METAL IONS RELEASED FROM COPPER BASED ALLOYS USED IN DENTISTRY AND THEIR EFFECTS ON CELLS

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HASAN HESHMAT

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ABSTRACT

Copper-based alloys have been used as an alternative to gold based alloys for dental crown and bridge applications. Their popularity is mainly due to their bright gold-like appearance and a lower cost when compared to alloys made from noble metals. Release of metal ions (Cu^{2+} and Ni^{2+}) and microparticles which may induce inflammation of the adjacent periodontal tissues and the oral mucosa has been documented in both *in vitro* and *in vivo* studies involving metallic dental devices. The primary aim of this research project was to elucidate the release of metal ions from metallic dental alloys in a simulated oral environment.

Discs of copper-based dental alloy (NPG^{TM+2}) with the following nominal composition [in wt %] copper 77.3: aluminium 7.8: nickel 4.3: iron 3.0: zinc 2.7: gold 2.0 and manganese 1.7 and NPG^{TM} alloy (without gold) were obtained from Dentech Dental, London, UK. Specimens of NPG^{TM+2} and NPG^{TM} alloys (32 mm × 10 mm × 1.5 mm) were totally immersed in 10 mL of sterile simulated saliva at both neutral (pH 7.0) and acidic pH (pH 4.0) (acidic milieu simulating the condition of plaque build-up in inter-proximal space between adjacent teeth). The tubes were sealed and incubated at 37^{0}C for 7 days with agitation. Saliva extracts were analysed for metal ion concentrations (Cu, Ni, Zn, Al and Mn) using Inductively coupled plasma mass spectrometry (ICP-MS)..

Metal ion release from both alloys in simulated saliva at pH 4.0 was significantly higher (for all the metals, and at all incubation times) when compared to the levels observed at pH 7.0 (2 sample T-test $p \leq 0.05$). The extent of corrosion was observed at both pHs 7.0 and 4.0 for the NPG^{TM} alloy, at incubation times 1, 3, 5 and 6 days. As would be expected from the composition, Cu ions were released to the greatest extent on corrosion. The greatest increase in corrosion, comparing pHs 7.0 and 4.0, was measured with Ni (200 fold increase), followed by Mn (90 fold increase) and Zn (30 fold increase). Au in NPG^{TM+2} showed no significant difference in ion release observed in the extent of corrosion between the two alloys at either pH 4.0 or 7.0.

In the *in vitro* study the potential toxicity of a mixture of 79 μM Cu, 25 μM Mn, 15 μM Al, 47 μM Ni and 12 μM Zn to immortalised mouse fibroblast (3T3 cell line) was

investigated over 4 weeks. The composition of this mixture was equivalent to the maximal ion release from the alloys. The following parameters were measured Lactate Dehydrogenase (LDH), total cell protein content using Lowry assay, activities and expression of glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT) and superoxide peroxidase (SOD).

Oxidation stress induced by incubating 3T3 cells in metal extracts media revealed upregulation of CAT compared to controls. Differences between control and metal treated cells showed transient decrease for GR, SOD and GPx. Marked decrease observed for GR at week 2, in contrast, CAT shown transient increase at week 2.

This study suggests that at least at the short term and despite considerable metal ion release from the two dental alloys, this did not lead to overt toxicity.

LIST OF ABBREVIATIONS

AB	Antibody
ADA	American Dental Association
ALS-	Amyotrophic Lateral Sclerosis
APS	Ammonium persulphate
CAT	Catalase
Cyt-C	Cytochrome C
dH2O	Distilled water
DMF	Dimethyl fluoride
EDTA	Ethylenediaminetetraacetic acid
GIT	Gastrointestinal tract
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
KOH	Potassium hydroxide
KCN	Potassium cyanide
LDH	Lactate Dehydrogenase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NR	Neutral Red
PPM	Parts per million
PVDF	Polyvinylidene fluoride membrane
Rh	Rhodium
ROS	Reactive oxygen species
RPM	Rotations per minute
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SS	Simulated saliva

SOD	Superoxide dismutase
SOD I	Superoxide dismutase I
SODII	Superoxide dismutase II
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
TNF	Tumor Necrosis Factor
Trx	Thioredoxin
TTBS	Tween tris-buffered saline
XO	Xanthine oxidase

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1. CHAPTER I: INTRODUCTION

1.1 Dental Alloy Composition

500 B.C. is the earliest known time when dental materials have been used. Etruscans used gold to fabricate the very first dental bridges. 2000 years later, when those bridges were discovered they were still gold coloured and free of corrosion. However, the first dental amalgam “Silver Filling” was created in France in the 1800s. The technology found its path to the United States of America in 1830 (Ferracane, 2001) . Since then there has been a huge rise in the use of base metal alloys (alloys with noble metal content of less than 25% w/w) in dentistry, and particularly in the past two decades as a result of the considerable increase in the price of gold. Copper-based alloys with copper concentrations as high as 87% and varying alloying additions of Al, Ni and Zn have been used as an alternative to the gold based alloys for restorative dentistry. For example, dental amalgam is basically an alloy consisting of Ag, Sn, Zn, Hg and Cu. Dental amalgam is defined as an alloy of mercury, and in the 1960s, it was determined that increasing the amount of Cu in the alloy produced an amalgam with improved clinical properties.

These High-Copper Amalgams (HCAs) were produced by mixing Hg with Ag powder of approximately 40-60%, and adding Sn 27% and Cu 13-30%. Most HCAs require less mercury. In contrast, Low-Copper-Amalgams (LCAs) contain only 4-5% copper and their corrosion resistance is inferior to high copper ones. When Hg is mixed with the amalgam alloy, Ag, Cu and Sn metal ions dissolve from the outer layers of the particles into the Hg. A chemical reaction takes place between Ag and Hg which forms the crystalline matrix of the amalgam called gamma-1, which occupies 40% of its total volume. Gamma-1 phase is more pronounced in HCA than in LCA where a Sn-Hg compound is formed and called Gamma-2. This represents the weak phase of the amalgam most susceptible to corrosion. In contrast, in HCA tin reacts with Cu instead of Hg to form Sn-Cu compounds that eliminate the gamma-2 phase, hence it has a better clinical performance (Ferracane, 2001). HCAs are preferred, as there is minimal marginal breakdown and corrosion in the oral cavity which is induced by the gamma-2 Sn-Ag present in LCAs (Scheller-Sheridan, 2010).

Cu based alloys are widely implemented in Full-Metal-Crown (FMC) and bridgework, onlays, posts /cores and dental prosthetic applications (Braemer, 2001).

Conventional dental restorative amalgam contains 68% Ag, 28% Sn, 4% Cu and 0-2% Zn. However, the high-copper dental restorations are now in favour over this conventional one. HCAs contain 60% Ag, 27% Sn, 13% Cu and 0% Zn. Cu is added to decrease the gamma-2 phase thus strengthening the alloy and minimizing corrosion (Mitchell *et al*., 2010) .

A palladium-gold-copper-gallium based alloy is frequently used in dental crown bridge and frameworks; the alloy is composed of 2% Au, 78% Pd, 10.5% Cu and 9.47% Ga (Rushforth, 2004). Generally, in dental alloys there are 4 categories to classify dental alloy used especially in inlays. Type I, II, III and IV alloys, basically, they are numbered according to their hardness with type I being the softest and IV is the hardest. Alloy specifications incorporate Cu, Ag and Zn in about 25% of the alloy, with the remaining 75% usually of noble alloys. (Table 1.1).

Types	I	II	III	IV
Copper	5 %	10 %	11 %	16 %
Silver	10 %	15 %	26 %	20 %
Zinc	-	1 %	1 %	2 %
Platinum	-	1 %	3 %	4 %
Palladium	-	4 %	4 %	6 %

Table 1.1: Dental Alloys types and related metal content percentage in each category.

Type I alloy contains only Cu and Ag; whereas type II alloy is the material of choice in dental inlay. Type III is widely used in crown-bridgeworks, extra-hard Alloy IV, previously was used in casting denture-frameworks, but recently it has been limited to clasp construction in partial dentures. In resin-bonded bridges Cu material is not being used wherever a porcelain facing is required as Cu tends to change the normal color of porcelain to green (Brown, 1988).

Another category of dental alloys is based on Au content. These are also called High-Gold-Alloys (HGA) “Specification Alloys”. Due to economic forces, Medium and Low-Gold Alloys (MGA and LGA) came to replace the HGAs. In medium, LGA it is

easier to reproduce the hardness levels of HGAs; however, this is at the expense of tarnish resistance which is inferior in the MGA, LGA.

In the United Kingdom to control the proliferation of the inferior alloys, these MGA, LGA should comply with the standard BS6042:1981, which describes the MGA as semi-precious and requires that they contain at least 30% of noble metal (Pt, Pd and Au), a maximum of 20% base metals (Cu and Zn) while the rest is Ag. They are generally called silver-palladium-gold-copper alloys.

In the United States, the American Dental Association (ADA) has an alternative classification for dental alloys; high-noble alloys involve alloys which contain 60% noble metals and at least 40% Au. Those of 25% Au, Pt or Pd are called Noble, and the predominantly base alloys are those containing less than 25% noble metals.

Noble alloys are really silver-palladium alloys; such alloys are called white-gold alloys and formerly have been used containing 30-45% Ag, 20-25% Pd, 15-30% Au, 15-20% Cu and 1% Zn. Recently used the White-Gold Alloys (WGA) or modern WGA used may contain as little as 2% Au together with base metals like Sn and In (Brown, 1988).

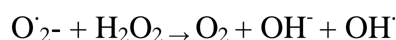
1.2 Toxicity Problems with Copper Containing Alloys

Copper containing materials might be able to produce DNA damage; human lung epithelial cell line A549 was exposed to the copper ions and cytotoxicity was measured by using trypan blue dye exclusion. At nanoparticle level, Copper oxide (CuO) was found to induce a considerable impact on DNA. At a concentration of 40μg/cm^2 DNA damage of about 40% of cell line A549 has been noticed for *in vitro* toxicity of CuO nanoparticles following 24 and 42 hours of exposure (Karlsson *et al.*, 2008). There is a potential risk of increased toxicity of Cu and Fe in the aging human population. In the development of the oxidative energy generation Cu and Fe redox have been used. These two elements contribute to the production of damaging oxidant radicals. Cu and Fe are essential elements for human vitality; our metabolic system maintains excessive storage of these two metals with reproductive life. Therefore with

aging, the oxidation damage may result from excess store increase, after age 50, since diseases at this stage do not contribute to the reproductive system; the natural selection of maintaining these metals ceases to act, this may lead to development of Alzheimer's disease; other neurodegenerative diseases, diabetes mellitus and arteriosclerosis (Brewer, 2009). Copper ions interact with amyloid β-peptides, the affinity determined at value 10^{10} M^{-1} range, and this is implicated in the neurotoxicity cascades of Alzheimer's disease (Hollenberg, 2010).

The metals (Cu, Ni) *in vivo* undergo redox cycling, which induces DNA damage by yielding oxygen species or metal ions (Bal and Kasprzak, 2002).

The mechanism of oxygen activation by Cu and Ni metal ions is provided by Fenton/Haber–Weiss and autoxidation reactions. It was shown that the interaction of hydrogen peroxide (H_2O_2) with superoxide ($O^{\cdot}_2-$) may result in generating reactive hydroxyl radical ($OH^{\cdot}$). The $OH^{\cdot}$ is normally produced by our biological system, as indicated in the Haber – Weiss reaction:



$$O^{\cdot}_2- + H_2O_2 \rightarrow O_2 + OH^- + OH^{\cdot}$$

This reaction may produce toxic radicals from less reactive $O^{\cdot}_2-$ and H_2O_2 that could be generated enzymatically in the biological system. Such reaction is unfavorable thermodynamically, having a second order rate constant in aqueous solution. To catalyse the reaction, metal ions are required to generate $OH^{\cdot}$ (Equation 3). The hydroxyl radical can be broken down into (Equation (1) and (2)). Different metal ions can catalyse this reaction, however, iron-catalysed Haber-Weiss reactions which utilise the Fenton Chemistry is the major mechanism by which biological systems generate highly reactive OH· radicals (Kehrer, 2000).

$$\mathbf{Fe^{3+} + O^{\cdot}_2- \rightarrow Fe^{2+} + O_2} \text{ ---------------------------------- } \mathbf{(Equation\ 1)}$$

$$\mathbf{Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}} \text{ ----------------------- } \mathbf{(Equation\ 2)}$$

$$\mathbf{O^{\cdot}_2- + H_2O_2 \rightarrow O_2 + OH^- + OH^{\cdot}} \text{ ------------------------- } \mathbf{(Equation\ 3)}$$

The most abundant molecule in a biological system is O_2. Oxygen exists as a di-radical, and therefore reacts extremely swiftly with other radicals. O_2 itself is often the source of such radicals, as partially reduced species are generated through normal metabolic

processes and some of these reactive species can escape. As a result prominent toxic intermediates appear; these are the reactive oxygen species (ROS) are involved in ‘oxidative stress’ (Kehrer, 2000). ROS has important role in haemostasis and cell signaling equally can induce structural damage when increased dramatically (Devasagayam *et al*., 2004).

Mitochondrial ROS involve a non-heme iron protein that transfers electrons to O_2. This occurs primarily at complex I and, to a lesser extent, following the autoxidation of coenzyme Q from the complex II and: or complex III sites (Figure.1). Once again, the exact contribution of each site to total mitochondrial ROS production is probably determined by local conditions including chemical or physical damage to the mitochondria, O_2 availability and the presence of xenobiotics (Kehrer, 2000).

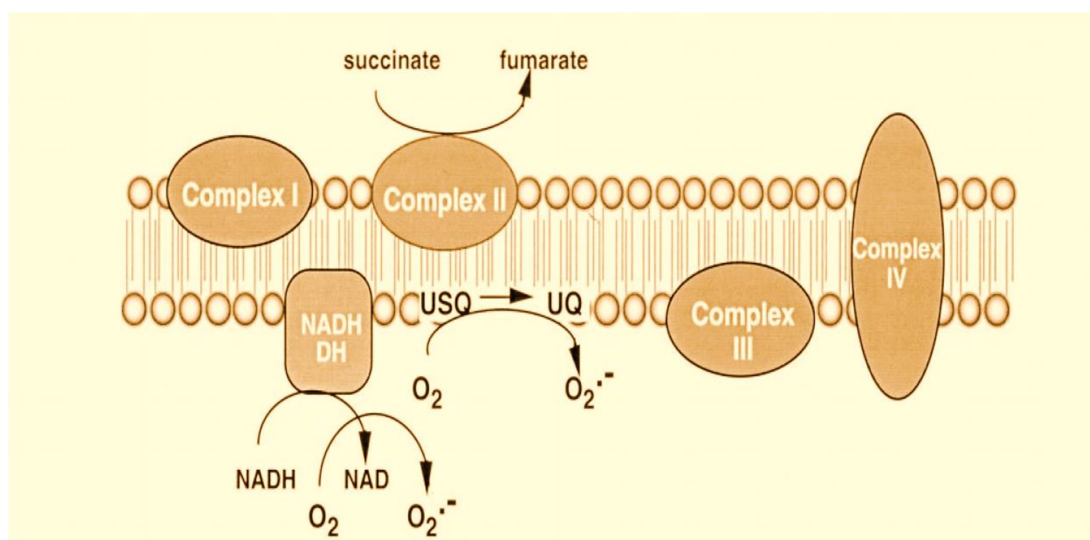


Figure 1.1: Mitochondrial ROS production sites. Donation of an electron to oxygen from NADH dehydrogenase at complex I can result in superoxide formation. Superoxide and/or hydrogen peroxide may also be formed following the autoxidation of reduced ubiquinone at Complex II (succinate-coenzyme Q) and/or Complex III (coenzyme QH2 - cytochrome c reductases) sites.

It could be difficult to differentiate between the free and metal-bound-oxygen radical species. Every case is specific and requires elaborate studies. For example, it has been established that metal-bound oxygen species are formed only in the reaction of Cu(I) aqua ion with H_2O_2 whilst the EDTA complex of Fe(II) catalyses H_2O_2 decomposition to free OH radicals (Bal and Kasprzak, 2002).

In an i*n vitro* experiment (Dreher *et al*., 1997) have used samples of 100 mM phosphate buffers, pH 7.4, and ambient O_2 were used, and incubated at 37°C for 16-24 hours. This has shown, Cu and Ni ions complex equally, promoted double-strand scission by hydrogen peroxide in calf thymus DNA.

Metal ions released from fixed dental prosthetics (dentures) showed a significant cytotoxic effect on a cellular level. Salt solution of Zn, Cu, Ni Pd, Ag, Cr, Mo, Be, Fe, Al, and K were exposed to, cultured mouse fibroblasts L-929. The percentage of viable cells for each metal or element was measured by implementing a trypan blue dye exclusion assay. Zn and Cu released from gold alloys, and Ni released from nickel–chromium alloys showed high evidence of cytotoxic activity on L-929 cell culture (San Miguel *et al*., 2013).

In synthesised terminally blocked peptides imitating the N-terminal histone-fold domain and C-terminal tail of histone H2B, Cu ions may bind with histone H2B (32-62) and (94-125) fragments, which may interfere with the structure of nucleosome and dynamics this includes the ubiquitination of lysosome 120 associated with silencing of gene (Zavitsanos *et al*., 2011).

Epidemiological studies suggest that paternal exposures to metal ions may potentiate the risk of cancer in the progeny. This may be concomitant with promutagenic damage to the spermatic DNA. Ni ion alone was found to stimulate oxidative DNA strand scission mainly single-strand breakdown and base damage, Cu ion alone produced similar effects, to a much greater extent. Both Ni and Cu metals were relatively more damaging to the pyrimidine bases than to purine bases (Liang *et al*., 1999).

Metal ions, which are released from alloys used in dental restorations, can incite local or systemic allergic or inflammatory reactions. A case has been reported of an individual who suffered from an immunoglobulin A nephropathy following placement of Ni alloy base crowns. Analysis of 139 published cases illustrating the allergic reaction to dental alloy metallic restorations has showed that patients developed local irritations primarily in the form of gingivitis and oral mucosal stomatitis (Doctors, 2004).

Another group extracted dental cast alloys into cell-culture media measured metal release, compared with the cytotoxicity of prepared metal salt solutions based on the metal ions measured in extracts. The metals Cu, Zn, Ni, and Ag were detected in extracts and Cu (+ve Control) using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). The extracted medium samples of alloys were non-toxic in mouse fibroblasts (L929 cells). The amounts of metal ions analysed in these extracts were weakly cytotoxic when tested as salt solutions prepared from chloride (Cu^{2+}, Zn^{2+}, Ni^{2+}) or sulphate (Ag^{1+}) salts. The concentrations of leaching metal elements that caused 50% cell death (TC50) were slightly less in analogous salt-solutions compared to those in the extracts. Cytotoxicity of extracts obtained from medium samples consistently proved to be slightly lower than that of the corresponding salt solutions (Schmalz *et al*., 1998).

Metal alloys used in dentistry contributed to a number of intraoral conditions such as gingivitis, tongue anomalies as in Lingua Plicata or Lingua Geographic also called Fissured Tongue (Figure 1.1); gingival discoloration, palate or tongue redness as well as oral mucosal lichenoid may develop in oral tissues adjacent to dental metal alloys (Garhammer *et al*., 2001).

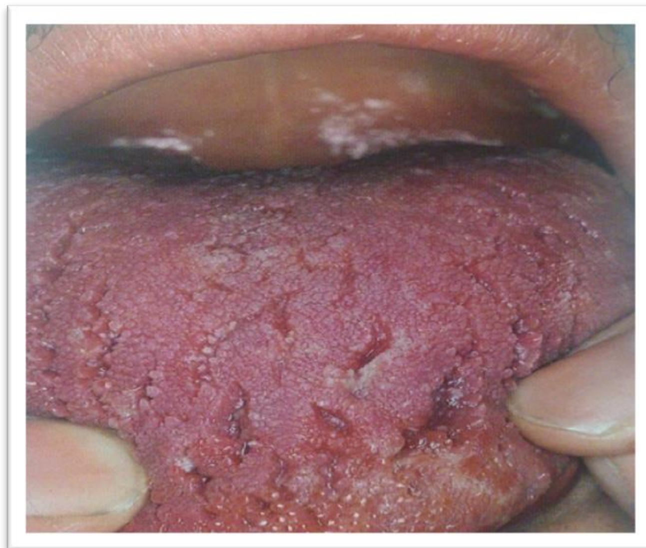


Figure 1.2: Fissured Tongue (Scully and Cawson, 2008).

In Fissured Tongue there are multiple numbers of fissures associated commonly with erythema migrans of no apparent consequence, nevertheless, the tongue is sore sometimes for idiopathic reasons (Crispian and Cawson, 1999).

The metal contents of dental alloys can accumulate in the adjacent oral tissue. The majority of cases revealed the presence of one or more of the dental casting alloys in the nearby oral tissue (Garhammer *et al*., 2003).

