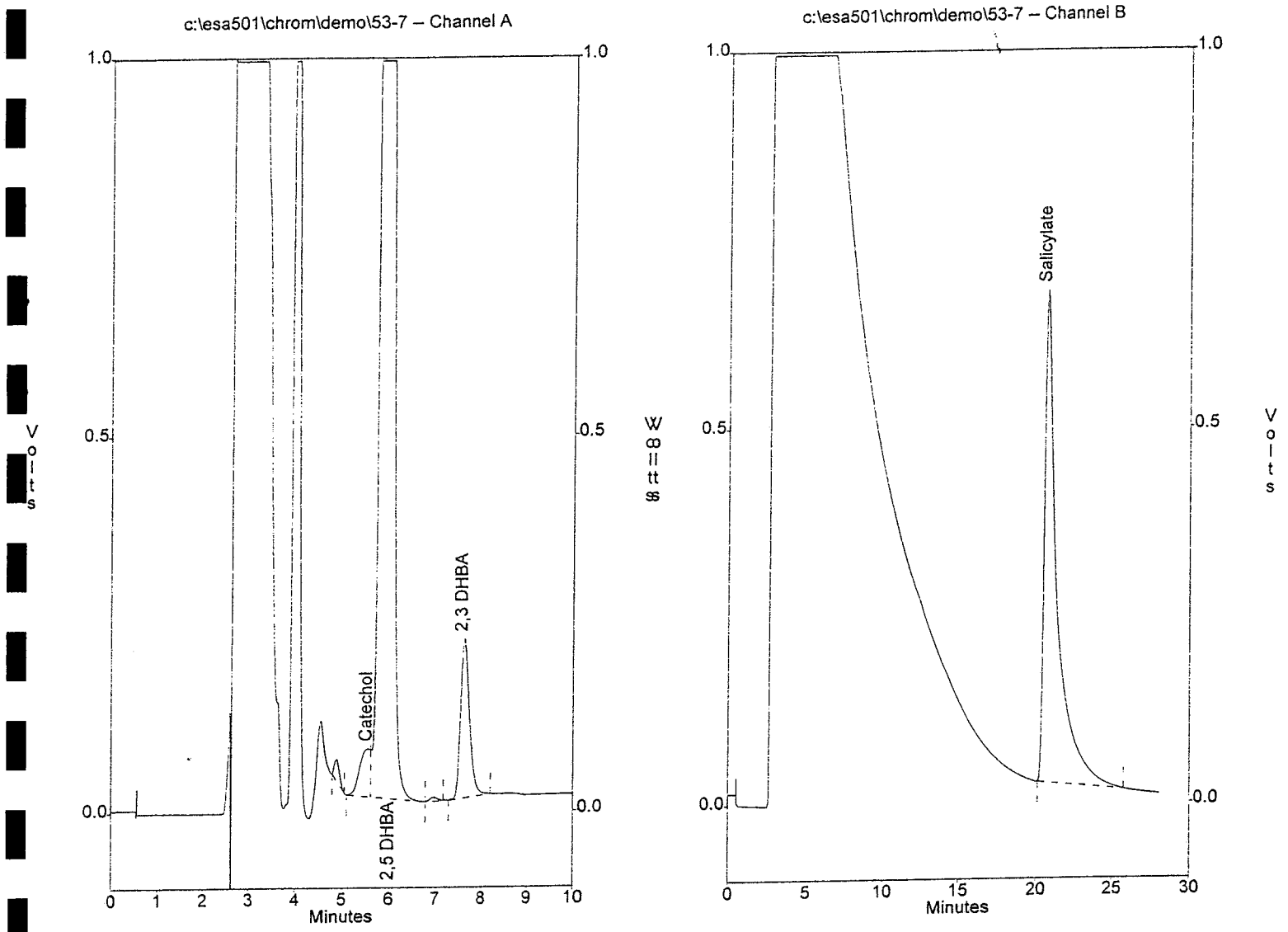
Channel B Results

#	Peak Name	Time	Peak Area	Area Percent	Peak Height
1	Salicylate	21.56	42527136	100.0	716446

DRVG

Figure 4

E1=250mV, R1=200nA; E2=750mV, R2=20uA
MP- 50mM NaAcetate, 50mM Citric Acid, 25% MeOH, 5% IsopropylOH (pH 2.5)
Flow 0.5 mL/min, 20uL injections
Sample ID :
Acquired : Feb 18, 2000 14:51:57



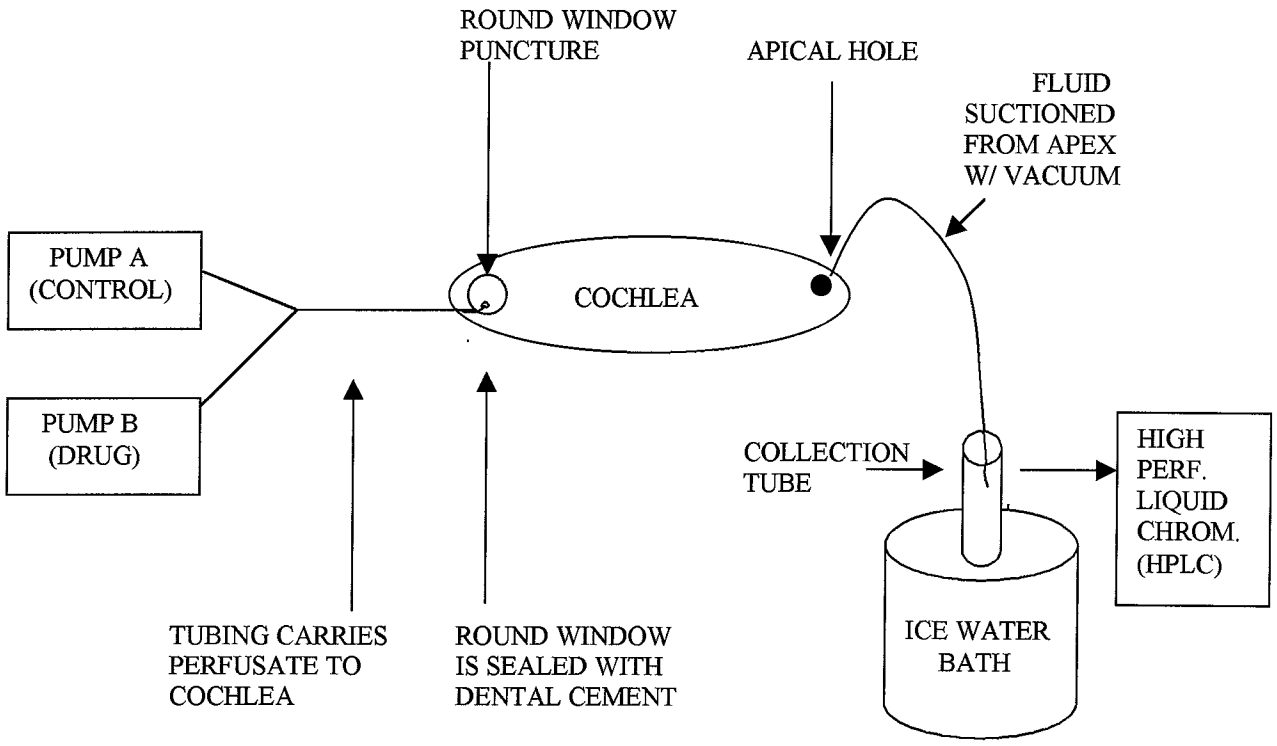
Channel A Results

#	Peak Name	Time	Peak Area	Area Percent	Peak Height
2	Catechol	5.58	984350	3.6	63605
3	2,5 DHBA	6.05	22836184	83.8	978234
5	2,3 DHBA	7.63	2846344	10.4	210710

Channel B Results

#	Peak Name	Time	Peak Area	Area Percent	Peak Height
1	Salicylate	20.73	38006764	100.0	654257

Figure 5



EXPLANATION:

1. Two pumps are side by side with different syringes in each, Pump A's syringe contains the control substance (artificial perilymph) and Pump B's syringe contains the drug solution (aminotriazol and mercaptosuccinate)

2. Perfusate runs through PE10 tubing that is joined by a "Y" conector such that tubing does not have to be changed when switching between pumps.

3. The tubing is placed into a puncture made in the round window and then the round window is sealed off with dental cement

4. Fluid is pumped via the tubing into the round window and then is collected from a hole made in the apex of the cochlea.