Using a mouse fibroblast cell line and the agar overlay test, using a MTT assay and millipore filter test, cytotoxicity of various metals, metal alloys, and ceramics for dental restoration were examined. Modification in the composition and the pretreatment handling of dental alloys can significantly control the metal cytotoxicity. The release of Cu and Zn ions seem to play a major role in development of the cytotoxic effect (Sjogren *et al*., 2000).

Exposure of (HepG2) human hepatoma cells to Ni ions induced stimulation of the Serine/threonine-protein-kinases (Ser/Thr kinase Akt) in a Phosphatidylinositol-3-Kinase- (PI3K) fashion, activation most likely being independent of oxidative processes. In contrast to the Cu ions, nickel-induced Akt activation is not propagated further downstream to FoxO-dependent signaling beyond the phosphorylation of FoxO1a and 3a. Cu ions were over 100 fold more toxic to cells than Ni, as shown from analyses of colony forming abilities. In spite of this absence of oxidative and cytotoxic action, exposure of human HepG2 cells to Ni^{2+} resulted in a significant activation of Akt that was abrogated by inhibitors of (PI3K) (Eckers *et al*., 2009) the cytoxicity of pure metals and their effect on murine fibroblasts (L929 cell line) and murine osteoblastic cells (MC3T3-E1) was observed to be greatly dependent on the concentration and the chemical state in the extracts. Anions such as chloride, nitrate, or sulphate ions are found to induce relatively smaller cytotoxic effect than those of metal cations (Takeda *et al*., 1989).

1.3 Metal alloys selected for the present study

The chosen dental alloys for study are NPG and NPG^{+2}. NPG is a non-precious alloy which consists primarily of 6 types of metals, and is yellow-gold coloured (Figure 1.3). NPG^{+2} classified as a base-metal alloy, contains a noble metal (Au) within its composition, and it involves 6 other metals, NPG^{+2} is yellow-gold coloured (Figure 1.4). Table 1.2 shows the composition of both alloys.

Alloy Type	Au	Cu%	Al%	Fe%	Zn%	Mn%	Ni%	Density g/cm^3	Melting Point °C
NPG	-	80.7	7.8	3	2.7	1.7	4.3	7.8	1,012-1,068
NPG^{+2}	2	78.7	7.8	3	2.7	1.7	4.3	7.8	1,012-1,068

Table 1.2: Dental Alloys NPG and NPG^{+2} Metal Composition. Technicare Dental Supplies Ltd, 1999, Alloys. [Online] Available at: <http://technicare-dental.co.uk/ALLOYS-c-32.html> [Accessed 15th October 2010].

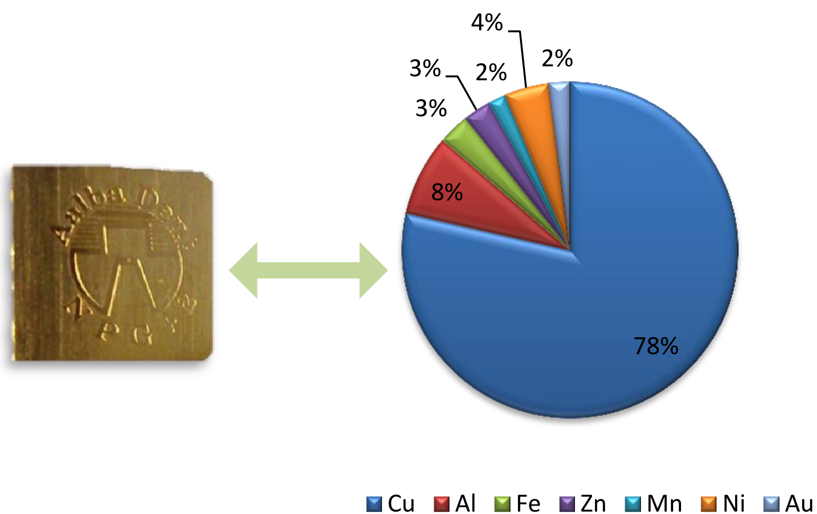


Figure 1.3: NPG^{+2} dental alloy composition, 7 metals are incorporated in this alloy including Au which forms 2%.

NPG alloy Composition

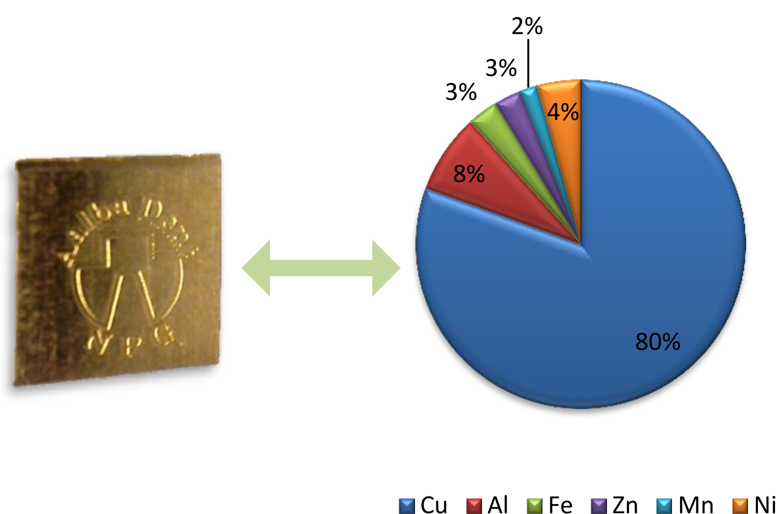


Figure 1.4: NPG dental alloy composition, 6 metals are incorporated in this alloy. It does not contain gold.

Both metals are widely used within the dental practice, especially in construction of dental crowns, short-span partial dentures, full veneer crowns, onlays, saddles and bridgework. These alloys are considered type III alloys classified as high-strength casting used in dental treatments and they provide considerable level of metal strength to withstand the mastication forces. Composition of the alloy should be within 0.5 % of the weight reported by the manufacturer or 0.1 % of the weight if selected as a hazardous element by National Institute for Occupational Safety and Health or the World Health Organization (WHO). Mechanical properties are assessed by measuring yield strength in megapascals, elongation percentage and hardness (JADA, 2002). NPG and NPG^{+2} alloys yield strengths of 265 MPa and 286 MPa, respectively (Dental, 2010).

1.4 Release of metal ions from Alloys

An in vitro study has showed that following 1, 2, 3, 4, 5, 6, 7, 14, 21 and 30 days of exposure of high-noble gold-platinum alloy samples to pH 6 phosphate buffer, metal ions leached at various rate and concentrations. Four metal ions were released, Zn, Cr,

Cu and Fe, among which Zn was the most released metal ions and Fe ions were the least ($p<0.01$) (Baucic *et al*., 2003).

Corrosion of metallic orthodontic brackets has also been reported following emersion of the appliances into pH4 and pH7 saliva. Brackets immersed into the acidic saliva corroded more than those in neutral pH. Metal (Ni, Cu, Mn, Co, Fe, and Cr) ions increased over the incubation time of 48 weeks. Ni ions were the predominant leached ions. Metallic orthodontic appliances corrosion may take place within neutral and acidic oral environment regardless of the metal alloys used or whether it is new or recycled (Huang *et al*., 2004).

Co-Cr alloys are widely used in dental prostheses especially partial dentures prostheses (Viennot *et al*., 2006). It was found that alloys of Co-Cr-Mo degenerate easily in cells, cobalt metal dissolves from the marginal areas of these alloys, whilst chromium remains inside cells. Cobalt-chromium-Molybdenum alloys are inducing a potential risk to human body (Ichinose *et al*., 2003). A number of alloys have been subjected to low pH environment, those alloys involved Zn, Cu, Pd, Ag, Ni and Cr. The alloys have been immersed in 1% lactic acid and 0.9% sodium chloride (NaCl), and were kept at 37 ºC for a week, (Elshahawy *et alet al*., 2009), and it was found that Zn and Cu ions released from gold alloys, and Ni ions released from nickel-chromium alloys, have shown evidence of a high cytotoxic effect on mouse fibroblast (L-929) cells.

Dental alloys metal ions are released into the oral environment, which may induce biological responses over different periods. Toxicity of metal ions to biological rho0-cells ranked in decreasing order: Hg >, Ag >, Au >, Cu, > Ni, Co, Zn. This outcome correlates with the level of metal ion toxicity to growth of HeLa mitochondrial DNA - rho0-cells (Yang and Pon, 2003).

Monocytes (THP-1 cells) could be influenced by the exposure to Ag, Au, Cu, Hg and particularly Ni. After 72 hours Ni ions raised the interleukin (IL)-1β and Tumor Necrosis Factor (TNF)-α level. With lipopolysaccharide (LPS) added, Ag, Cu, and Ni markedly augmented the LPS-induced production of IL-1β but only Ni amplified the TNF-α response. It was observed that 50μmol/L of Au^{3+} and 50% lyses took place at

concentration of 290 μmol/L. After 1 week of exposure, 85 μmol/L of Ni^{2+} decreased the induction of IL-1β release by 70%. After a 4-week exposure, as little as 2 μmol/L of Ni^{2+} reduced the LPS induced cytokines by 50%. For TNF-α, 85 μmol/L of Ni^{2+} induced a 50% reduction in the LPS response after seven days, and nearly 100% decrease after 28 days. These alterations in cytokine response occurred with metal ion concentrations, which have been previously shown to be released from dental alloys *in vitro* and *in vivo* (Nelson *et al.*, 2001).

Cu and Ni ions were not noticeably toxic over a short period of time i.e. 24-72 hours; however, longer exposure of cells to such ions had a toxic effect on cell viability. THP-1 monocytes were exposed for 4 weeks to four metal ions that are recognised to leach from dental alloys such as Ni, Cu, Ag and Hg (Wataha *et al.*, 2000). Cell proliferation was measured for up to 28 day concentrations at 1–10% of the cytotoxic value. Prolonged duration of exposure to low doses is of relevance for the clinical use of dental and other biomedical alloys. High concentration effects of Cu^{2+} and Hg^{2+} paralleled the lower concentrations; metal ions might be capable of inducing temporary increase in cellular proliferation. Ag^{1+} at 4 mmol/l increased the cell count in 21 days, and Ni^{2+} at 5 mmol/l augmented proliferation in 14 days. The duration of cell exposure to metal ions is inversely proportional to cell proliferation, more than 4 weeks exposure duration resulted in decline in cell count. Metal ions have an individual distinctive effect on cell proliferation. Cu^{2+} at 20 μmol/l, and Ni^{2+} at 5μmol/l were the lowest metal ion concentrations that caused biological impact on prolonged exposure for a period of more than 4 weeks (Wataha *et al.*, 2000).

Cu and Zn act as elements that bind to a protein and are necessary for the protein biological activity, i.e. they work as cofactors. Peripheral Blood Mononuclear Cells (PBMC) were examined for Cu toxic effects, and the cyto-protective effect of Zn on Cu induced toxicity was also studied. Cu uptake by the PBMCs was directly related to the concentration of the metal in the medium, whilst there was no significant increase in Cu uptake by PBMC in the presence of Zn. PBMC proliferation plummeted with an increase in Cu concentration, in contrast, the cells treated with Zn were similar in terms of proliferation when compared with the control as shown on Table 1.3. (Singh *et al.*, 2006).

PBMCs	LD50	Cu Uptake by cells	DNA	Glutathion GSH	metallothionein MT	Cell Membrane	Mitochondria
Cu LD50 without presence of Zn	115 µM	Increase with time	Fragmentation occurs	↓ with Cu↑ presence	↓ at high Cu levels > 250 µM	bound cyctic spaces	disrupted cristae
Cu LD 50 with presence of Zn	710 µM	No change in Cu uptake in presence of Zn	Protection against Fragmentation	↑with Zn presence	↑ in presence of Zn	Improvement in cyctic spaces	Improvement in Structure

Table 1.3: Cytological effect of Cu^{2+} and Zn^{2+} on PBMC cells (Singh *et al*., 2006).

PBMC viability was significantly affected by the increased concentration of Cu in the media, the LD_{50} of (PBMC) was observed at 115µM. Figure 1.3. (Singh *et al*., 2006)

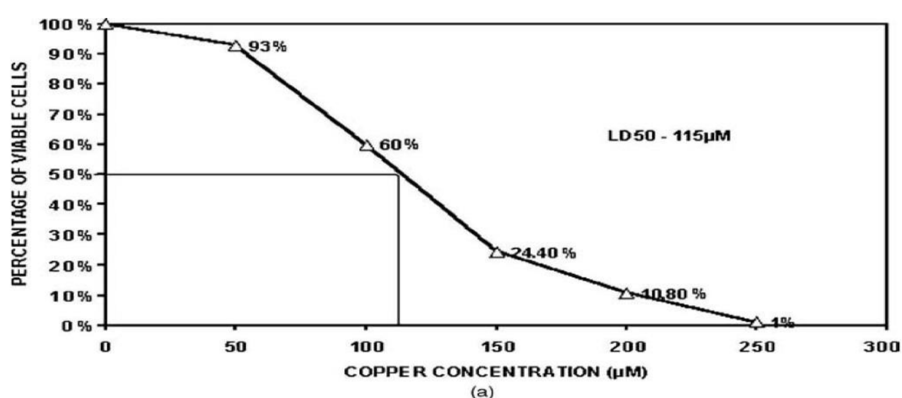


Figure 1.5 LD_{50} of PBMC measured in relation to Cu concentration in media. (Singh et al., 2006)

Copper is a redox-active metal and is an essential element that is required for normal cellular function. Our defenses against such radicals are electron transfer Cu^{2+}-containing proteins such as cytochrome oxidase for electron transport, Cu^{2+}-Zn^{2+} SOD and ceruloplasmin for iron homeostasis. Patients with Wilson's disease (a rare autosomal recessive disorder) caused by mutations in the gene ATP7B, located on chromosome 13 (Pfeiffer, 2011), suffer from loss of function hepatic copper disposition a mutations in a P-type copper ATPase, which leads to copper toxicity. Patients who have impaired Cu^{+2} homeostasis mechanism may suffer of cell death in the liver and central nervous system.

Radical inhibition of pyruvate dehydrogenase (PDH) and alpha-ketoglutarate dehydrogenase (αKGDH) significantly mediate Cu neurotoxicity and thiamine or dihydrolipoic acid reduces copper neurotoxicity by ameliorating this inhibition. To measure the 50% lethal dose (LD_{50}) following 23 hour exposure to Cu, near-pure neuronal cultures, glial cultures, mixed neuronal/glial, and HepG2 cultures were analyzed, , after which the LD_{50} was determined by lactate dehydrogenase release from dose titrations. Duplicate cultures were also exposed to 5µM Cu^{2+}in the presence of 0.3µM disulfiram (DiS) for 23 hours, and death was determined as shown on Table 1.4 (Sheline and Choi, 2004).

Cell types	50%LD_{50} µM Cu ions	% Cell death +/- Standard Error of Mean 5µM Copper ion + 0.3µM DiS
Glia / Mixed Neurons 13 Days *in Vitro*	17	77 +/- 7.9[a]
Mixed cells 8 Days *in vitro*	75[b]	73 +/- 8.2[a]
Pure Glia 28 Days *in vitro*	135[b]	49.2 +/- 6.4
Pure Neurons 13 Days *in vitro*	>600[b]	61.9+/-6.8
Pure Neurons 8 Days *in vitro*	>600[b]	59.1+/-7.9
Hep G2	340[b]	41.8 +/- 9.7

Table 1.4: Neuronal and hepatic cells LD_{50} after 23 hours Cu^{+2} exposure (Sheline and Choi, 2004). (a) The cell death determinations shown here represent approximately 50% death of both the neurons and the glia in these mixed cultures as determined by cell counting after trypan blue staining (data not shown). (b) Cultures differ significantly from, $p < 0.05$ statistical analysis by one-way analysis of variance followed by a Bonferroni test.

Electron transfer by key Cu^{2+}containing proteins: cytochrome-c oxidase for electron transport, Cu^{2+}/Zn^{2} SOD for defense against free radicals, and ceruloplasmin for iron homeostasis, all are redox properties of Cu^{2+} and are essential for cytoprotection, They can also induce lipid and protein oxidation by formation of free radicals and DNA cleavage which may lead to development of certain genetic disorders like Menkes disease (Ch X) or Wilson disorder (Ch XIII) as shown in (Figure 1.4) (Cox, 1995).

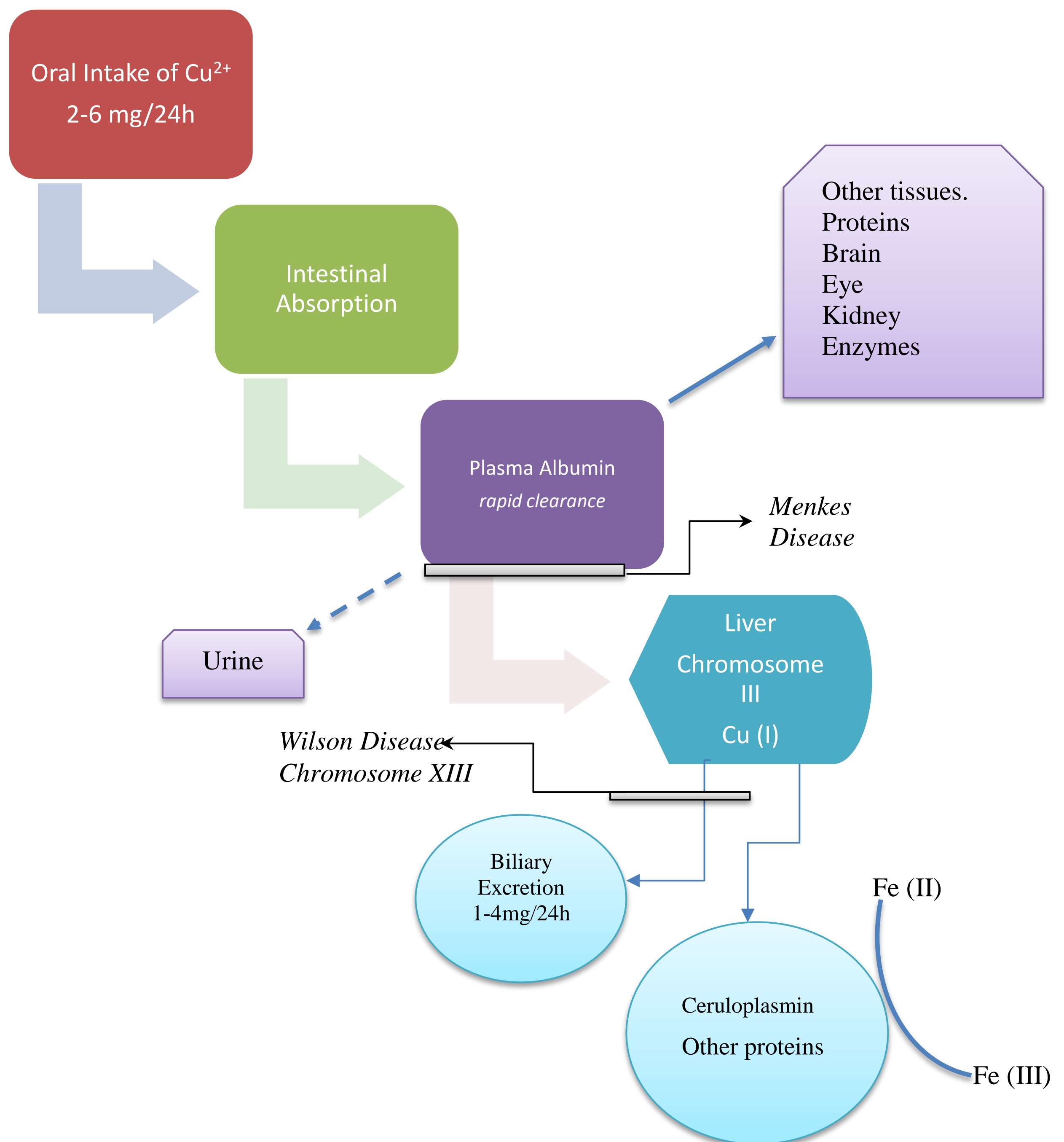


Figure 1.6: Pathway overview of Cu ions transport; it shows that dietary intake of copper generally exceeds the trace amount required, and mechanisms to control influx and efflux from cells have been developed to preserve a proper balance.

Cu^{2+} preferentially facilitates the formation of hydroxyl free radicals via the Fenton reaction, and copper redox cycling through glutathione, cysteine, or ascorbic acid can potentiate radical generation. ATP loss and death that were attenuated by mitochondrial substrates, activators, and cofactors occurred following the exposure of mixed neuronal cultures to 20 µM Cu (Sheline and Choi, 2004).

1.5 Effect of pH of the leaching ions from the alloys

The pH of saliva is normally between 6.2-7.4, but in the dental plaque (a biofilm formed by colonising bacteria on the surface of a tooth), specific types of acid-producing bacteria most notably *Streptococci mutans* and *Lactobacilli*, contribute to decreasing the oral cavity pH by producing acids, such as lactic, formic, and acetic acids in the presence of fermentable carbohydrates such as sucrose, fructose and glucose (Lagerlof and Oliveby, 1994) and a rapid pH drop in plaque bio-film down to a pH of 4.3 after consumption of sugar containing foods and beverages has been reported in extreme caries active subjects (Heasman, 2008).These acids diffuse into the enamel through the water-filled pores of the enamel or dentin leading to demineralisation (loss of mineral structure on the tooth's surface) and development of tooth decay.