5. PE10 tubing collects the fluid exiting the apex , suctioning it with the use of a vacuum.

6. Samples are dripped into a collection tube placed in an ice water bath.

7. Samples are then analyzed for content using High Performance Liquid Chromatography (HPLC)

Figure 6

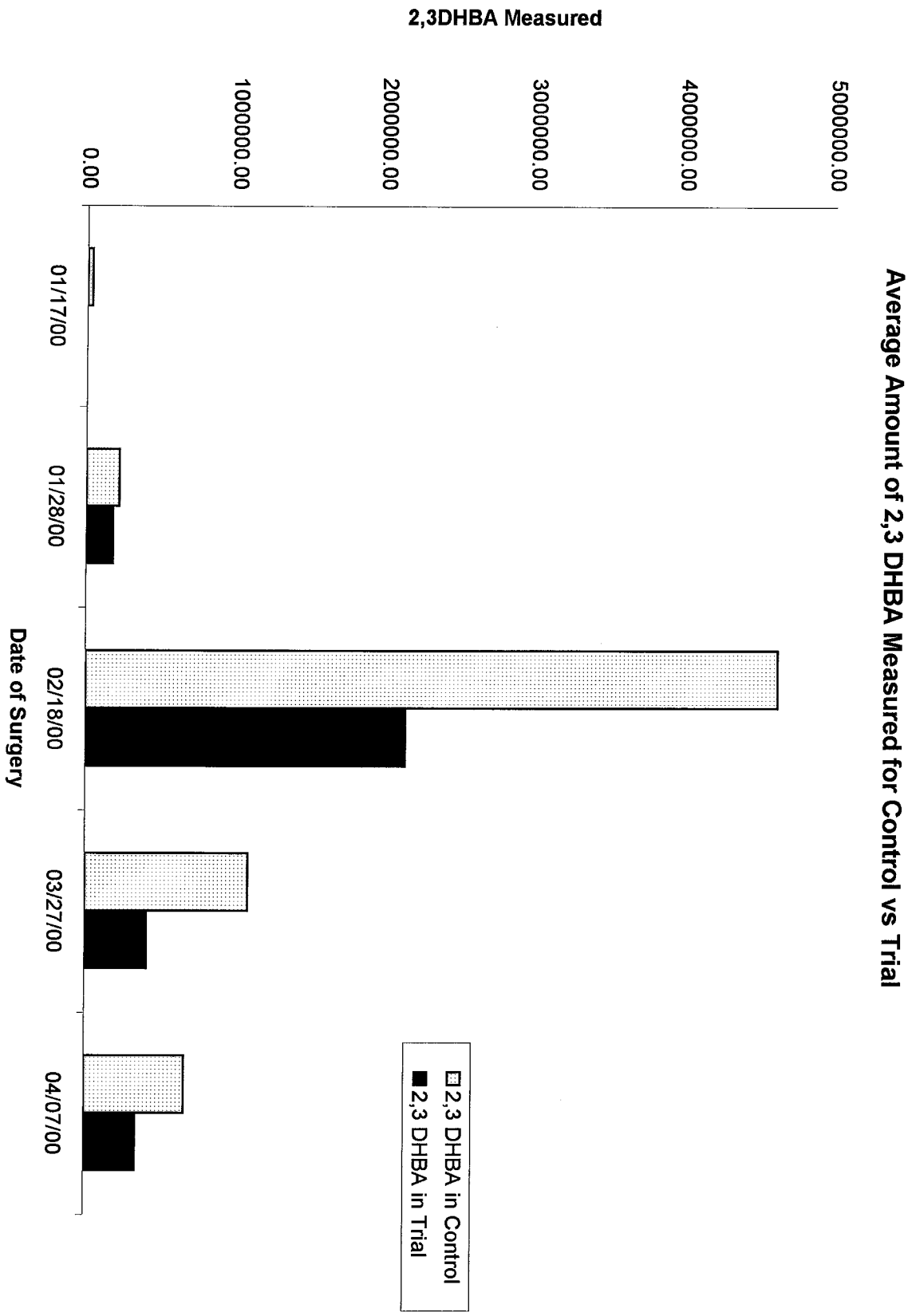

2,3DHBA Measured
Average Amount of 2,3 DHBA Measured for Control vs Trial
5000000.00
4000000.00
3000000.00
2000000.00
1000000.00
0.00
01/17/00
01/28/00
02/18/00
03/27/00
04/07/00
Date of Surgery
2,3 DHBA in Control
2,3 DHBA in Trial

Figure 7

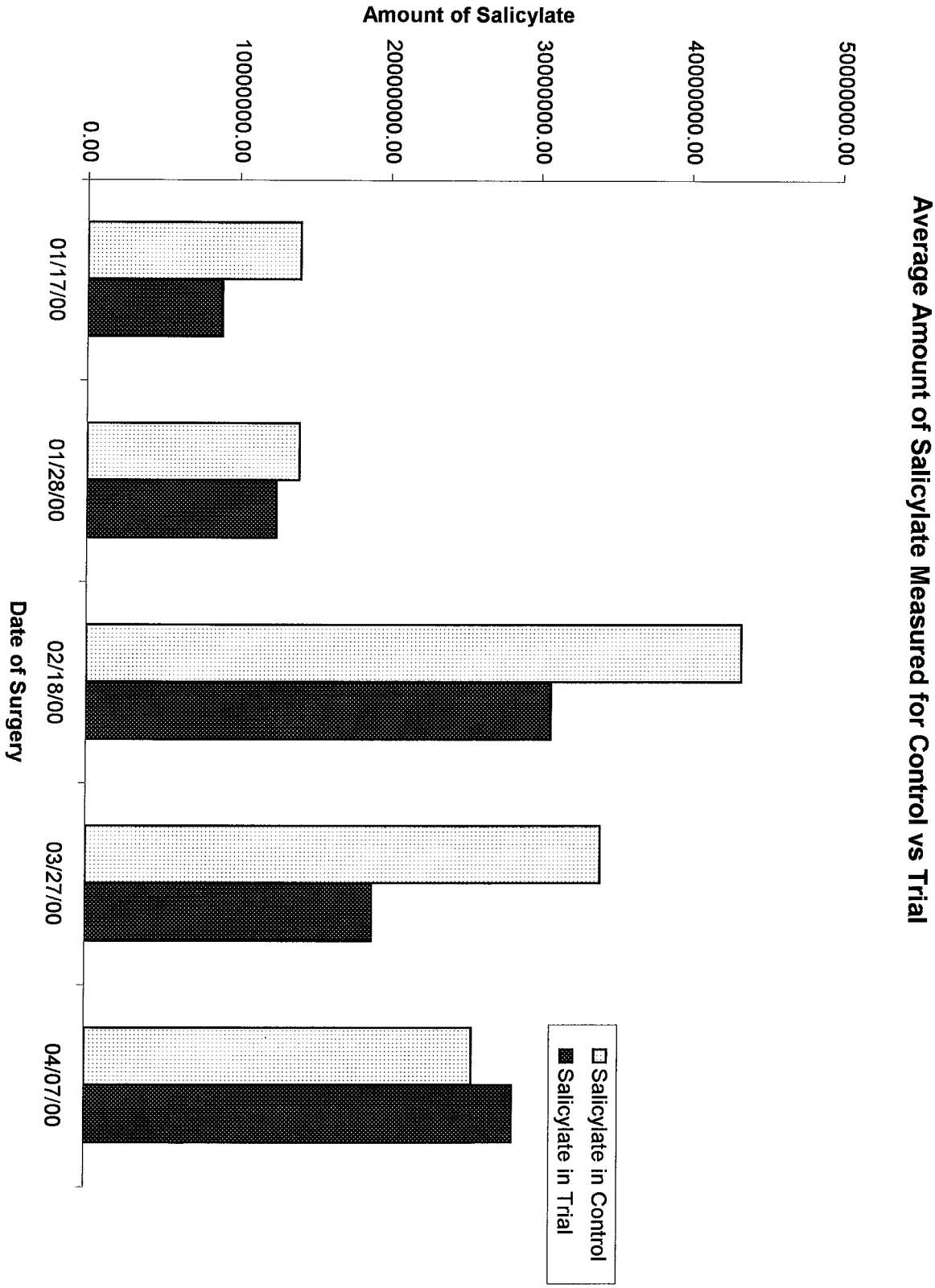

Average Amount of Salicylate Measured for Control vs Trial
Amount of Salicylate
0.00
10000000.00
20000000.00
30000000.00
40000000.00
50000000.00
01/17/00
01/28/00
02/18/00
03/27/00
04/07/00
Date of Surgery
Salicylate in Control
Salicylate in Trial