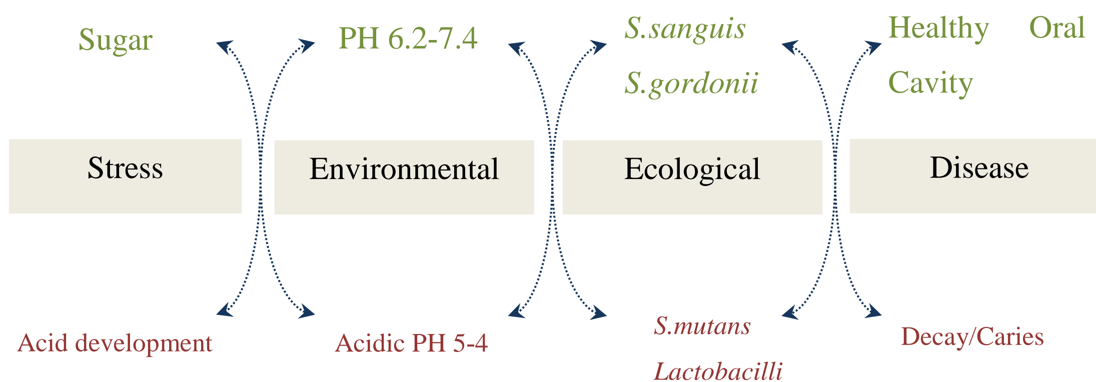


Figure 1.7: Factors affecting the development of dental caries including pH.

Control of oral pH is crucial for oral tissue health, in that the mineralisation process take place when the oral pH is neutral whilst demineralisation occurs when pH drops below 5 (Hurlbutt *et al*., 2010).

With the presence of a considerable load of potentially toxic metal ion leaching from dental alloys pH levels can be a cofactor affecting the biological viability and/or biological balance within the oral cavity. These two elements (pH and metal ions) can have a detrimental influence on oral cavity cells, shifting the oral ecological and environmental factors towards developing oral cellular lysis or abnormal cellular behavior.

1.6 Studies of the cytotoxicities of leached metals

Release of ions from the metallic elements as in high-noble -gold-platinum alloys (Cr, Cu, Mg, Zn Al, Ag, Co, Mo, Au, Cd, Ni, Pd, Ti Ca, and Pt) could be contributing to the development of cytotoxic reaction leading to imitation of contact allergy response (Celebic *et al*., 2006).

IMR-32 Human Neuroblastoma, J774A.1 Murine Monocyte-Macrophages, Human Uterine Cervix Epitheloid Carcinoma HeLa S3, and IMR-90 Human Pulmonary Diploid fibroblasts were evaluated for metal cytotoxicity. The metal salts used were: $K2Cr_2O_7$, $AgNO_3$, $CuCl_2$, $NiCl_2$, $CoCl_2$, VCl_3, $FeCl_3$, Cr $(NO_3)_3$, $SbCl_3$, $TiCl_4$, $ZnCl_2$ and Al $(NO_3)_3$. To compare the metal salt cytotoxicity between cell lines, cell viability index was used (IC_{50}) sensitivity of each cell line differed in terms of cytotoxicity to metal salts. The close relationship of the values for the IC_{50s} of the metal salts in the cell lines suggested the presence of common tendency of cytotoxicity of the metal salts. $K_2Cr_2O_7$, followed by $AgNO_3$ expressed the highest IC_{50} cytotoxicity to cell lines. IMR-32 was the cell line with the highest sensitivity (Yamamoto *et al*., 1999).

To assess the cytotoxic effect of different concentrations of several metal salts human oligodendrocytes (type of brain cells MO3.13) and human gingival fibroblasts (HGF) were used. The MTT test was implemented to measure the mitochondrial dehydrogenase activity. MTT analysis revealed that metal ions provoke reproducible cytotoxic effects in MO3.13 oligodendroglia and human gingival fibroblasts, which is related to the dose of the examined agent. The MO3.13 cells responded differently compared to the HGF cells, the former were more sensitive than the latter to most of the metals, however, *in vivo* the normal levels of these metals are lower than those determined as toxic *in vitro* (Issa *et al*., 2008).

Metals such as Cr, Ti, and Co all have toxic effects as the evidence suggest. However, there is little attention paid to the common other minor elements involved in the implant metal alloy such as Ni and V. The concentration of these metals is not found frequently raised in the serum, but chronic low levels of exposure induce harmful effect. Levels of metal ion in serum, when they are below 0.001–0.005 µg/mL, are considered normal, and

the detection limit is at 3 x 10^{-4}μg/mL. Ni ions serum levels are typically less than the levels of V. Ni levels in serum have been calculated to vary from 0.0009 - 0.0195 μg/mL (0.9–19.5 μg/L). It was observed that V concentration in tissue surrounding hip implants at 2.9 and 220μg/mL (2.9-220 μg/g) were comparable to V metal concentration level in implants themselves (Agins *et al*., 1988). Jurkat Human T-lymphocytes were examined for cytotoxic effects of leached Ni and V ions and to find out whether cell death occurs via the apoptosis pathway. It was revealed that both Ni and V are cytotoxic to Jurkat T cells. Vanadium is more toxic than the Ni. Lethal effects were demonstrated by reduction in cell viability and considerable reduction in DNA synthesis. Moreover, the mode of the lethal effect involves induction of Caspase-3 to induce apoptotic cell death, production of nuclear DNA condensation and fragmentation, apoptotic body formation, and shrinkage of cells (Au *et al*., 2006).

1.7 Measuring metals leaching into simulated saliva

Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) is a sensitive method to detect metals and non-metals at very low concentrations of 10^{12} ppm (Jenner *et al*., 1990), ICP-MS facilitates measuring more than one metal ion in a single analysis; measurements take place in the plasma matter stage. ICP-MS is the most versatile atomiser and element ioniser available. In contrast to low-temperature ion sources for molecular ions, in plasma all bonds are broken irrespective of their chemical bonding. Hence, the data acquired from a plasma ion source correspond to the total content of an element in the sample. This allows excellent analysis of metal content in the simulated saliva.

1.8 Safety of dental alloys

Until now studies have shown no observed evidence of patients developing direct health complications because of dental alloys or Ni-containing dental appliances and fillings. Individuals suffering hypersensitivity reactions to Ni often show with prior sensitisation to the element not related to dental alloys. Exposure to small doses of Ni may develop tolerance to this allergen (Setcos *et al*., 2006). The growth of L-929 fibroblasts exposed to metal ions Cu^{+2}, Zn^{+2}, Ni^{+2} and Au^{+2} was inhibited only at the highest concentration

(1mmol/L) tested but showed no effects between 0.0033 and 0.33 mmol/L (Schedle *et al.*, 1995).

Healthy individuals could survive the oxidation levels after exposure to metal induced stress. However in immune compromised people this can have an adverse effect (Lindh *et al.*, 2002) and it was observed that metal exposure from dental amalgam can cause ill health in a susceptible part of the exposed population.

By using ICP-AES, it was observed that Cu, Ni, Zn and Ag were detected in extracts of dental alloys. These were non-toxic in mouse fibroblasts L-929 cells (Schmalz *et al.*, 1998). Metals are part of the food chain; these can be found in saliva of people with even intact teeth, having a single metal alloy filling in the oral cavity may increase the content of metal elements in the saliva. Levels of metal ions in the saliva of individuals with metal dental reconstruction related problems do not correlate with occurrence of lowered metal tolerance (Kueerova *et al.*, 2002).

1.9 Prospective assays for cellular viability

Measuring cell viability is an important aspect in toxicological experiments. This will provide information about the extent of effect induced by elements on cell proliferation and membrane integrity. Cell viability indices include number of assays; the MTT assay is a commonly used assay to assess cell viability in cultured mammalian cells (Berridge *et al.*, 2005). MTT assay is a colorimetric assay system, which detects the reduction of the yellow tetrazolam salt, MTT, into the purple insoluble formazan product. This reaction takes place in the mitochondria and cytosol, of viable cells. MTT is added to cells and allowed to incubate for 2-4 hours, and then detergent solution is added to solubilise the coloured crystals and lyse the cells. Using 570nm wavelength, samples are examined in an ELISA plate reader. Amount of colour change produced is directly proportional to the viable cell count (Mosmann, 1983).

Neutral Red (NR) assay is another widely used assay to determine cell viability (Repetto *et al.*, 2008), and it is the *in vitro* assay which is mainly implemented to quantify cells viability/survival exposed to oxidation stress. The NR assay can be performed to develop

a linear relationship between cell concentration and absorbance (Valdivieso-Garcia *et al*., 1993). NR was developed as chemosensitivity assay; it is based on the ability of cells to bind the red dye to lysosomes. As a weak cationic dye, NR can readily penetrate cell membranes by non-ionic diffusion. A matrix lysosomal anionic site binds with NR intracellularly. The cytoplasm appears clear whilst the cationic probe NR appears bright red as a result of retention by the lysosome. With time progressing cells with damaged membranes show leakage of the dye, turning the cytosol into tinged pink color. Two other common methods used in this study to determine cell viability are measurement of cell protein content and lactate dehydrogenase (LDH) cytosolic enzyme leakage (Drent *et al*., 1996) (Macnair *et al*., 1997). LDH leakage from the cell membrane is one of the initial indicators of cell membrane disruption, because of its simplicity, and ability to measure LDH in culture medium over prolonged time, this was used as an index to determine the potential cytotoxic effect of metals leaching from NPG and NPG^{+2} dental alloys in 3T3 fibroblasts.

The Lowry assay is sensitive method used to measure cell total protein content. In this assay only viable cells remain attached to the culture surface; The Lowry assay measures the number of viable cells by measuring the number of cells attached to the flask. Compared to LDH leakage, the Lowry assay is more sensitive (Macnair *et al*., 1997).

To evaluate the effect of metals leached into the saliva from NPG and NPG^{+2} we have used antioxidants enzymes GR, GPx, SOD and CAT activities and expression in 3T3 fibroblasts as stress markers. This was done by measuring enzyme activities individually, and examining the molecular expression of the enzymes in a Western blot assay. Similar procedures were carried out in other studies to investigate the response of the antioxidant defense system to two oxidative stressors, hydrogen peroxide and tert-butyl hydroperoxide, in HepG2 cells in culture (Alía *et al*., 2006).

1.10 Aim of the study

The aim of this project was to study the effect of pH levels on metals leaching from NPG and NPG^{+2} dental alloys, and observe the potential cytotoxic effects of leached ions in 3T3 fibroblast cells. Once Cu, Zn, Mn, Ni ions leached from the alloy *in vivo*, they could enter the body system, and I have investigated the effect of these metal ions on detoxifying mechanisms.

The specific objectives of this project were:

- To measure the effect of pH levels on dental alloy corrosion in simulated saliva.

- To observe whether adding noble elements to dental alloys stabilises the degradation of dental alloys in acidic environment.

- To measure the cytological effect of metal ions leaked on cellular viability by:

    - MTT cell viability assay.
    - NR quantitative cell viability assay.
    - LDH cell membrane damage.
    - Lowry total cell protein content assay.

- To determine the contribution of glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) to allow 3T3 fibroblasts to cope with the effects of metals.

2. CHAPTER II: GENERAL MATERIALS AND METHODS

2.1 Introduction

This chapter illustrates the general methods and materials used during the course of this project. The methods start with preparation of simulated saliva, extraction of metal ions from metallic samples; measurement of ion levels in both neural and acidic pH-saliva, observing cell proliferation, examining cell morphology, carry out tests for cytotoxic effects with MTT and NR, measuring the comparative cytotoxicity at pH 7 / pH 4 and correlate this with metal concentration; then measuring cell cytoprotection mechanisms under oxidative stress.

In order to determine the release of metal ions from copper based dental alloys and the resultant *in vitro* cytotoxicity, two popular copper-rich dental alloys were chosen. Discs of copper-based dental alloy ($NPGTM^{+2}$) with the following nominal composition [in wt %] Cu 77.3: Al 7.8: Ni 4.3: Fe 3.0: Zn 2.7: Au 2.0 and Mg 1.7 and NPGTM alloy (without gold) were obtained from Dentech Dental, London, United Kingdom. Standard corrosion test methods as prescribed for testing dental metallic materials by the International Standards (BS EN ISO 10271) from https://bsol.bsigroup.com/ accessed on 06/08/2010 have been used in this project.

All extraction incubations for the determination of potential metal ion leakage from the alloys in oral cavity conditions were carried out in standard synthetic saliva solution. This provided a milieu which simulates the normal clinical conditions in oral cavity and acidic conditions have been generated (pH 4.0) in order to determine the release of metal ions in a milieu simulating the condition of plaque build-up in inter-proximal space between adjacent teeth.

Tests for *in vitro* cytotoxicity (ISO 10993-5:2009) were used to assess the toxicological effects of the metal ions that may be released *in vivo* from the dental alloys. The effect of specimen immersion test extracts on cell death, cell growth and cell proliferation of cultured cells (fibroblasts) was elucidated using standard cytotoxicity tests, the MTT and NR assays that were well established in our research group. The morphology of treated cells was assessed by light microscopy. The redox status of the cells was

examined by measuring the glutathione levels, this is by oxidation-reduction reactions with the metal ions, and cytoprotection mechanisms against oxidative stress were assessed.

2.2 Simulated Saliva (SS)

1000 mL sterile water autoclaved in 121°C and 15 PSI was added to 0.260g sodium biphosphate Na_2HPO_4, 0.700g sodium chloride NaCl, 0.200 potassium dihydrogen phosphate KH_2PO_4, 1.500g sodium bicarbonate $NaHCO_3$ and potassium chloride KCl (BSI, 1988). The pH of the saliva was adjusted to 4 and 7, using 1M HCl or 1M NaOH as appropriate.

2.3 Dynamic immersion test in synthetic saliva

Four specimens of NPG^{+2TM} and NPG^{TM} alloys (32 mm × 10 mm × 1,5 mm) were totally immersed in 10 mL of sterile synthetic saliva at both neutral (pH 7.0) and acidic pH (pH 4.0) acidic environment simulating the condition of dental plaque pH in inter-dental areas (Ardlin *et al*., 2009). They were incubated at temperature 37°C, and agitated at 35 RPM on an orbital shaker for 7 successive days. The extracts obtained for each metal alloy and pH level each day for a complete week were collected; extract/unit surface area was $1ml/cm^2$. Extracts were then placed in a -70°C freezer until analysis was implemented.

2.4 Metal ion leakage

At the conclusion of the exposure, aliquots (1 mL) of each extract were taken and frozen at -70°C until analysed for metal ion concentrations (Cu, Ni, Al, Zn and Mg) using ICP-MS method with appropriate standards. The analytical method has been quality assured [by Trace Elements External Quality Assessment Scheme (TEQAS)]. The metal ion analyses were carried out by Dr. Grace Alfolaranmi in department of Pure and Applied Chemistry, University of Strathclyde.

2.5 Determination of metal ions levels - ICP-MS

Inductively coupled plasma mass spectroscopy was used for detection of metal ion levels leached out NPG™+2 and NPG™ following immersing in both acidic and neutral pH. 5 mL of 500 µg/L, 100 µg/L, 50 µg/L, 10 µg/L, 5 µg/L, 1 µg/L and 0 µg/L standard solutions of Zn, Cu, Al, Ni and Mg were prepared by serial dilution. 1:5 fold dilution of 1 mL metal samples extracts were used with 1.95M Nitric acid. 1mL (100µg) scandium (Sc) + 1mL (100µg) rhodium (Rh) were used as internal standards for ICP-MS.
In order to detect the presence of any visible sign of change or deterioration, the test specimen was washed using deionised water and dried at the end of the exposure period and visually examined without magnification.

2.6 Cell culturing materials

2.6.1 Versene EDTA in PBS, pH 7.2

Compounds used to prepare a standard stock of versene EDTA in PBS at pH 7 are listed in appendix A section 1. This was dissolved in 1.5l of distilled water, aliquoted in 500ml bottles, and sterilised in an autoclave at 15lb (1.05 Kgf/cm^2) pressure for 15 minutes. Fungi growth was checked with Sabouraud medium, pH 6.5 (SAB), and Brain Heart Infusion (BHI) was used to check for bacterial growth for at least 3 days. Then the versene was made into aliquots in universal containers (20 ml), and stored at room temperature or $4^{\circ}C$.

2.6.2 TRIS Buffered Saline (TBS) for trypsin stock

Reagents and compounds used to prepare TBS for trypsin stock are presented in appendix A section 2.

This was dissolved in 500ml of distilled water. The pH was adjusted to 7.7 at 25°C (with 0.94ml of concentrated HCl). It was sterilised in an autoclave for 15 minutes at 15 lb pressure. Fungi growth was checked with Sabouraud's medium, pH 6.5 (SAB),

and Brain Heart Infusion (BHI) was used to check for bacterial growth for at least 3 days. Sterile trypsin solution was added to give a final concentration of 0.25 % (w/v), (50ml of 2.5% stock). The final solution was aliquoted into universals (20ml) and stored at -20°C.
Trypsin in versene was then prepared by diluting the trypsin in TBS 1:5 with versene, to give a working solution of 0.05%. This was aliquoted into 20ml universals and stored at -20°C.

2.7 Freezing Medium

Freezing medium was prepared by adding 5ml Dimethyl Sulphoxide (DMSO) to 20ml sterile medium and 25ml sterile Foetal Calf Serum (FCS). This compound then can then be stored for about a week at 4°C; reagents used are listed in appendix A section 3.

2.7.1 Cell culture media preparation

3T3 cell lines were grown in Dulbecco's Modified Eagles Medium (DMEM). This is purchased as a 1X stock and is stored in the cold room. Added to the 1X bottle are, 50ml sterile FCS, 5ml penicillin 50 units/mL and 50 µg/mL streptomycin (PEST), 5ml Non-Essential Amino Acids (NEAA).
Contamination was checked by adding 1ml of the medium to both a universal with BHI and SAB broth. These were then placed in the 37 °C incubators for 2-3 days. If the solutions remained clear, the media were sterile.

2.8 Mammalian Cell Culture

This section gives details of culturing cells of mammalian origin, and cell viability assays used. Fibroblast Swiss 3T3 cell line was the mammalian cell used. 3T3 cells express a wide range of receptors and second messenger systems and for these reasons are used as a standard cell line for many biochemical and cell biological studies, including studies of growth factors and cytokines and their signal transduction

mechanisms. They are widely employed in research laboratories, and are recommended by ISO 10993 for testing the safety of medical devices.

2.8.1 3T3 Cells culture

3T3 fibroblast cells were cultured as a monolayer in DMEM supplemented with foetal bovine serum 10% (v/v), antibiotic (PEST 50 units/mL, 50 μg/mL streptomycin) and 1% (v/v) NEAA at 37°C with 5% CO_2. Swiss 3T3 cells were passaged once every 3 days. A split ratio of 1:10 was performed. The growth medium was removed, and the confluent layer of 3T3 cells washed with 0.02% (v/v) versene.

To detach the cells from the flask, 1 mL of trypsin (0.05% w/v) in versene was added. This was left to cover the cell layer until detachment occurred. Approximately 5 mL of fresh medium was added once 3T3 cells detached, this is to inhibit the trypsin and it was pipetted up and down to break-up any cell clumps. In this experiment the seeding density was fixed ($7x10^3$ cells/cm^2). 3T3 cells were counted using suspensions of cells and diluted appropriately in medium. A Neubauer haemocytometer counting chamber was used to count cells, and cells were then passaged to a new flask after counting, or they were seeded at $7x10^3$ cells per cm^2 for experimentation.

The area of a 96-well plate well is 0.32cm^2/well, so the seeding density was 2.54 $x10^5$ cells/well. Two T25 flasks were set-up over 4 weeks, with seeding density of 7 x 10^3 cells /cm^2 in 10 ml medium (in total 3.36 x 10^5 cells in total in the flask). One of the T25 flasks had control (C) medium DMEM with 10% (v/v) FCS, and the other has the metal mixture in DMEM (M). These were left to grow without changing the medium. And then 1ml of the medium was taken from each T25 flask before passaging the cells to new flask, measuring the LDH leakage was performed within 24h, keeping the medium at 4°C.

2.8.2 Metal solution used in culture medium

The levels of leached metal ions from the alloy discs into saliva determined by ICP-MS were used to make the mixture of metals added to cell culture media. Metal levels were calculated based on the maximal leached concentrations at pH 4. The cytotoxic effect of these maximal leached metal concentrations was determined in cultured 3T3 cells. As shown in Table 2.4 below, the made up solution of Al, Mn, Cu, Zn and Ni was representative of leached metals of NPG and NPG^{+2} alloys.

Metal	Molecular Weight	leached concentration mg/l	Used solution µM
Aluminium	26.98	0.4	15
Manganese	54.93	1.4	25
Nickel	58.70	2.75	47
Zinc	65.38	0.77	12
Copper	63.50	5	79

Table 2.1: Metal extracts used to make up the solution of metals corresponding to concentration levels of measured metal ions leached from NPG and NPG^{+2} analysed by ICP-MS.