Figure 8

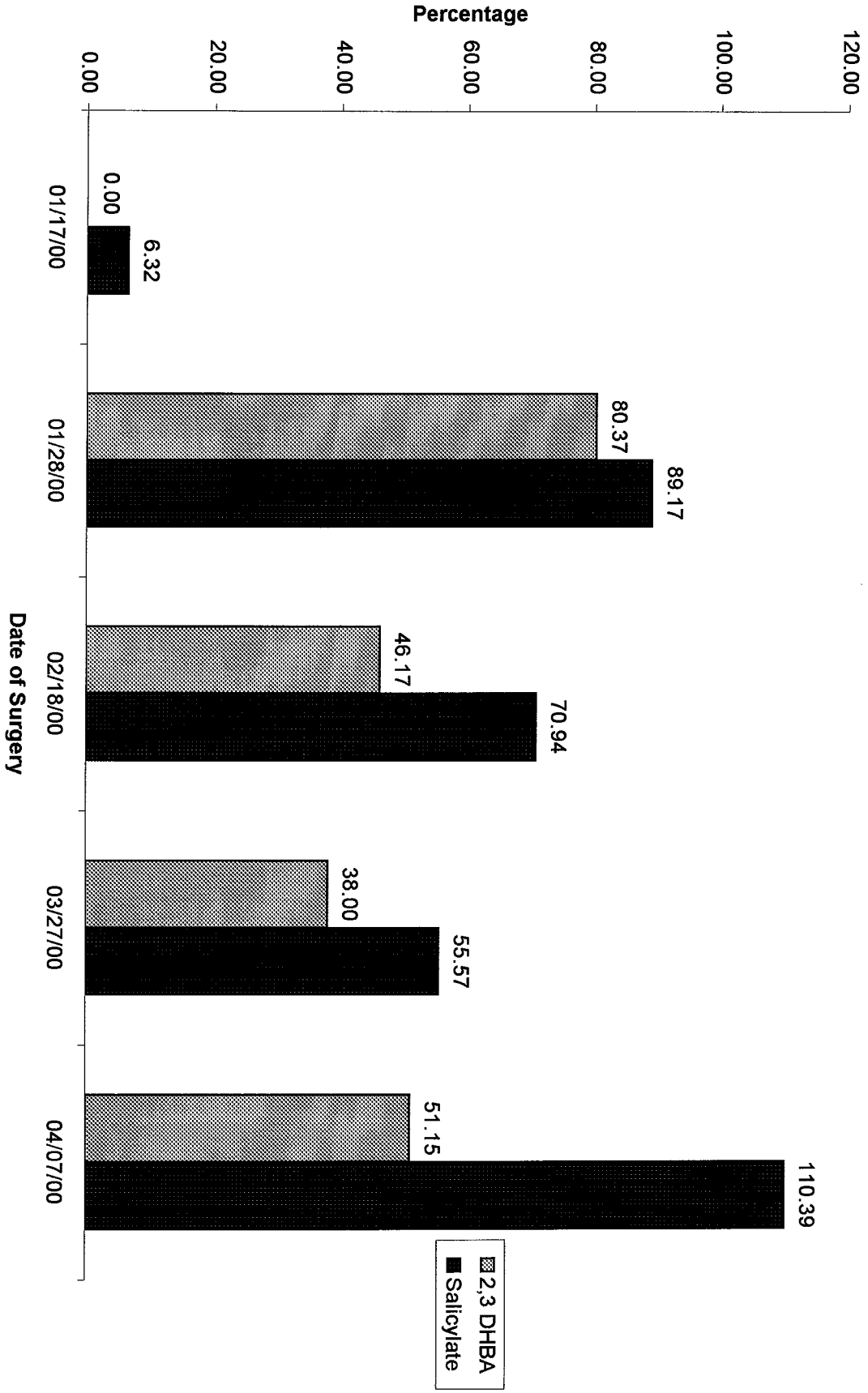

Percent of Control 2,3 DHBA and Salicylate Measured in Trials
Percentage
0.00
20.00
40.00
60.00
80.00
100.00
120.00
0.00
6.32
80.37
89.17
46.17
70.94
38.00
55.57
51.15
110.39
01/17/00
01/28/00
02/18/00
03/27/00
04/07/00
Date of Surgery
2,3 DHBA
Salicylate