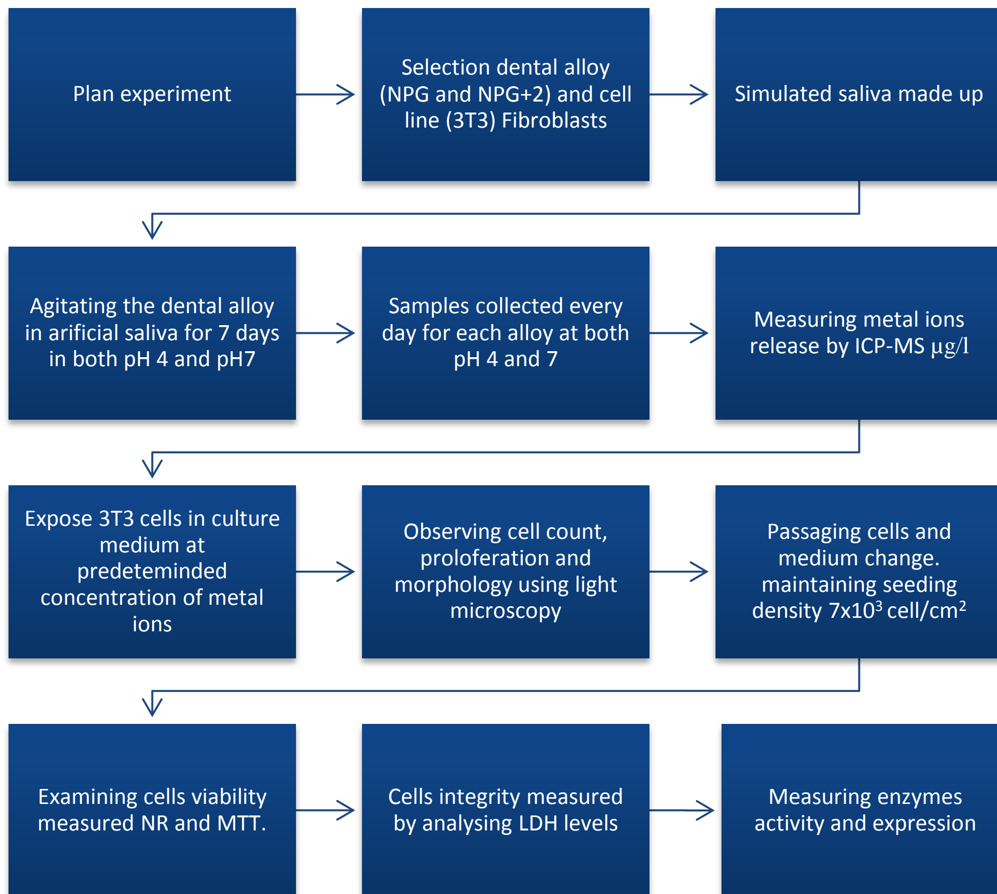


Figure 2.1:Diagram illustrates the main steps made to design this experiment. Cell redox activity measured by analysing 3T3 cells viability, enzymes activities and expression. The predetermined concentration of metal ions was taken from the maximal concentration of metal ions leached from the alloy discs into the simulated saliva over 7 day incubation period.

2.8.3 Monitoring 3T3 proliferation rate and morphology

Using light microscopy at x100 magnification, cells incubated in culture medium at pH4 and pH7 were examined. Observing cells proliferation and morphology, microscopic photos were taken to illustrate these characteristics.

2.8.4 Setup 96-well plate for MTT and NR assays

Using a seeding density 2.54×10^5 cells/well, volume of 200µl of (3T3 fibroblast/media), plate was divided to accommodate cells for MTT and NR assays (Figure 2.2). 3T3

cellular activity was measured, cells were exposed to metals containing medium for 4 consecutive weeks.

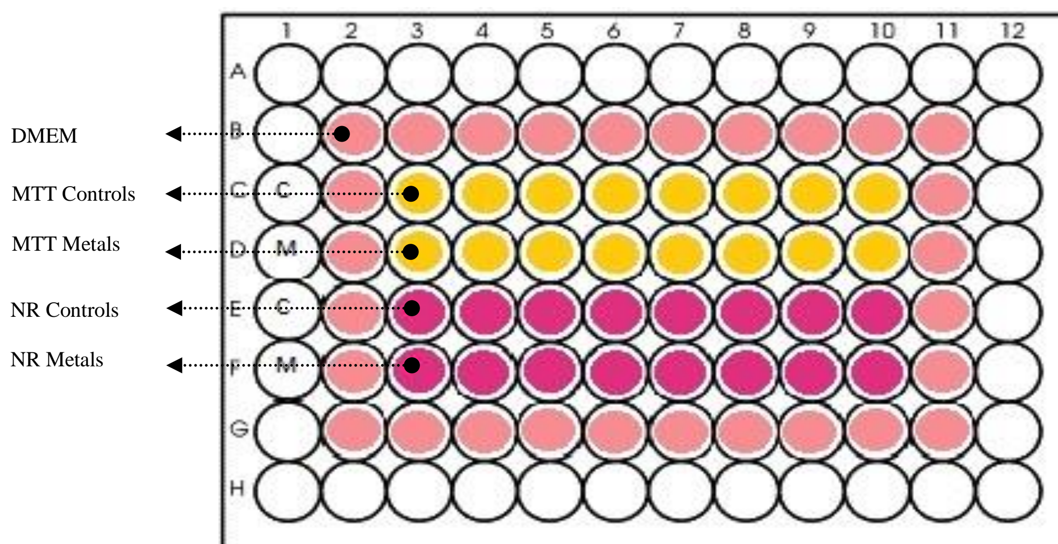


Figure 2.2: To assess the effect of the metal on growth, MTT and NR assays carried out for week 1, 2, 3, and 4. 96-well plate, control and metal exposed cells of 200 µl / well added, seeding density$2.54x10^5$ cells/well. DMEM wells had no cells, added to prevent dehydration of cells containing wells.

2.9 MTT assay a tetrazolium-based colorimetric assay for cell viability

To measure the cytotoxic effect of leached dental alloys metals the MTT assay was performed. The MTT assay measured cell respiration and the amount of formazan produced is proportional to the number of viable cells existing in culture, in terms of the amount of metabolically active cells. This assay is based on the uptake and the reduction by mitochondrial succinic dehydrogenase and the other intracellular reductase of the soluble yellow tetrazolium dye to an insoluble purple formazan product. This reaction induces change in colour, and hence MTT assay is a colorimetric system (Mosmann, 1983).

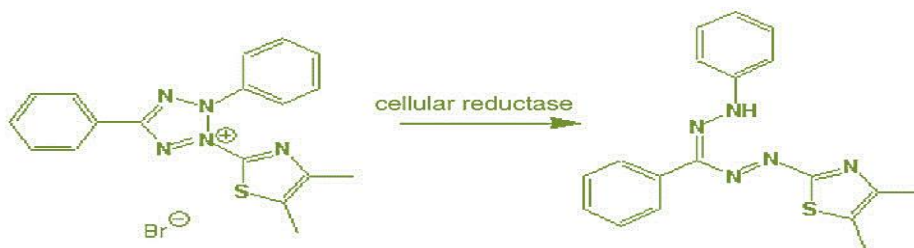


Figure 2.3: MTT, yellow tetrazole is reduced to purple formazan in living cells by cellular reductase enzyme.

A concentration of 10 mM (0.41g/100ml) MTT solution was made up in PBS at pH 6.75. This was then filtered through a filter of 0.2µm and stored for maximum of two weeks at 4°C.

Cells were incubated for 48 hours within the 96-well plate, their medium was removed and 50µl MTT solution added to each well and the plate incubated at 37°C for 4 hours. After incubation, the MTT solution was removed and 200 µl of DMSO (Sigma-Aldrich, UK) added per well. In order to dissolve the blue formazan product, the added DMSO was pipetted up and down. The formation of purple formazan was assessed spectrophotometrically (Bio-Rad 450) at 540nm and the data recorded.

2.10 Neutral Red (NR)

The NR viability assay provided a quantitative estimation of the number of viable mammalian cells in the culture. NR is one of the most used cytotoxicity tests with many biomedical applications. It is based on the ability of viable cells to incorporate and bind the supravital stain NR in to the lysosomal matrix (Borenfreund and Puerner 1985).

The solution of NR was made by dissolving 5 mg of NR powder in PBS of 100ml volume. This was then incubated for 24 hours at 37°C and a 0.2 µm filter was used to filter the solution and remove any undissolved crystals. The filtered solution was stored at

4°C. The destain solution of NR was made up by mixing 49 ml distilled H_2O, 50 ml alcohol (ethanol) and 1 ml glacial acetic acid.

After exposure to the metals, analysis of cells involved removal of medium and a solution of 100 µl NR solution was added per well of 96-well plate. After 3 hours incubation at 37°C, the NR solution was discarded and wells washed with 200 µl PBS pH 7.2 per well. Afterwards 100 µl of NR destain was added to each well. Then the 96-well plate placed on an orbital shaker for 30 minutes until a homogenous colour was obtained within each well. Using a spectrometer (Bio-Rad 450) the absorbance was measured with a filter of 540 nm.

2.11 Lactate Dehydrogenase Assay - LDH

The reaction that this assay measures is:

The method of Anuforo *et al.*, 1978 was implemented to measure LDH in 3T3 cytoplasm. Solutions used for this assay are listed in appendix A section 4.

40µl of NADH/Pyruvate solution and 0.86ml of buffer were added to a 2.5ml cuvette. The reference cuvette contained 1ml of buffer. The reaction was initiated by addition of 100µl of medium. Solutions were mixed and the decrease in absorbance was measured at 340nm for 1 minute on Shimazu UV-2101PC spectrometer.

Absorbance decrease rate at 340nm, resulting from the oxidation of NADH is a measure of LDH activity. This is also directly related to material toxicity. To calculate the activity of the enzyme, the extraction coefficient of NADH ($6.22 mM^{-1}cm^{-1}$) was used. Before being passaged, 3T3 cells were incubated for 72h without changing the medium. Then 1ml of the medium was taken from each T25 flask to measure the LDH within 24h. At this point T25 flasks for both control and metal contained medium were washed gently with 5ml PBS, this is then discarded, and 1ml ice-cold Na phosphate was added. To detach the 3T3 cells, a scraper was used carefully, keeping flasks at 4°C.

These were then homogenised with 7 strokes of the motor driven homogeniser. Aliquots of 50µl obtained and placed into a 13 ml plastic tube for protein assay by the Lowry assay, stored this at -20°C.

2.12 Lowry Assay

Cell protein content was measured by the Lowry assay (Lowry *et al.*, 1951). As a standard, bovine serum albumin was used. Estimating the cellular protein was done colorimetrically after sequential addition of Ciocalteau reagent and alkaline copper sulphate and Folin. To produce the blue colour, aromatic amino acids, tyrosine and tryptophan, react with Folin's reagent, after the treatment of protein with alkaline cupper. Lowry Assay standards in appendix A section 5 used to setup the standard curve using specified reagents and concentrations.

From 25cm^2 flasks, cell samples were diluted by 10 fold with 0.5M NaOH (1ml : 100 µl). 5ml of solution "A" added to both standards and samples, they were mixed and kept a room temperature for 10 minutes. Then 0.5ml of solution "B" was added, samples were mixed immediately. Before the absorbance rate was read, mixed samples were left for 30 minutes or at most 90 minutes at room temperature. Wavelength used for reading absorbance rate was 725nm against distilled water on a UV-2101PC spectrophotometer. From the standard curve the cellular protein concentration was directly interpolated.

2.12.1 Preparation of cell homogenates for enzyme activities and expression

For preparation of homogenate, 3 control and 3 metal treated flasks were taken. Each were washed gently with PBS (5ml), this was then discarded. After this 1ml ice-cold 0.1M Na phosphate buffer was added, pH 7.4.

Maintaining flask on ice at 4°C, 3T3 cells were scraped into the flask carefully. Each flasks was treated as a separate sample, they were not combined at any stage. Using motor driven homogeniser, homogenates obtained after 7 strokes, 30s each. These were frozen in 100 µl aliquots at -80°C. Also 50 µl aliquots into a 13 ml plastic tube for protein

assay by the Lowry assay, stored at -20°C. The cell homogenates were used to determine enzyme activities and expression.

2.13 Enzymatic assay for measurement GR activity:

The (Carlberg and Mannervik, 1985) method was used to measure the GR activity in the cell homogenates. GR catalyses the reduction of GSSG to GSH using NADPH as an electron donor.

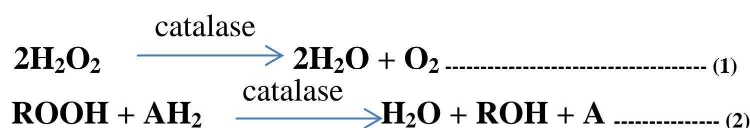
Solutions used in preparing GR assay are listed in appendix A section 6.

Procedure:

- Cell homogenates were added to 0.5 ml phosphate buffer, 0.3 ml distilled water and 50µl GSSG in quartz cuvettes, to measure the glutathione reductase activity.
- Addition of NADPH initiates the reaction in sample cuvette; change in absorbance was measured at 340nm over 30 min for 3T3 cells at 37°C.
- To determine linearity of the reaction, absorbance of the samples was read at intervals of 5 min. The extinction coefficient for NADPH of 6.22mM^{-1}cm^{-1} was used to calculate the rate of reaction, and the activity of enzymes was corrected for protein content.

2.14 Enzymatic assay for measurement of catalase activity CAT

Catalase activity assay was measured (Aebi, 1984). Catalase exerts a dual function: (1) Decomposition of H_2O_2 to give H_2O and O_2 and (2) Oxidation of H donors with consumption of 1 mol of peroxide. Several substrates (AH_2) can be oxidised *in vitro* by CAT and H_2O_2, including methanol, ethanol, formic acid, thiols and phenols.



Initiation of the reaction was started by addition of hydrogen peroxide, see appendix A section 7 for concentrations used to prepare this assay.

- In the blank 1ml phosphate buffer was substituted for H_2O_2. The wavelength used was 240nm.
- 250µl cell homogenate, 1.65ml phosphate buffer, 100µl 0.8% Triton X-100 and 1ml H_2O_2 were added to the reaction cuvette.
- The final volume in quartz cuvette was 3ml and the activity was calculated using extinction coefficient used 3.94mM-1 cm-1 for H_2O_2.

2.15 Enzymatic assay for measurement of SOD enzymatic activity:

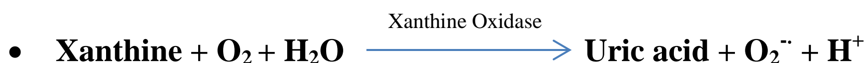
The (McCord and Fridovich, 1969) method was used to measure the SOD activity in cell homogenates. See appendix A section 8 for concentrations used to prepare this assay. Superoxide dismutase catalyses the reaction:

- $\mathbf{2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2}$

SOD activity was measured as the inhibition of the rate of reduction of Cytochrome-c by superoxide radical, this was observed at 550nm.

- **Cytochrome c (oxidized) + $O_2^{\cdot-}$ → Cytochrome (reduced) + O_2**

By the below reaction, the superoxide is produced enzymatically:



- $\mathbf{Xanthine + O_2 + H_2O \xrightarrow{Xanthine\ Oxidase} Uric\ acid + O_2^{\cdot-} + H^+}$

2.8ml reaction buffer solution was pipetted into 2 quartz cuvettes; absorbance rate was monitored at 550nm for 5min as baseline/blank. 0.2ml distilled water was added to reference cuvette and then placed in reference position. The uninhibited reaction was created by adding 0.1ml distilled water and 0.1 ml XO, these were mixed immediately and measured absorbance at 550nm for 5min.

To produce the standard curve, inhibited reaction with known SOD was used, XO and SOD were added to each cuvette individually immediately before reading absorbance for 5 min at 550nm.

Calculations:

The ΔA550nm for each inhibited test fall within 40-60% the uninhibited rate. Values outside this range were excluded.

$$\% \text{ Inhibition: } \frac{(\Delta A550nm/min\ Uninhibited - \Delta A550nm/min\ Inhibited)x(100)}{(\Delta A550nm/min\ Uninhibited - \Delta A550nm/min\ Blank)}$$

$$\text{Units/ml Enzyme: } \frac{(\% \text{ Inhibition}) \text{ x } (\text{Dilution Factor})}{(50\%) \text{ x } (0.10)}$$

50% = Inhibition of the rate of cytochrome c reduction per the unit definition
0.10% = millilitres of enzyme used in each test

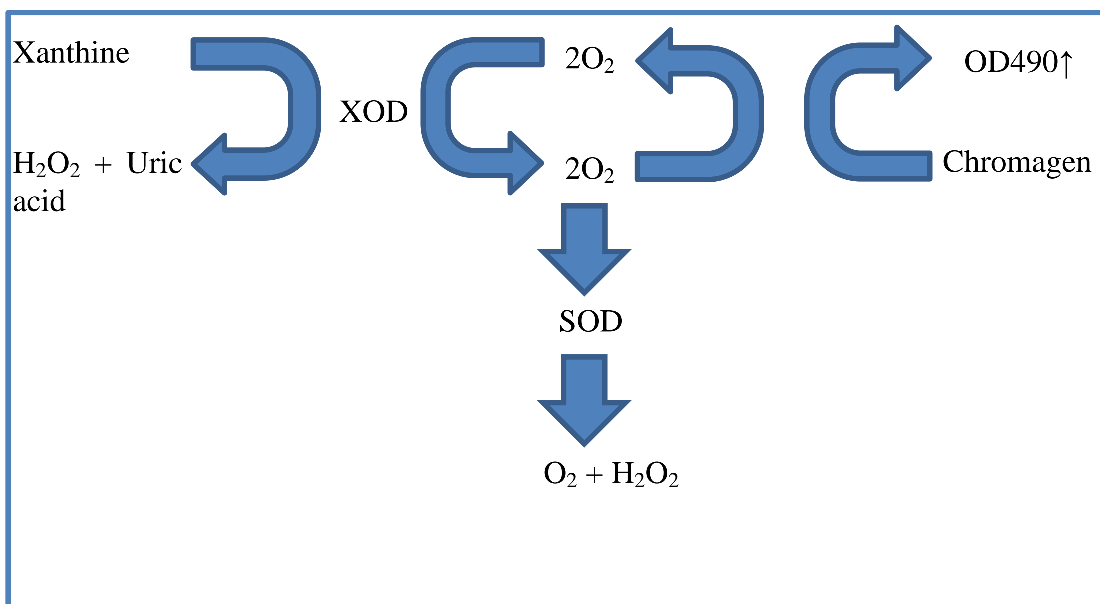


Figure 2.4 Assay Principle for measuring total activity of SOD enzyme

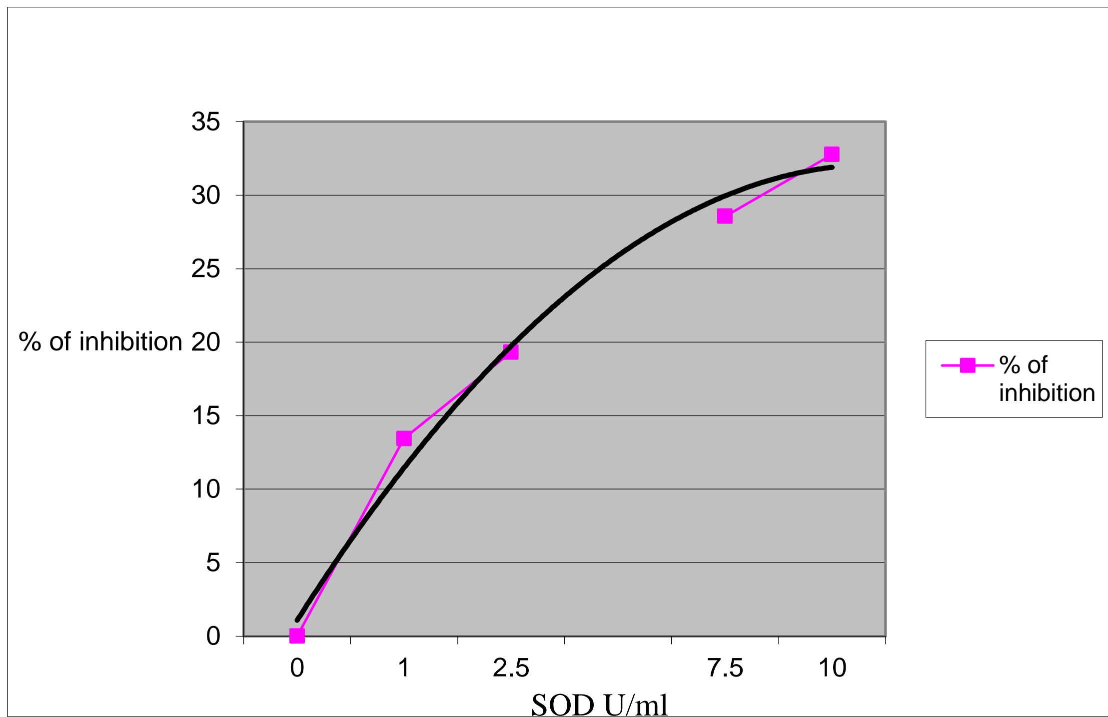


Figure 2.5: Producing the SOD standard curve, inhibited reaction with known SOD was used; XO and SOD were added to each cuvette individually immediately before reading absorbance for 5 min at 550nm.

The reaction buffer consisted of 12.5 ml phosphate buffer, 0.5 ml EDTA, 0.5 ml cytochrome-C, 11.5 ml dH_2O_2, and 25.0 ml Xanthine. 2.8 ml of reaction buffer was added to two cuvettes, reference and sample. The uninhibited reaction was observed upon addition of 0.1 ml (dH_2O_2) distilled water and 0.1 ml to XO to sample cuvette. Over 5min, the absorbance was read at 550 nm. To measure cytochrome-C inhibition rate of reduction, SOD standards/cell homogenates were added to sample cuvette along with xanthine oxidase (XO). The standard curve for inhibited reaction was determined with SOD standards. Measuring the SOD activity in cells carried out by substituting standards with cell homogenates for the assay. The change in absorbance was measured as mentioned above.

2.16 Enzymatic assay for measuring glutathione peroxidase activity

Method used to determine the GPx activity in cells homogenates was reported by (Carmagnol *et al.*, 1983). GPx reduces hydrogen peroxide to water and lipid hydroperoxides to alcohol.

$$2GSH + H_2O_2 \xrightarrow{GPx} GS\text{--}SG + 2H_2O$$

Concentrations and volumes of reagents used in the GPx assay are presented in appendix A section 9. In a 1ml glass cuvette the following were 0.8ml phosphate buffer at 37°C, 50µl GSH (5mM), 30µl glutathione reductase (1.4U), 30µl aminotriazole (3mM), 10µl NADPH (0.28mM), 20µl KCN (1.5mM) and 20µl cell homogenate.

The reaction was initiated by adding 50µl H_2O_2 to the cuvette. At 37°C the decrease in absorbance was measured at 340nm, and the extinction coefficient used was $6.22mM^{-1}cm^{-1}$.

2.17 Western Blotting

Whole cell extracts were fractionated by Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane using a transfer apparatus. After incubation with 5% nonfat milk in TTBS for 60 min, the membrane was washed once with TTBS and incubated with antibodies (GR,GPx, SOD and CAT.

In order to investigate the effect of metal ions on the expression of the enzymes (GR, GPx, SOD and CAT) in both control and metal treated 3T3s, chemicals, reagent and solutions used are listed in appendix A section 10.

Western blotting procedure:

This was carried out in two phases, firstly, the proteins were separated using SDS-PAGE then localisation was carried out by Immunoblotting.

Sample preparations:

Laemmli buffer was used to prepare cell homogenate samples from control and metal treated 3T3 cells, giving final loading concentration of 40µg protein / well. Determination of protein concentration (mg/ml) was based on the Lowry assay. This was used to define the required volume required to be added into each well.

Where; X_1 = Volume of sample (100µl)

Y_1 = Protein concentration of sample (mg/ml)

X_2 = Concentration / well

Y_2 = Volume to be added to get 40µg of sample/well

$$[X_1][Y1] = [X_2][Y2]$$

The required volume of Laemmli buffer was added to 100µl of the cell homogenate to yield a loading concentration of 40µg protein / well. Labelled grip-lock Eppendorfs were used, for these the samples prepared with Laemmli buffer. These tubes were boiled in water for 3 minutes approximately.

Phase 1 – SDS-PAGE:

The stacking gel and resolving gel were prepared using the solutions listed in appendix A section 10.

Reagents and solutions used in preparation of immunoblotting assay, APS was freshly made, TEMED was added before casting each gel.

Gel cassettes and the electrophoresis system were checked for leakage. As described above the samples were prepared and loaded into each well. For 15 minute a current of 50mA was applied. This was to stack the protein samples; protein separation based on molecular size was performed with current applied 100mA this was until the dye front was observed at the bottom of the gel.

Phase 2 – The Immunoblotting (Protein Transfer):

Transfer of protein from SDS-PAGE gel onto PVDF membrane was made. PVDF membrane was cut to the correct size for the gel and prepared for transfer by soaking in methanol. Sponges and blotting paper were soaked in cold transfer buffer (0.25 M Tris base, 1.92 M Glycine, 600ml methanol). Gels were removed from the electrophoresis tank and carefully removed from the glass plates. They were sandwiched in a transfer cassette as shown in Figure 2.6:

Figure 2.6: Preparation of immunoblotting cassette for protein transfer.

Before transferring the gels, filter papers, transfer membranes, and scotbrite pads all were soaked into Towbin's transfer buffer for a period of 15minutes. The transfer was set on 200mA for 3 hours.

After the transfer, the PVDF membranes were placed in 3% (v/v) gelatin solution, for 1 hour at 37°C this was after the transfer phase. Membranes were placed into containers placed on orbital shaker and then washed using Tween tris-buffered saline for 5min. Primary AB (dilution 1:1000) prepared in 1% (v/v) gelatin in TTBS, was added to these membranes and they were incubated overnight at temperature of 4°C.

After this period, the PVDF membranes were washed three times using TTBS. Each wash was for 5 min, and this took place on an orbital shaker. Following this, membranes were incubated in alkaline phosphatase conjugated secondary antibody (dilution 1:30000), and this was prepared in 1% (v/v) gelatin in Tween tris-buffered saline, for two hours at 37°C.PVDF membranes were then washed twice for 5 minutes each with Tween

tris-buffered saline on an orbital shaker. The third wash was performed using TBS for another 5 minutes.

The detection system of alkaline phosphatase composed of 2 reagents (A and B) and 1 development buffer. The later was prepared from 25x concentrate.

i. Solution A : Nitroblue tetrazolium in aqueous DMF
ii. Solution A: 5-bromo-4-chloro-3-iodolyl phosphate in DMF

Detection system preparation was as appears in appendix A section 10.

Detection solution was prepared; and the membranes were incubated in this for 5 – 10 minutes on an orbital shaker. This was performed until colour developed. After washing the membranes with dH_2O, the solution was discarded.

To avoid any excess background, careful handling of the membrane was carried out. Using NIH Image J 1.37 version, the generated blots were analysed and quantified according to their optical density.

3. CHAPTER III: RESULTS

3.1 Determination of leached metal ions

The two copper-based dental alloy specimens were immersed for 7 successive days with shaking (35 rpm) at 37°C in 5ml synthetic saliva as described in the methods. Tables 3.1 and 3.2 show the extent of corrosion observed at both pHs 7.0 and 4.0 for the NPG and NPG^{+2} alloy, at incubation times 1, 3, 5 and 6 days. Metal ion release from both alloys in simulated saliva at pH 4.0 was significantly higher (for all the metals, and at all incubation times) when compared to the levels observed at pH 7.0 (2 sample T-test $p \leq 0.05$).

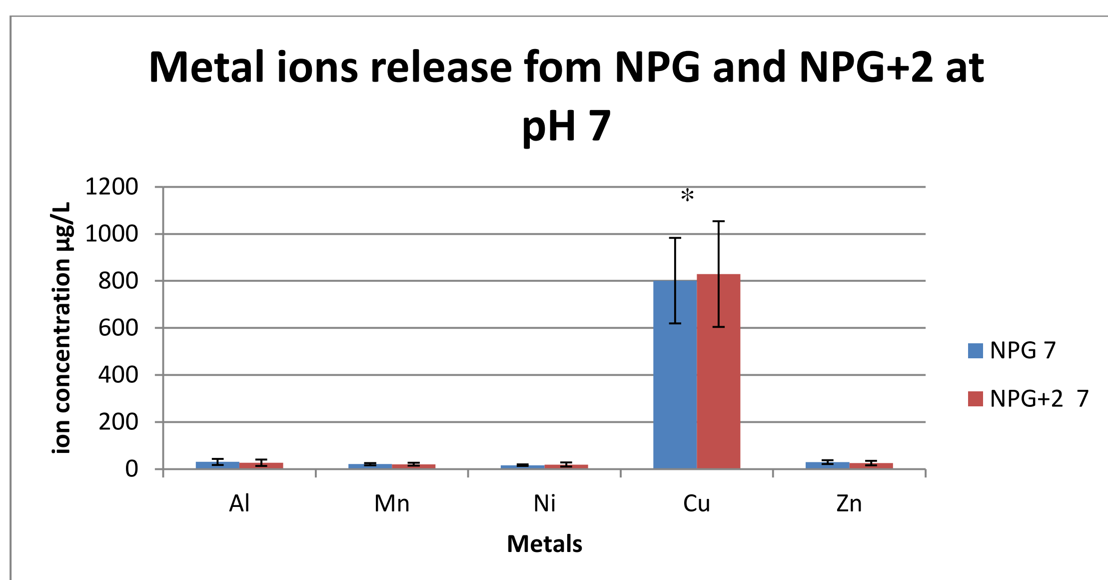


Figure 3.1: NPG and NPG^{+2} alloys metal ion release at pH 7, Cu ions leached despite incubation in neutral pH, results are means ± SEM, n= 3, comparing metal ions leaching at pH 7 in 4 week by ANOVA, $^{*}p<0.05$.

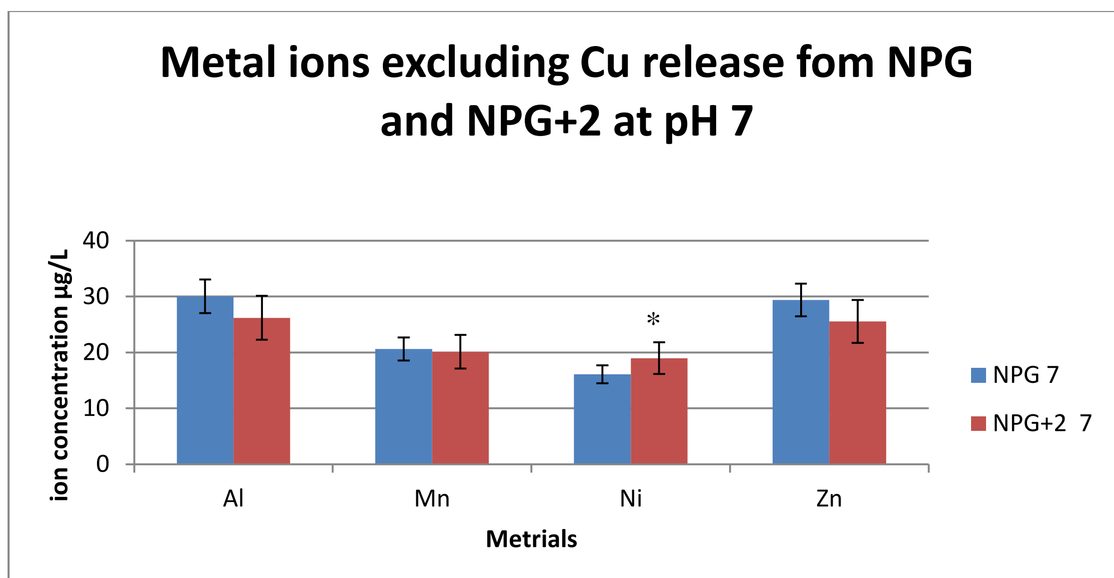


Figure 3.2 NPG and NPG^{+2} alloys metal ion release at pH 7. Cu ions were excluded, significant results observed with Ni ions. Results are means ± SEM, n= 3, comparing metal ions leaching at pH 7 in 4 week by ANOVA $*p<0.05$.

pH 7			
Metal ion	**Max release from NPG and when**	**Max release from NPG^{+2} and when**	**Ratio of max NPG^{+2} / NPG release**
Al^{3+}	**37.7 at day 1**	**36.2 at day 5**	**1:1.04**
Mn^{2+}	**25.8 at day 2**	**39.3 at day 4**	**1:1.5**
Ni^{2+}	**18.1 at day 2**	**31.7 at day 4**	**1:1.75**
Cu^{2+}	**846.9 at day 4**	**884.8 at day 6**	**1:1.04**
Zn^{2+}	**38.9 at day 3**	**31.5 at day 4**	**1:1.2**

Table 3.1: Maximum metal Ions release µg/L after immersing in simulated saliva, 1-7 days NPG, NPG^{+2} placed in pH7. Au has no significant effect on metal liability to leak.

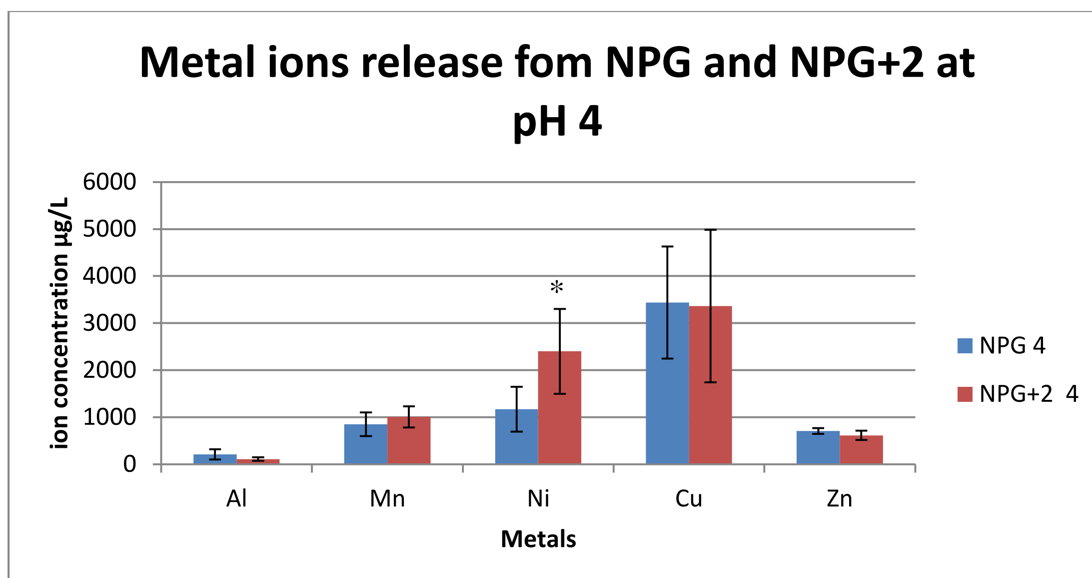


Figure 3.3: NPG and NPG^{+2} alloys metal ion release at pH 4, all metals showed increased level of ion leaching, this might be related to acidic environment at pH4. Significant results observed with Ni ions. Comparing values for alloys exposed to acidic pH4 with those in pH7 by t-test for each group, followed by ANOVA Results are means ± SEM, n= 3, *$p<0.05$.

pH 4			
Metal Ion	**Max release from NPG and when**	**Max release from NPG^{+2} and when**	**Ratio of max NGP^{+2} / NPG release**
Al^{3+}	**410.6 at day 5**	**184.7 at day 6**	**1:2.2**
Mn^{2+}	**1413 at day 6**	**1302.3 at day 5**	**1:1.08**
Ni^{2+}	**2747 at day 6**	**3925 at day 7**	**1:1.4**
Cu^{2+}	**4833 at day 1**	**7152.8 at day 4**	**1:1.5**
Zn^{2+}	**772.9 at day 5**	**860.7 at day 4**	**1:1.11**

Table 3.2: Maximum metal ions release µg/L after immersing in simulated saliva, 1-7 days NPG, NPG^{+2} placed in pH4. Au has no significant effect on metal liability to leak.

As would be expected from the composition, Cu ions are released to the greatest was measured with Ni (200 fold increase), followed by Mn (90 fold increase) and Zn (30 fold increase). No significant difference in ion release was observed in the extent of corrosion between the two alloys at both pHs (2 sample T-test $p \geq 0.05$). As shown in figures above (3.1, 3.2 and 3.3).

3.2 Incubation of 3T3 cells with metal extracts

A mixture of metal ions was made up for the cytotoxicity experiment, this was then sterilised by filtration through a 0.22μM filter as described in the methods. The levels of leached metal ions from the NPG and NPG^{+2} discs into simulated saliva determined by ICP-MS were used to make the metal extract mixture; this was added to cell culture media. The mixture was determined based on the maximal leached concentrations at pH 4. The cytotoxic effect of this maximal leached metal concentration was determined in cultured 3T3 cells.

Fibroblast cells incubated with metal showed decreased numbers of proliferation rate compared to controls. Cell count was less for metal treated flasks 5-8 x 10^5/cm^2 compared to 11-16 x 10^5/cm^2 for control. Light microscopic pictures for cells dividing in metal containing medium have demonstrated gaps between cells, cells in control medium, have showed higher proliferation with less gaps existed compared to those containing metals (Figure 3.4).

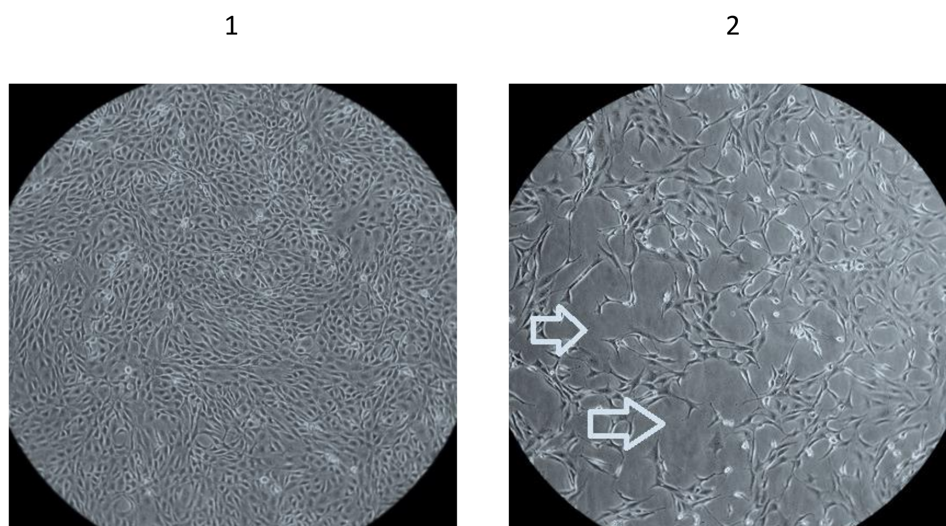


Figure 3.4: Photo 1, Control untreated culture of 3T3 cells in pH 7, x100 magnification, black and white photograph wide field view. Photo 2, 3T3 cells proliferating, 48 h post incubation in metal containing medium, light microscopic black and white photograph x100 magnification power, wide field view used to illustrate the voids spaces. Seeding density for both 7x10^3 cells/cm^2 flasks. White arrows are showing the gaps between cells as an indication of lower proliferation rate.

3T3 cells at metal ions made in medium (Photo 1), showed less proliferation activity being under high concentration of metal ions stress. Control cells (Photo 2) at neutral pH appear normally. This is condensed cells ready for passage 48 hours incubation at 37°C. At some places, cell morphology was irregular and larger than those in control. Microscopic photos shown some changes in the morphology of the cells, for those in metal ions containing medium have irregular and larger outer shape while 3T3s in pH7 where with normal outlines (Figure 3.5).

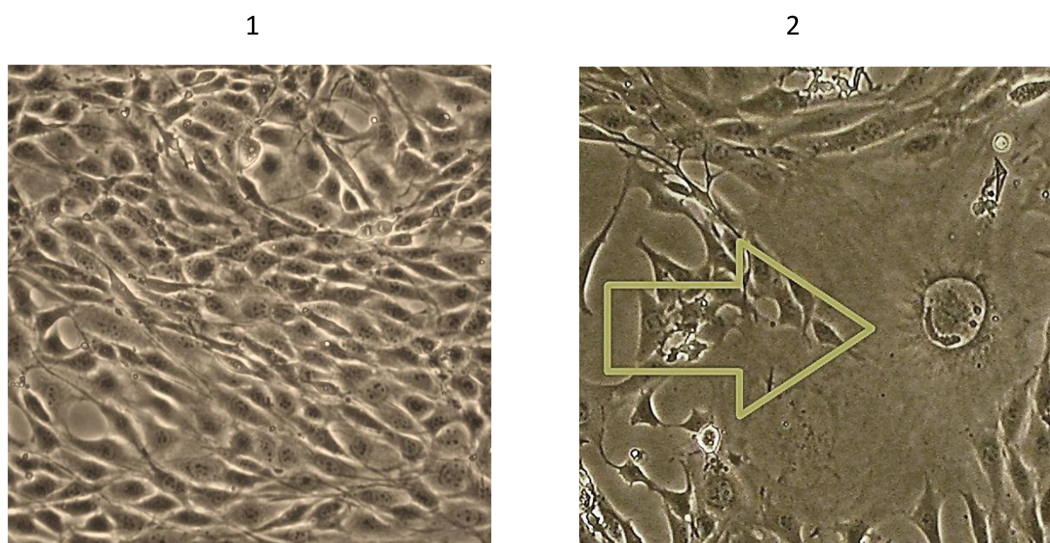


Figure 3.5: Photo 1 Untreated control 3T3 cells were of normal outer shape and growing normally. Photo 2 3T3 cells treated with metal extracts, light microscopic black and white photograph, magnification 135X, close field view. These cells multiplying in high metal concentration show some morphological changes.