Figure 9

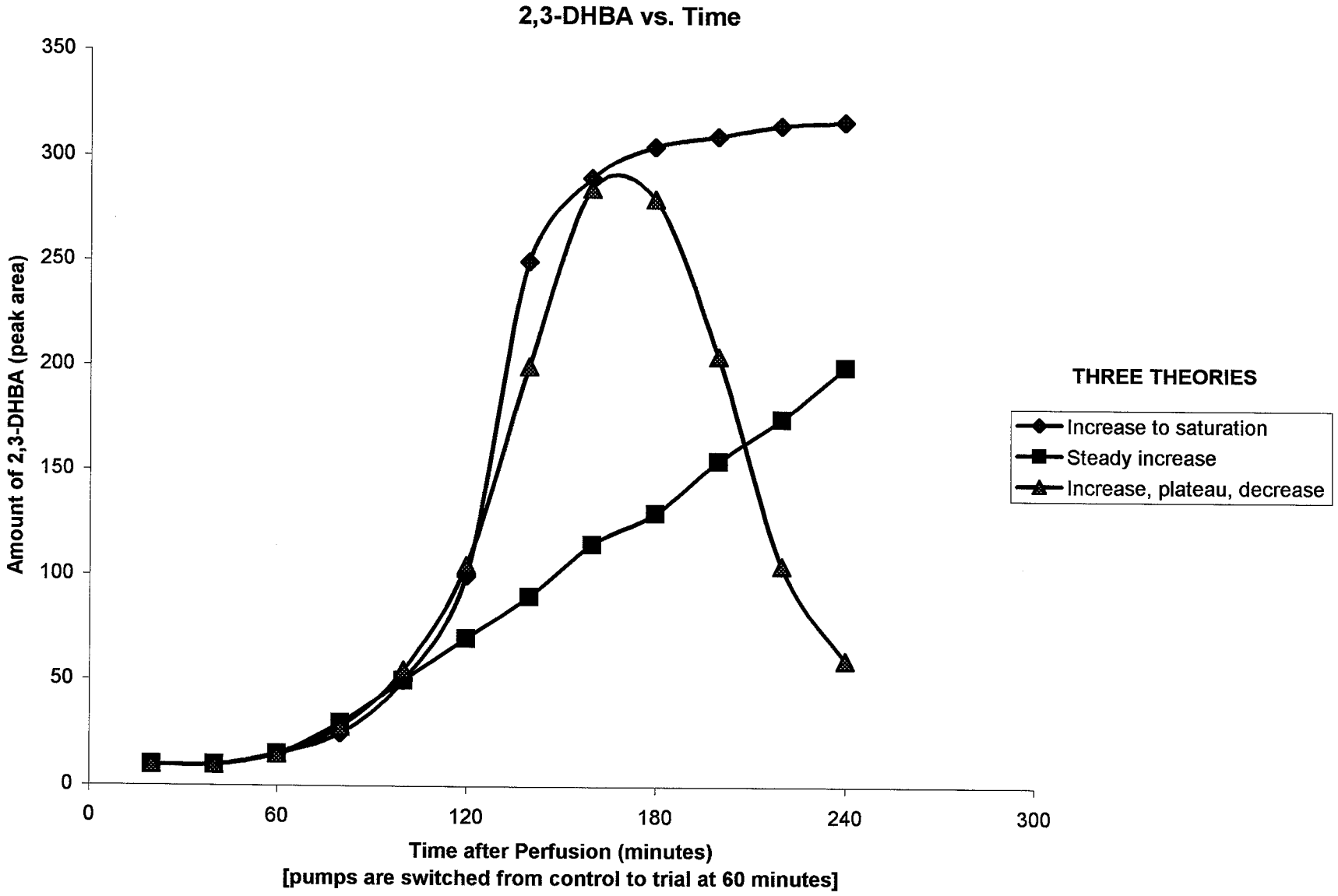
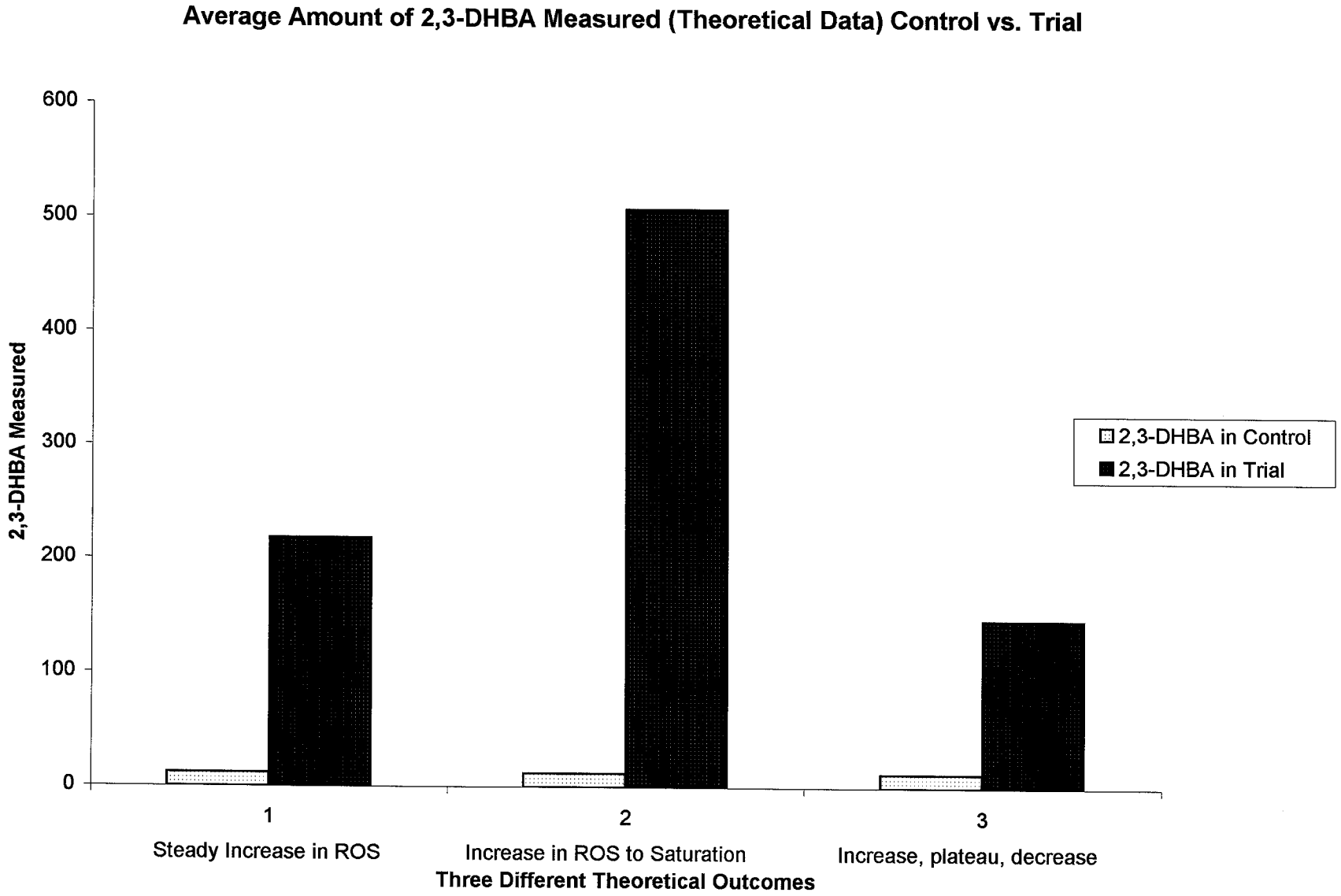

2,3-DHBA vs. Time
Amount of 2,3-DHBA (peak area)
350
300
250
200
150
100
50
0
0
60
120
180
240
300
Time after Perfusion (minutes)
[pumps are switched from control to trial at 60 minutes]
THREE THEORIES
Increase to saturation
Steady increase
Increase, plateau, decrease

Figure 10


Average Amount of 2,3-DHBA Measured (Theoretical Data) Control vs. Trial
2,3-DHBA Measured
600
500
400
300
200
100
0
2,3-DHBA in Control
2,3-DHBA in Trial
1
2
3
Steady Increase in ROS
Increase in ROS to Saturation
Increase, plateau, decrease
Three Different Theoretical Outcomes