Seeding density was constant at $7x10^3$cell/cm^2. Metal ions media incubated cell proliferation rate declined from day 1 to 7, for that last day being lowest. Microscopic photos were taken prior to cell passage. 3T3s incubated in high metal concentration media have shown decreased count numbers.

3.3 MTT assay:

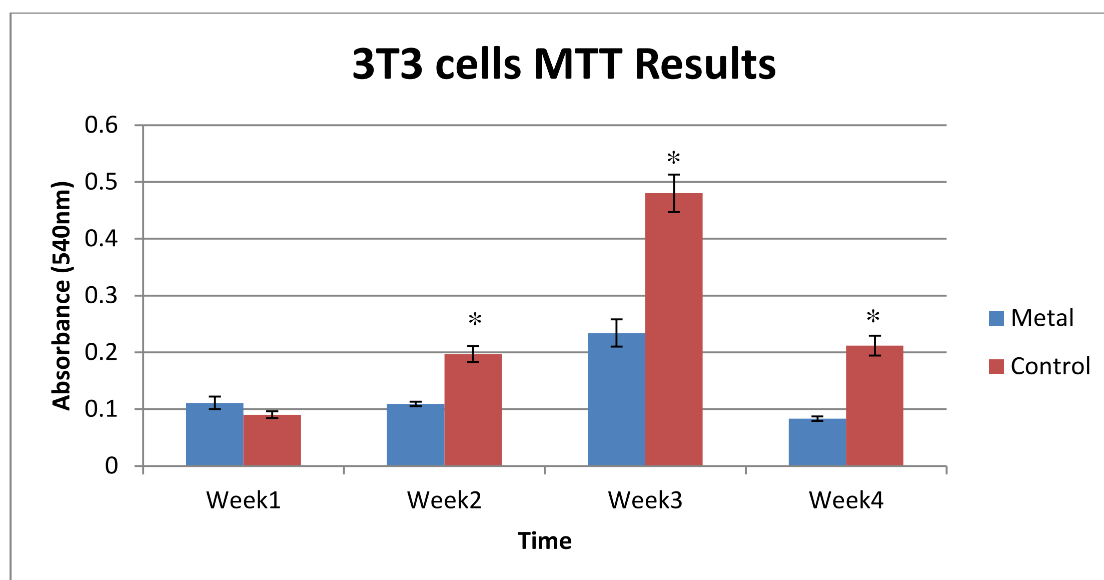


Figure 3.6: 3T3 cells MTT assay, measured over 1, 2, 3 and 4-week exposure. Results are the mean ± SEM, n=3, statistical differences detected at week 2,3 and 4. Comparing values for cells exposed to metal ions with those in control cells in the absence of metal ions by t-test for each group, followed by ANOVA *p-value <0.05.

3T3 cellular enzymatic activity was measured over 4 weeks for both metal treated and control cells as described in methods chapter. Cells exposed to metal ions showed less viable activity compared to controls for all weeks part from week 1.

Statistical analysis shown significant difference between the cells over the last three weeks, cells exposed to metal extracts were less viable compared to controls. These data from the mixture of metal ions made up were used to approximate the maximum concentration eluting, and to assess the impact of exposing cells to high level of stress.

3.4 NR assay

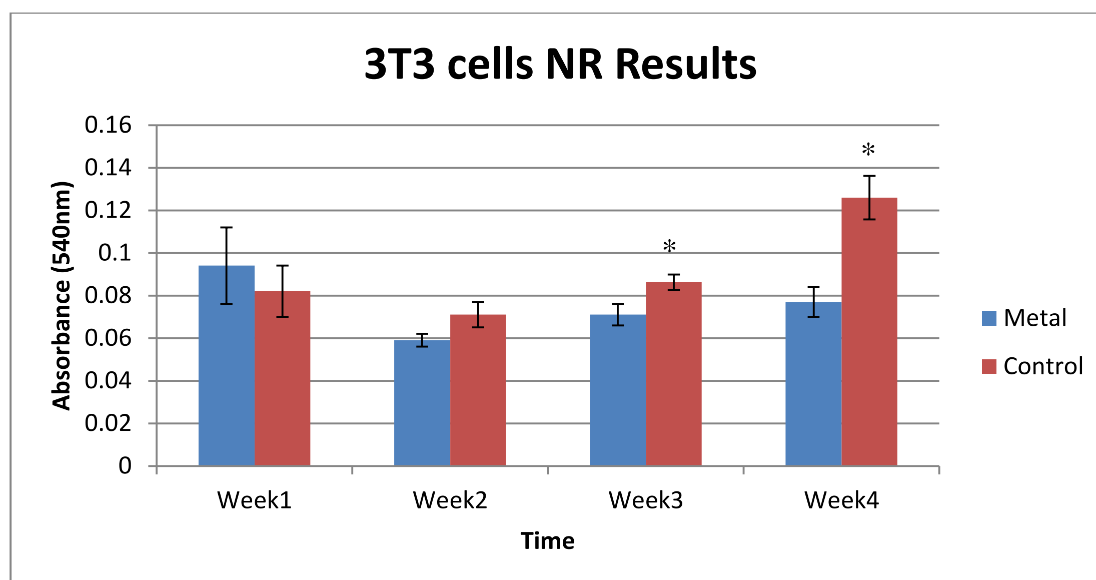


Figure 3.7: 3T3 cells NR assay, measured over 1, 2, 3 and 4-week exposure. Results are the mean ± SEM, n=3, statistical differences detected at week 3 and 4. Comparing values for cells exposed to metal ions with those in control cells in the absence of metal ions by t-test for each group, followed by ANOVA *p-value <0.05.

Measuring the ability of viable 3T3 cells to incorporate and bind the vital dye to lysosomes showed increase of control cell viability for the first two weeks compared with metal treated 3T3s.

During week 3 and 4, the numbers of viable fibroblasts in samples exposed to metal ions were lower than in control cells. However, the statistical analysis carried out revealed no significant differences for the first two weeks. In contrast week 3 and 4 have shown statistical significance, p-value <0.05.

3.5 Lactate dehydrogenase (LDH) enzyme leakage

Fibroblasts were maintained in culture for 4 weeks, and the effect of the metal ions on the cell membranes was monitored by measuring the LDH leakage from the cells.

Figure 3.8 shows that exposure of cells to metal ions had shown significant effect in week 2 on the LDH activity leaking out of the 3T3 cells compared to control, at any time point measured. After 1 week the activity in the medium was 0.50 ± 0.08 (mean ± SEM, n=3), compared with the control level was 0.7 ± 0.04 (mean ± SEM, n=3). When these cells were exposed for a longer period to the metal extracts LDH levels increased. Week 2 showed increased levels for metal treated cells 0.90 ± 0.08 (mean ± SEM, n=3), compared with control 3T3s which had LDH activity in their cytoplasm 0.73 ± 0.04 (mean ± SEM, n=3).

LDH levels for week 3 and 4 also showed no significant differences. Control LDH measured was 0.8 - 0.9 ± 0.04 (mean ± SEM, n=3) μmol/ml/min and metal exposed cells showed an activity slightly lower than this of 0.7 - 0.8 ± 0.08 (mean ± SEM, n=3) μmol/ml/min. Total LDH activity for control was 0.772 μml/ml/min and 0.725 μml/ml/min for metal.

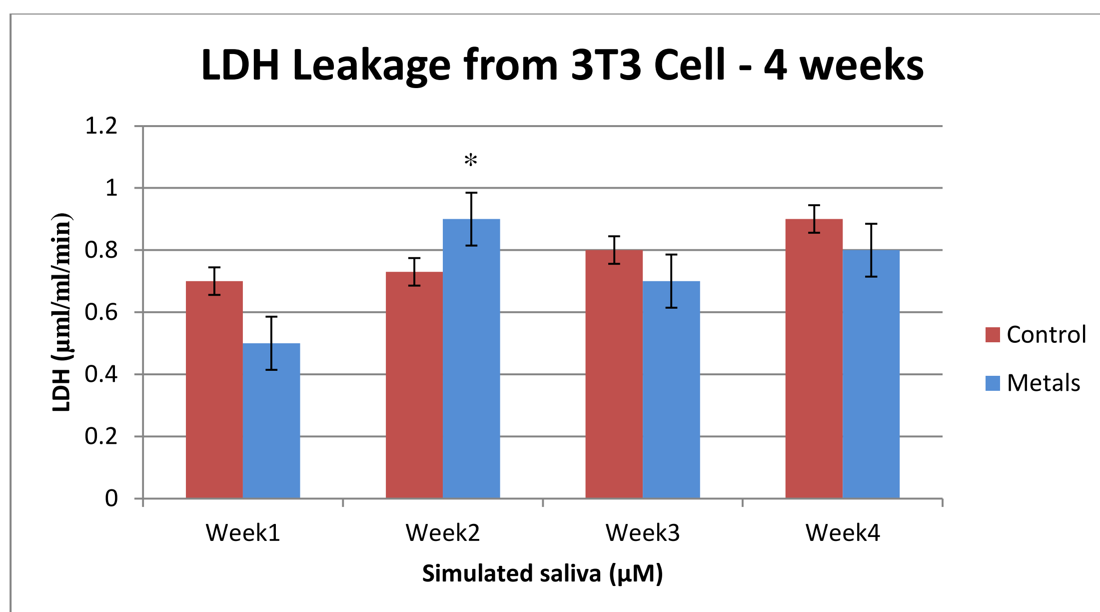


Figure 3.8: LDH leakage from 3T3 Cells exposed for 4 weeks to metal containing medium. Results are means ± SEM, n=3. Comparing values for cells exposed to metal ions with those in control cells in the absence of metal ions by t-test for each group, followed by ANOVA, *p-value <0.05.

3.6 3T3 fibroblasts protein concentration – Lowry assay

3T3 fibroblast protein concentration was measured for four consecutive weeks; cells incubated in metal containing medium contain higher level of protein compared to control (Figure 3.9).

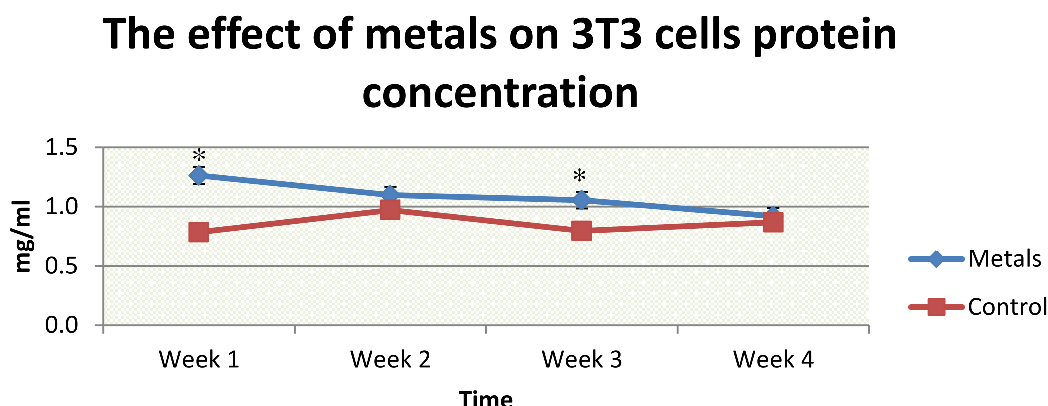


Figure 3.9: 3T3 fibroblasts protein concentration measured using Lowry assay. 3T3 cells incubated in metal containing medium show increased levels of protein compared to control. Control cells contained 0.784 - 0.971mg/ml over 4 weeks. Metal exposed cells showed significantly higher levels of protein concentration (0.918 – 1.261 mg/ml) compared to control. Results are mean ± SEM, n=3, comparing values for cells exposed to metal ions with those in control cells in the absence of metal ions by t-test for each group, followed by ANOVA, *p-value <0.05.

Summary of the Assays used to detect effects on cell viability:

The following methods were used to measure the effects if the metal ions on cell viability (MTT. NR, LDH) and total adherent protein assay (Lowry).

Assay	Result
MTT	transient decrease in MTT reduction after 2 weeks
NR	transient decrease in number of viable cells after 1 week
LDH	transient increase in LDH at week 2
Adherent Protein	metals cause increase in adherent protein at all time points

Table 3.3: Effects of the metal on cell viability.

It is apparent that exposure to the metal ions at concentrations used certainly affects the cells, but it is not overtly toxic.

3.7 Western blotting and enzyme activity

Measurement of enzyme expression of GR, CAT, GPx and SOD was performed using Western blots.

3.7.1 GR – Western blotting

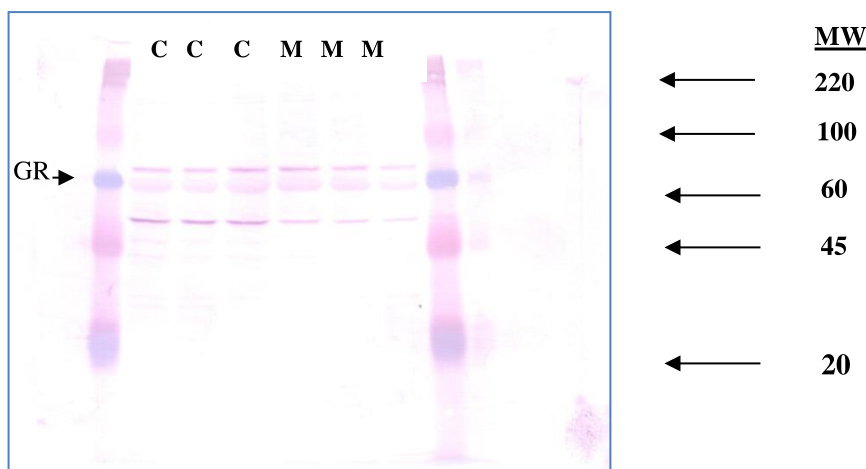


Figure 3.10: GR enzyme expressions. GR bands are shown at 58KDa.

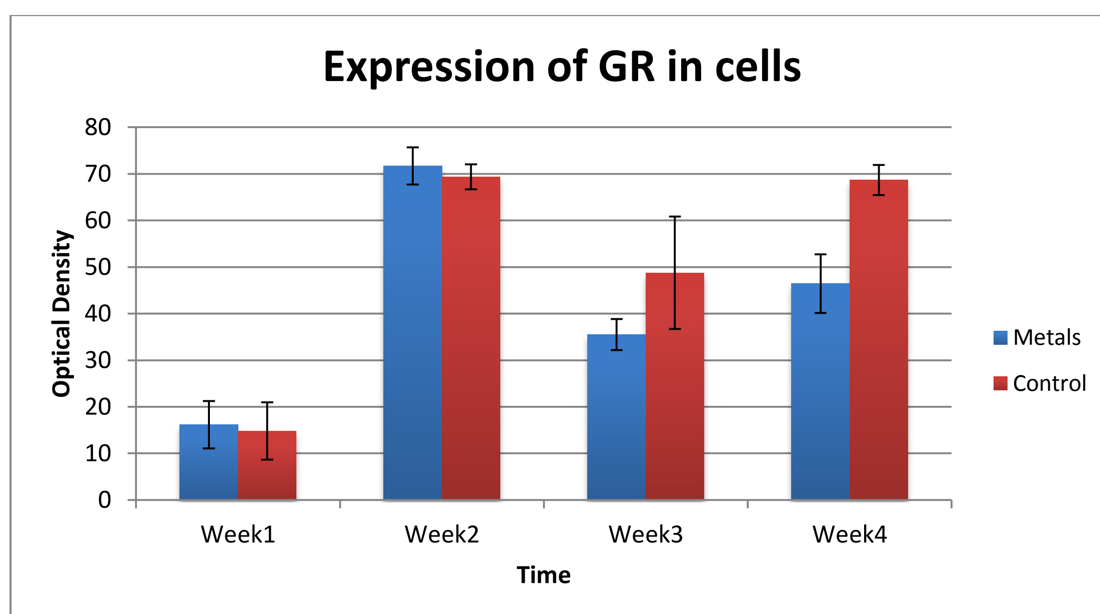


Figure 3.11: GR levels expressed in 3T3 cells incubated in metal containing media were higher than control in 1,2 weeks only, results are mean ± SEM, n=3, no statistical differences were detected, comparison by t-test for each group followed by ANOVA for all groups.

A representative Western blot of GR expression in 3T3 cell line exposed to metal mixture at the end of week 4 is shown (Figure 3.10). Lanes C-C: cells in control medium and

lanes M-M: cells exposed to metal ions. Control cell optical densities at end of week 1: 14.7 ± 6.15; week 2: 69.3 ± 2.68; week 3: 48.7 ± 12.0; week 4: 68.8 ± 3.2 (n=3; Mean ± SEM) Predicted molecular weight: 58 kDa (Abcam, 2011c).

Figure 3.10, shows expression levels of GR enzyme in both control and metal containing medium incubated cells. The level for both groups of cells appeared to be increased in week 2; the levels in cells incubated in metal containing medium was 71.73 ± 4.01 (mean ± SEM, n=3) and controls were 69.36 ± 4.65 (mean ± SEM). For GR expression for both groups cells at week 2 were the highest compared to week1, 3 and 4. It is important to note that exposure to metals caused no significant alteration to the expression of the GR protein at any time point.

GR activity is suppressed at week 2 after treatment with metal containing (Figure 3.12), and a comparison with figure 3.9 reveals that this does not correlate with a decrease in the amount of the GR protein.

3.7.2 GR Activity

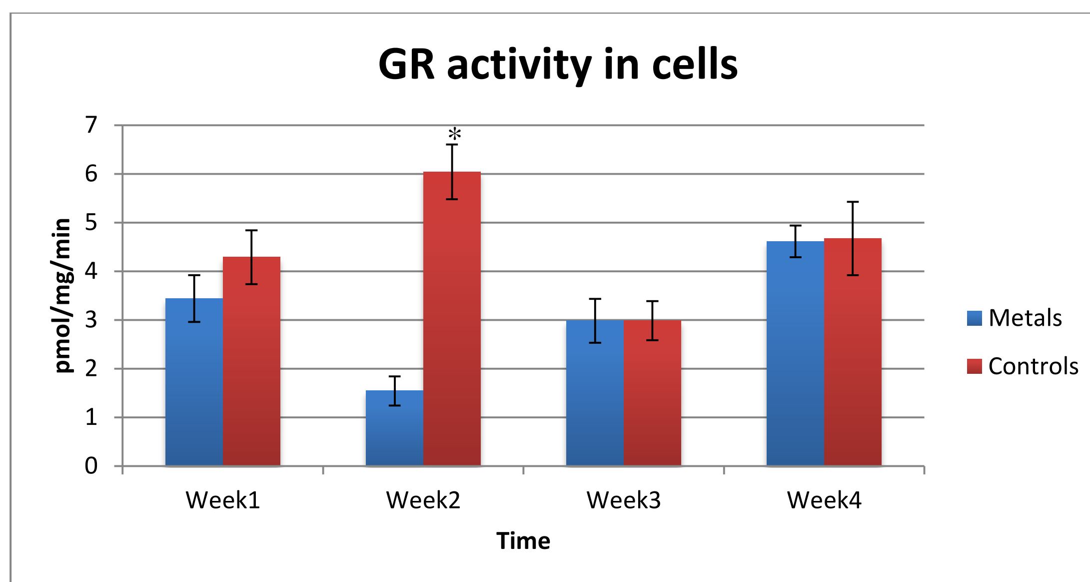


Figure 3.12: GR activity pmol/mg/min.3T3 cells incubated in metal containing media expressed lower levels of GR activity compared to control at 1,2,3 and 4 weeks of exposure. Results are means ± SEM, n=3, comparing values for cells exposed to metal ions with those in control cells in the absence of metal ions by t-test for each group, followed by ANOVA to compare all groups, *p-value <0.05.

Figure 3.12 shows that GR activity are levels higher for control cells compared to cells incubated with metal extracts except for week 3, for example, at week 2, control cells GR levels 6.04 ± 0.56 pmol/ml/min (mean ± SEM, n=3), fibroblasts incubated in metal extract media showed lower levels, 1.54 ± 0.30 pmol/ml/min (mean ± SEM, n=3). This was statistically significant, p-value 0.002.

3.7.3 CAT - Western blotting

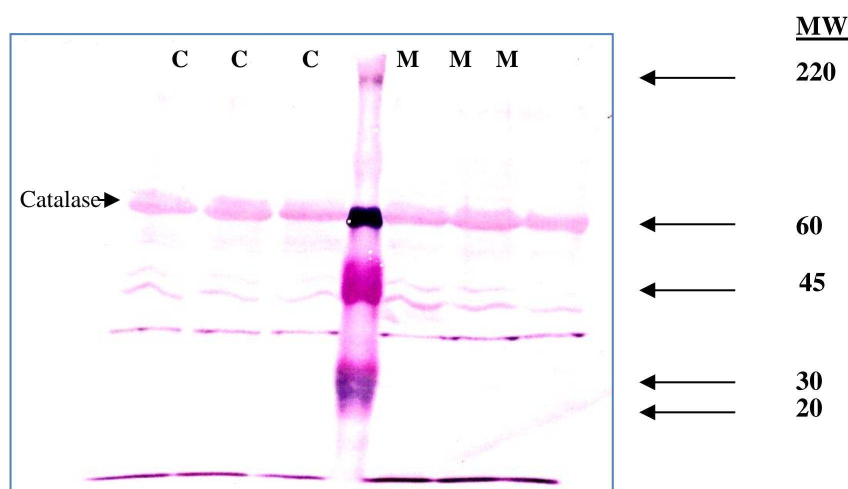


Figure 3.13: CAT enzyme expressions. CAT bands are shown at 60KDa.

A representative Western blot of CAT expression in 3T3 cell line exposed to metal mixture at the end of week 4 is shown (Figure 3.13). Lanes C-C: cells in control medium and lanes M-M: cells exposed to metal ions. Metal exposed cells optical densities at end of week 1: 116.7 ± 6.3; week 2: 89.4 ± 3.8; week 3: 124± 0.4; week 4: 110.6 ± 22.2 (n=3; Mean ± SEM) predicted molecular weight: 60 kDa (Abcam, 2011a)

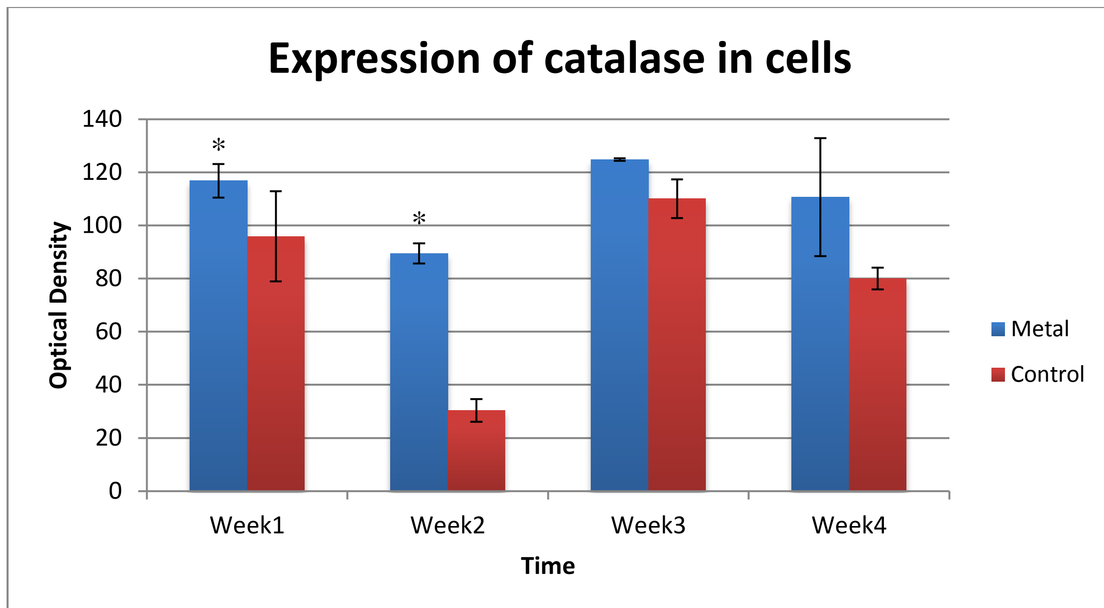


Figure 3.14: CAT levels expressed in 3T3 cells incubated in metal containing media were higher than control in week 1,2,3 and 4. Results are mean ± SEM, n=3, however, no statistical differences were detected at week 3 and 4, comparison by t-test for each group followed by ANOVA for all groups, *p-value <0.05.

Changes in CAT expression observed with 3T3 cell exposed to incubation with metal extracts (Figure 3.14). CAT levels were greater for all 4 weeks with metal incubated cells. CAT levels were highest at week 3. 124.7 ± 0.5 (mean ± SEM) in metal treated cells compared to 110.3 ± 7 (mean ± SEM) in the control. However, statistical significance observed at week 1and 2 only, registered levels first two weeks were, week 1, controls 95.85 ± 16.98 and metals exposed cells116.78 ± 6.34 (mean ± SEM) and week2, controls 30.36 ± 4.25 and 89.44 ± 3.81 in metal exposed cells (mean ± SEM).

3.7.4 CAT Activity

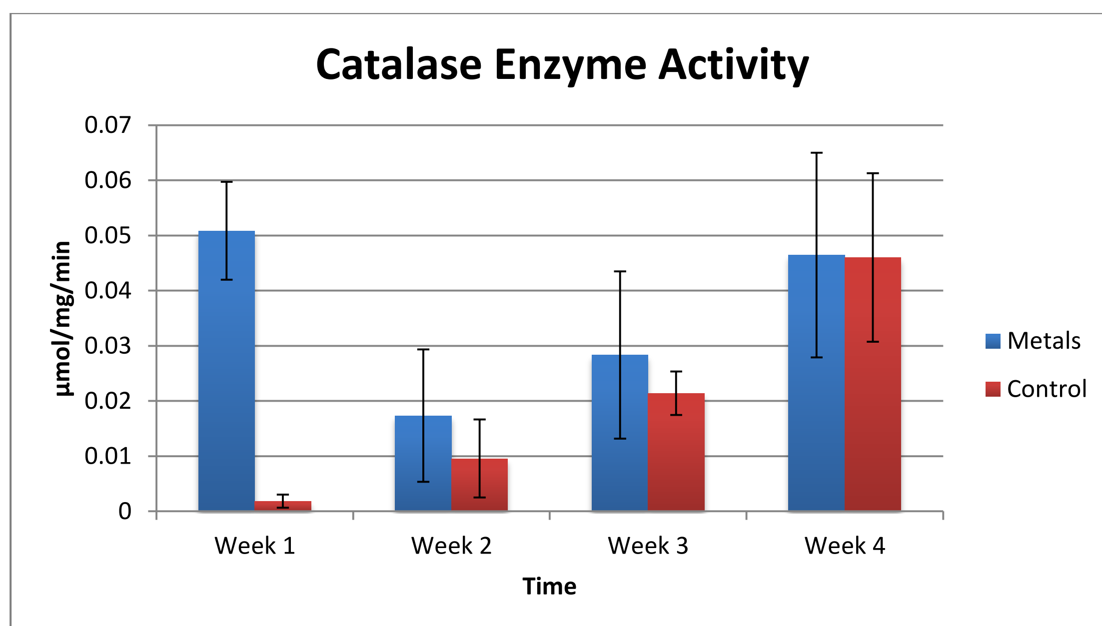


Figure 3.15: CAT enzyme activities µmol/mg/min, in 3T3 cells incubated in metal containing media. Statistical difference was observed in week 1 only. Results are means ± SEM, n=3, Comparing values for cells exposed to metal ions with those in control cells in the absence of metal ions by t-test for each group, followed by ANOVA to compare all groups, *p-value <0.05.

Figure 3.15 shows CAT activity measured over the 4 weeks. Metal exposed 3T3 cells showed greater levels of CAT activity at all-time points compared to controls, with the highest readings observed in the first week 0 0.0405 ± 0.00594 (mean ± SEM, n=3) µmol/mg/min protein respectively. Controls CAT activity was measured highest in the fourth week at 0.046 ± 0.152 (mean ± SEM, n=3) µmol/mg/min protein.

Within 1 week of exposure to the metal ions, the CAT activity in the cells had increased several folds over that in the controls. 0.0508 ± 0.008 (mean ± SEM, n=3) µmol/mg/min in metals, and 0.002 ± 0.011 (mean ± SEM, n=3) µmol/mg/min in control.

This large increase was however, transient, and activity declined (relative to control) thereafter. This large increase in catalase activity was not accompanied by an increase in

catalase protein expression at week 1, but interestingly the expression was dramatically increased at week 2. See Figure 3.14

3.7.5 Super Oxide Dismutase (SODI) – Western blotting

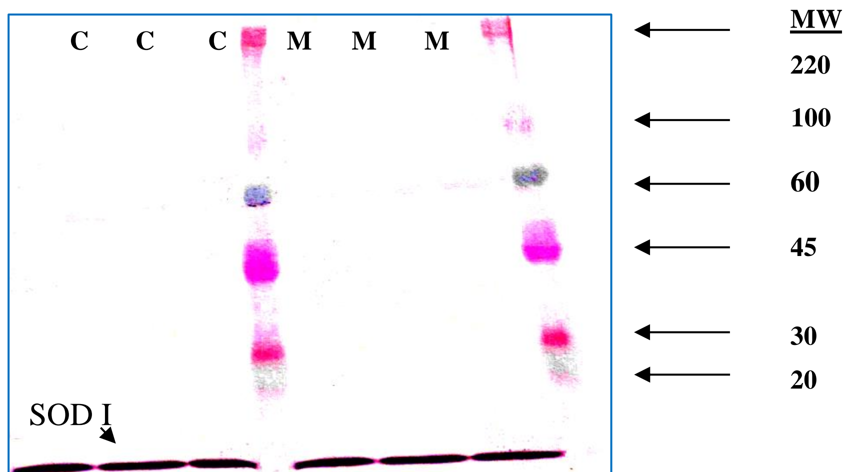


Figure 3.16: SODI enzyme expressions. SODI bands are shown at 17KDa.

A representative Western blot of SODI expression in 3T3 cell line exposed to metal mixture at the end of week 4 is shown (Figure 3.16). Lanes C-C: cells in control medium and lanes M-M: cells exposed to metal ions. Metal exposed cell optical densities at end of week 1: 129.3 ± 2.5; week 2: 132.9 ± 8.7; week 3: 121.7± 2.7; week 4: 155.8 ± 12.05 (n=3; Mean ± SEM) predicted molecular weight: 17 kDa (Abcam, 2011e).

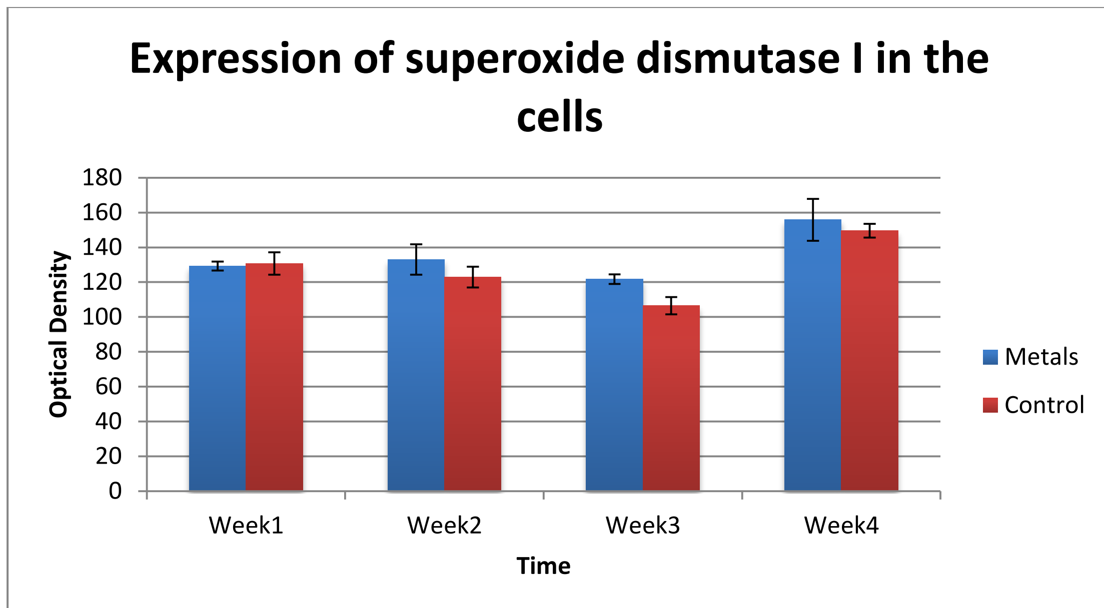


Figure 3.17: Shows that SODI expression is not affected significantly by exposure of the cells to metal ions. Results are mean ± SEM, n=3, no statistical differences were detected, statistical analysis carried out t-Test: Paired Two Sample and ANOVA.

Changes in SODI expression observed with 3T3 cell exposed to incubation with metal containing medium (Figure 3.17). SODI levels were greater for all 4 weeks with metal incubated cells. no statistical significance observed at 4 week. SODI levels were highest at week 4. 155.8 ± 12.05 (mean ± SEM) in metal exposed cells compared to 149.5 ± 3.9 (mean ± SEM) in the control.

3.7.6 SODII - Western blotting

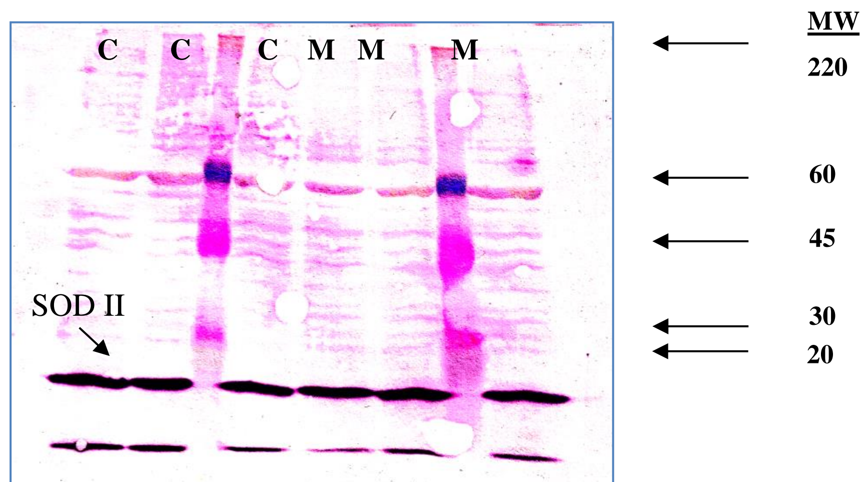


Figure 3.18: SODII enzyme expressions. SODII bands are shown at 25KDa.

Western blot of SODII expression in 3T3 cell line exposed to metal mixture at the end of week 4 is shown in Figure 3.18. Lanes C-C: cells in control medium and lanes M-M: cells exposed to metal ions. Metal exposed cells optical densities at end of week 1: 31.9 ± 5.3; week 2: 4.76 ± 0.31; week 3: 1.62 ± 0.35; week 4: 3.61 ± 5.27 (n=3; Mean ± SEM) Predicted molecular weight: 25 kDa (Abcam, 2011d).

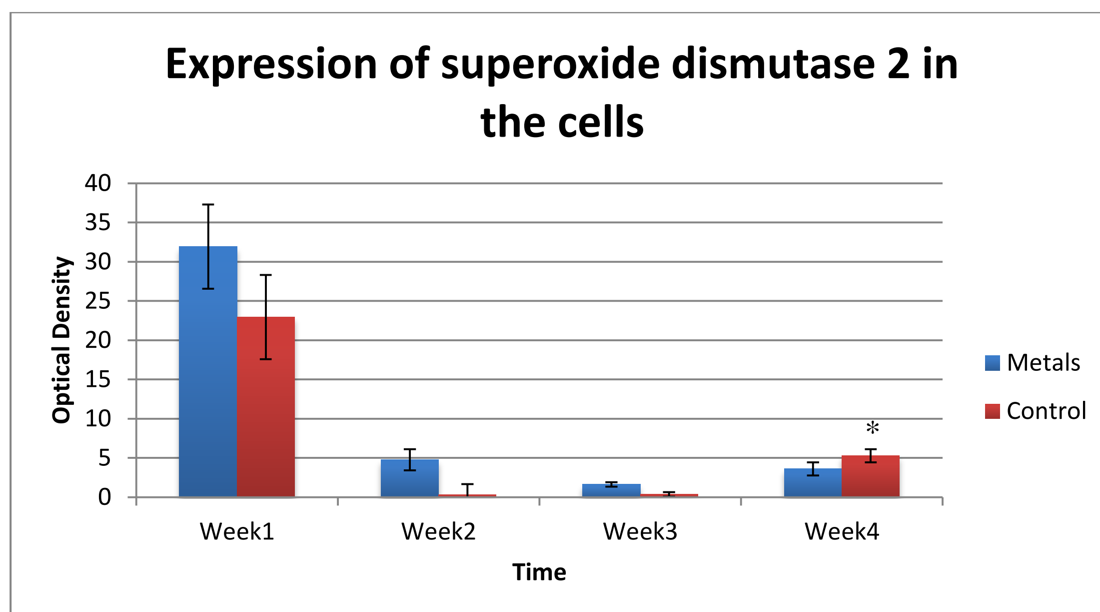


Figure 3.19: SODII expressions in the cells. Results are SEM±, n=3, no statistical differences were detected at week 1,2 and 3, comparison by t-Test and ANOVA, *p-value <0.05.

Interestingly, as shown in Figure 3.19, SODII expression declined as the time in culture increased. The reason for this is not clear, but exposure to the metal ions made little difference to the expression compared to control cells.

3.7.7 Super Oxide Dismutase Enzyme Activity

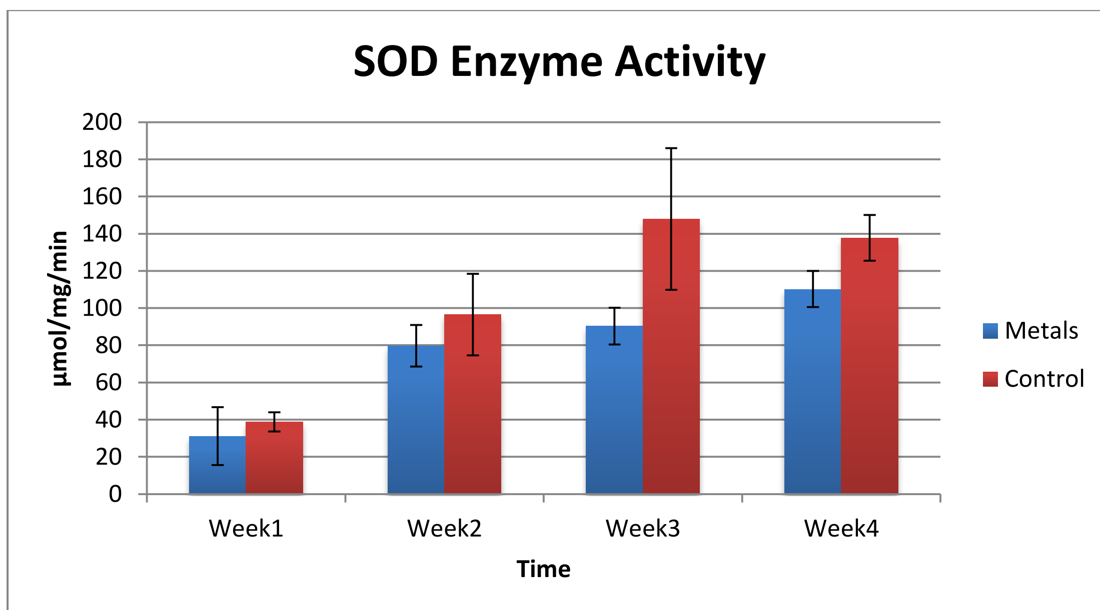


Figure 3.20: SOD enzyme activity, in µmol/mg/min, in the 3T3 cells incubated in metal containing medium. Results are means ± SEM, N=3, no statistical differences were detected, comparison by ANOVA and t-test, p-value <0.05.

SOD activity levels were lower in metal exposed 3T3 fibroblasts at all-time points compared to control. But this effect was not significantly different at any time point. This assay measured the total SOD activity in our samples.

3.7.8 GPx - Western blotting

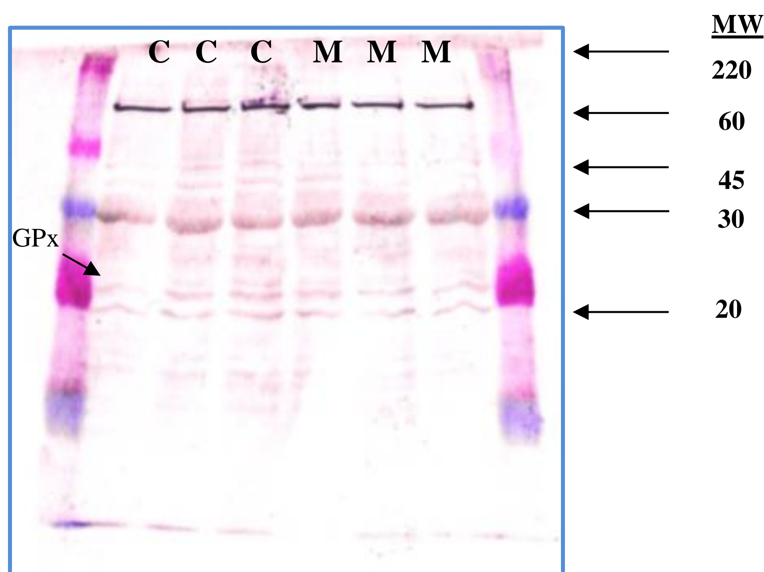


Figure 3.21: GPx enzyme expressions. GPx bands are shown at 22KDa.

Western blot of GPx expression in 3T3 cell line exposed to metal mixture at the end of week 4 is shown in figure 3.21. Lanes C-C: cells in control medium and lanes M-M: cells exposed to metal ions. Metal exposed cells optical densities at end of week 1: 84.2 ± 4.7; week 2: 63.1 ± 1.3; week 3: 84.6 ± 3.1; week 4: 54.8 ± 5.1 (n=3; Mean ± SEM) Predicted molecular weight: 22 kDa (Abcam, 2011b).

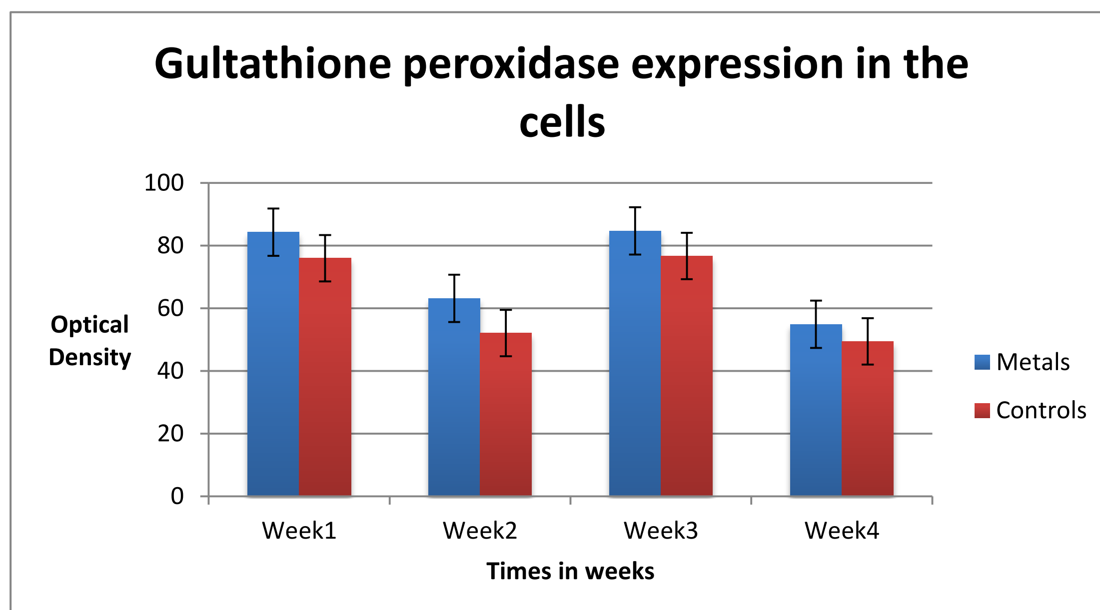


Figure 3.22: Expression of GPx in the cells. Results are SEM±, n=3, no statistical differences were detected, comparison by t-Test and ANOVA.

Figure 3.22 illustrates that exposure to the metal ions for up to 4 weeks has no significant effect on the expression of the GPx relative to that in control cells.

3.7.9 GPx Enzyme Activity

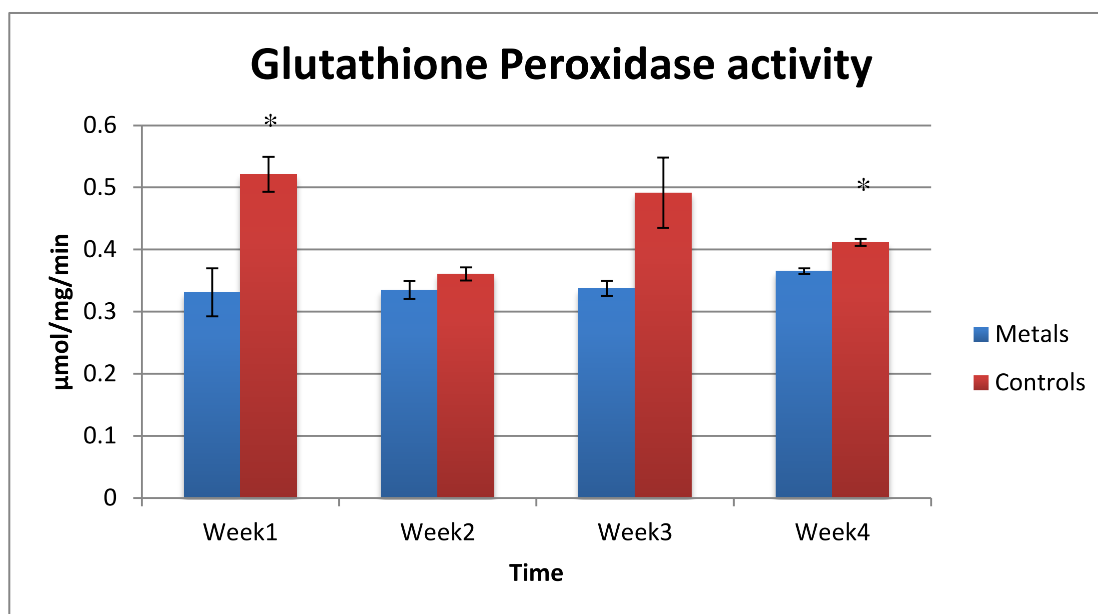


Figure 3.23: Glutathione Peroxidase activity, in µmol/mg/min. Results are means ± SEM, N=3, statistical differences were detected at week 1 and 4, comparison by ANOVA and t-test, *p-value <0.05.

GPx enzyme activity for control 3T3 fibroblasts was significantly higher in weeks 1 and 4 only compared to 3T3s incubated in metal containing medium. Levels of GPx activity for the later increased gradually with time. 3T3s exposed to metal contained highest activity at week 4 0.335 ± 0.009 (mean ± SEM, n=3) µmol/mg/min protein, this was still lower than control 3T3 cells at the same time point 0.356 ± 0.011 (mean ± SEM, n=3) µmol/mg/min protein.

3.8 Summary of metal ions effects on expression and activities of enzymes

Enzyme	Expression	Activity
GR	no effect	transient overall decrease ↓, marked decrease↓ at week 2
CAT	higher in presence of metal, marked increase at week 2	transient increase at week 2
SOD I **SODII**	no affect no affect	activities slightly lower, ↓ but no significant affect
GPx	no affect	activity decreased ↓, but not dramatically

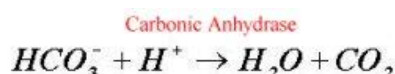
Table 3.4: Expression and activity of summary of antioxidant enzymes

As shown in Table 3.4, only catalase expression was induced by exposure to metal ions, and this was characterised by marked increase at week 2.

This was accompanied by transient increase in catalase activity at week 2. The activity of the GR also had shown marked decrease at week 2, with overall transient decrease. For SOD, there were no effects on enzyme expression with a small decrease in activity. For GPx there was no significant effect on enzyme expression with a small reduction in enzyme activity especially at week 1 and 4. This suggests that in the presence of metal ions the activities of all enzymes are decreased to some extent, a part from the catalase. But the effect was only marked for both GR and GPx, and it is noteworthy that this was not accompanied by a decrease in protein expression.

4. CHAPTER IV: DISCUSSION

In this study, metal ions leaching from copper-based dental alloys NPG / NPG^{+2} (Alba Dent, USA) were quantified and the effects on cells then was evaluated. Dental material specimens were immersed in simulated saliva for 1 week at pH 4, simulating the oral environment that favours dental decay, and at pH 7, neutral conditions when saliva bicarbonate compensates to balance into pH 7. The action of bicarbonate ion to neutralise acid is shown below.



The occurrence of acidogenic, aciduric bacteria in sheltered sites of the oral environment with a fermentable carbohydrate such as glucose, would produce a pH in the region of 4.5, or even lower (Newcastle, 2012). Leaching of the metal ions was quantified at both pH values to simulate different oral conditions and help to predict any cytotoxic effects.

One of the major concerns is the cytotoxic impact of dental metal restorations on patients receiving crowns, crown-bridges and onlays.

3T3 fibroblasts were used in this research. Cell viability after exposure to metal ions was measured by performing sensitive cytotoxic assays, LDH, MTT and NR (Lobo *et al*., 2009). Microscopic evaluation of the cells, and the number of adherent cells at different time points was measured in terms of total cell protein.

The relationship between exposure to metal ions and oxidation stress was assessed (Matés *et al*., 2000). To do this GR, GPx, CAT and SOD, expression levels and activities were measured.

4.1 Metals in the dental alloys investigated and their release into simulated saliva.

NPG and NPG^{+2} dental alloys are used in dental operative procedures. NPG^{+2} considered as a premium base metal type III casting alloy, with 2% Au, mainly for crown preparations, crown-bridges and onlays. NPG is used as premium non-precious type III casting alloy, also used in crowns, crown-bridges and onlays.

ICP-MS was used to assess release of all components of each alloy. This sensitive technique was used to determine which elements were primarily released from dental alloys. In addition, ICP-MS techniques allow less sample throughput this technique has been used in number of studies as sensitive procedure for multi-element determination (MacDonald *et al*., 2004).

The pattern of metal ions leached illustrates clearly that although Cu^{2+} is a prominent ion released on corrosion at both pH values, the increase in corrosion of Ni^{2+}, at pH 4 is more dramatic. The day on which the maximum corrosion occurs is also affected by pH. The least release was observed with Al^{3+}. It is of considerable concern to observe the propensity for Ni^{2+} to be released from the metal alloys. >10% of the population of the UK/USA show allergic response to Ni, and this gives a considerable risk of adverse reaction. (Doctors, 2004)

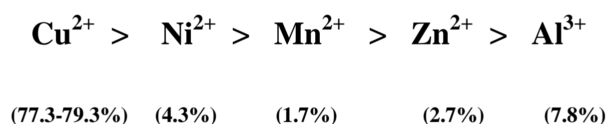
These alloys are still in service despite being categorised as copper-based alloys. However, due to their potential cytotoxicity these materials are not accepted in some EU countries as biomaterials (Rosca *et al*., 2006).

In this study we have found that the presence of gold in NPG^{+2} does not have an effect on the extent of corrosion-metal ion release. Saliva pH has a significant effect on the extent of metal ion release with more ion release measured at pH 4.0 compared to pH 7.0. Adding noble elements to NPG had no advantage on diminishing metal ions leaking. Gold (Au) in NPG^{+2} exhibited no significant effect on increasing the stability of the elements at either pH. Cu^{+} ions were the most abundant leached in simulated saliva at both pH 4.0 and 7.0, considering the amounts of copper in NPG and NPG^{+2} were 80.7

% and 78.7% respectively, this result is to be expected. However, Ni^{+2} reached only 4.3% in both alloy types and showed a 200 fold increase in release at pH 4 compared with pH 7. The increase in release at pH4 was 90 fold in Mn^{2+} and 30 fold for Zn^{+2} compared with pH7. It was observed that the metals have increased leaching pattern in acidic environment.

Former studies have reported several observations; first, metal ions (Ni^{2+}, Cr^{2+}, Mo^{2+}, Fe^{2+} and Al^{3+}) release from nickel-based alloys into the biological medium (α-MEM supplemented with 5% foetal calf serum at 37°C in a humidified 5% CO_2 atmosphere) is higher at the initial stages (Messer and Lucas, 2000), (Lappalainen and Yli-Urpo, 1987). This result was observed and reported for a diversity of dental casting alloys. Secondly, Ni ions are liable to leach at higher rate in contrast to other elements in dental alloys(Covington *et al.*, 1985). In a previous study, it has been shown that alloys with high content of noble elements such as Au, have relative stability in low pH environments (Wataha *et al.*, 1992). In the present study I did not observe any significant effect of Au on the maximum release of the metal ions from the alloys.

The pattern of leaching of each individual metal ion is shown in the results chapter and it has been shown that pH4 has considerable effect on the leaching of the ions. In all cases leaching was increased as shown in the results section on Table 3.2. In order of increasing ion release in corrosion of the alloy at either pH:-



In brackets under each metal is given the percentage in terms of the alloy composition. By comparing the order in terms of release on corrosion of the alloy, and its composition, the major conclusions are:

(i) Cu^{2+} is the predominant metal in the alloy, and that it is released to the greater extent is not unexpected.

(ii) Ni^{2+} and Mn^{2+} are released to a greater extent than might be predicted from alloy composition.

(iii) Al^{3+} is stable alloy component, and release is less than expected, it is the same case with Zn^{2+}.

In summary:

- pH 4.0 increases the leaching of all metals from the alloy compared with pH7.
- The presence of gold in the alloy does not markedly alter the leaching of the other ions.
- Each metal has different pattern of leaching from dental alloys.
- This may also suggest different cytological effect on fibroblasts.
- Cu^{2+} and Ni^{2+} being most observed leached metal ions.

The effects on cell viability were assessed using the MTT assay, the NR assay, LDH leakage and total adherent protein levels.

4.2 Observing 3T3 fibroblast viability

Cells incubated in both metal and control media were examined for viability. It was originally my intention to use the metal extracts generated in the simulated saliva to measure the effect on 3T3 cells, but when cells were exposed to the extracts at pH 4.0, the pH caused a cytotoxic/cytostatic effect.

To separate the effects of pH *per se*, and metal ion concentrations, a mixture of metals was prepared in PBS, and this was used to assess potential cytotoxicity. The concentrations of the metals were chosen to represent the maximum leaching observed from the alloy at pH4.

In order to ensure that the concentrations of the metals in this mixture were not overly toxic to the cells, preliminary experiments were carried out by Mrs Katie Henderson, and indicated that the LD_{50} concentration of Cu^{2+} to 3T3 cells was 115µM. Based on these data a 4 week exposure to the metal ion mixture was planned, the design of which is shown in the methods chapter.

In this study it was observed that fibroblasts incubated in metal containing medium have shown lower MTT reduction compared to control in week 2, 3 and 4 (0.109, 0.234 and 0.083).

3T3 fibroblasts exposed to metal ions showed less viable activity compared to controls at each time point apart from week 1. Low cell viability been observed in previous studies, Ni^{+2} can act as an antagonist to essential metal ions like Mg^{+2}, Ca^{+2}, and Zn^{+2} and disturb the biological processes.

Similarly, Cu^{+2} (26.6 µg/l in 7 days) also induces oxidative stress within the cell, which results in severe loss of cell viability; this was determined by (Haider *et al*., 2011) using SRB (Sulforhodamine B cytotoxicity screening) assay. Loss of viability may also be related to Ni^{2+}, which is a potent cytotoxic element. A study by (Bumgardner *et al*., 1989) has revealed that cellular viability and proliferation are affected by the presence of 14.70 ± 2.51 ppm Cu^{2+} and Ni^{2+} in medium. Cell count may be affected by such levels of metal stress existed in the culture medium. A study carried out on epit**h**elial cell has revealed that Zn^{+2} concentrations of 100 mM were sufficient for induction of apoptosis following 24h exposure (Untergasser *et al*., 2000).

Zn^{+2} was observed in several previous studies to be corroding at certain pH levels ranging from 1.0-8.0 (Geurtsen, 2002). Zn^{+2} ions were observed to induce a decrease in mitochondrial succinate dehydrogenase activity and cell viability. Studies have suggested that aluminium may promote cell prolifer**a**tion by triggering a decrease in protein and mRNA EpoR levels and inhibiting Epo anti-apoptotic action (Vittori *et al*., 2005). On the contrary other studies have observed the opposite, after exposure to Al^{3+} parathyroid glands cells in rats showed lower cell proliferation than control without apoptotic events being detected (Gonzalez-Suarez *et al*., 2003).

Results have shown that MTT reduction decreased markedly with time in culture. It is not clear why the MTT reduction was decreased so markedly after 1 week exposure, but we propose that oxidative stress occurred within the cells, and lead to a disturbance in the availability of NADH/NADPH. Reduction of MTT is absolutely dependent on the availability of these cofactors for electron transfer to the dye.

The effect is significant, and the cells continued to demonstrate reduction in viability and deal with the oxidative imbalance. After week 1 the MTT reduction declined, and was opposite to the situation in control cells. The decrease after the first week of exposure may be a function of chronic cytotoxicity, either through oxidation or other mechanisms.

When exposure time extended from day1 to day 7, manganese, zinc and aluminium ions (Liu and Ng, 2000) may have induced anti-proliferative effects *in vitro*. Further studies have shown that Mn^{+2} at concentration of 250 mM (MnCl2) may induce DNA aggregation (Polyanichko *et al*., 2004). In a former study the effect of aluminium ions on fibroblasts at 10 μM was shown. This significantly reduced the growth of fibroblast-like marrow stromal cells within 3 days when they were cultured and the effect was reliably seen in 1 week and 2 weeks of culture (Kidder *et al*., 1993).

Analysing 3T3 fibroblast survival with NR assay revealed that cells incubated in metal containing media demonstrate a transient decrease after 1 week exposure in NR binding to lysosomes, which appears to be normalised at week 2-3, then continued to decline during week 4.

Cells demonstrated lower ability to bind NR to their lysosomes compared to that in control. In (Paasche *et al*., 2011) study, the reduced cell (NIH/3T3 and L-929 fibroblasts and PC-12) survival after 48h exposure to copper, zinc and silver ions at concentration of 0.3 μmol/l and 10 mmol/l, indicating that these ions might be promising for reducing tissue growth on the surface of CI (Cochlear Implantation) electrode arrays. These effects were also observed when combinations of two of these ions were investigated.

It has also been suggested that cells grown in metal containing medium had intact cell membrane with viable lysosomes (Repetto *et al*., 2008). Further evidence for this comes from the LDH assay. Lactate dehydrogenase protein molecules will leak once the cell membrane damaged. In this study, LDH leakage was taken as an index to analyse the cytological effect of metals in fibroblasts.

The viability of cultured 3T3 monolayer was measured non-invasively by measuring LDH activity in culture medium (Decker and Lohmann-Matthes, 1988). In this study the LDH levels for 3T3 cells incubated in metal containing medium were surprisingly slightly lower than those in control. Increased levels of LDH enzyme in medium indicate cell damage or lysis (Fotakis and Timbrell, 2006). These results suggest that 3T3 cells exposed to metals in their medium retained cell membrane integrity and tolerated the levels of metal ions presented in culture medium.

A more sensitive index of cytotoxicity compared to LDH activity is protein content measurement (Macnair *et al*., 1997). This measures the number of cells that are attached to the flasks, 3T3 cells damaged by metals leaking from NPG and NPG^{+2} dental alloys floated in culture medium after detaching from the surface of the flask.

In this study we used Lowry assay (Lowry *et al*., 1951) to measure the concentration of attached protein in both control and metal extracts treated 3T3 fibroblasts. Although a trend of decline in **a**ttached protein content was observed during time of exposure for metal exposed cells, these results suggest that during 4 weeks these fibroblasts sustained their viability.

4.3 The effect of exposure to metal ions on enzymes expression and activity

In this research the measured enzymes were used as indices of oxidative stress. Their roles are summarized in results chapter. The evidence that oxidative stress has occurred in the cells after exposure to metal ions is that the expression and activity of CAT is increased after 2 weeks of exposure to metals. Maximum effect is reached at week 3 (124.8 ± 0.5 mean ± SEM). The expression of rest of the enzymes is not affected, and their activities are decreased.

In the case of SOD and GPx, the decrease in activity is slight. GR activity is decreased transiently at week 2 (1.53 pmol/mg/min ± 0.30 mean ±SEM), and this may be due to a decrease in availability of GSH for the reaction. As NR assay is highly reliable, (Garcia-Fernandez *et al*., 2002) had shown using fractions of the NR_{50} concentrations ($NR_{6.25}$, $NR_{12.5}$ and NR_{25}) to determine the biochemical parameters, the total GSH

which fell below control values at NR_{25} concentrations of metals, and cellular GSSG content declined significantly at all metal concentrations (Hg^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+}).

GSH as an antioxidant against toxicity is also a cellular target for metal toxicities. *In vivo* experiments had shown that exposure to metals decreases the levels of GSH.

This decrease is related to the formation of metal-GSH complexes, and/or consumption by GSHpx reaction under metal induced oxidative stress (Massaro, 1997).

It was observed in this study that GR levels for 3T3 cells incubated in metal containing medium were lower than control. A sharp decrease in GR levels was noticed after 14 days of exposure to 0.0015μmol/mg; controls have shown 0.0060 μmol/mg for the same period. However, GR levels start to increase with time for cell in metal treated medium, the opposite occurred with controls. At week 4 both cells had similar GR levels 0.0046 μmol/mg.

Oxidative stress generated from adding metal extracts to 3T3 culture medium has increased generation of free radicals and/or the impaired antioxidant defences of endogenous compensatory response, oxidative stress could lead to decreased activity of antioxidant enzymes including GR, SOD and Vit E (Taheri *et al*., 2012).

Cu, Co and Ni each can form a complex with GSSG (Formicka-Kozlowska *et al*., 1979) vanadyl cation can bind to both GSSG and GSH (Cohen *et al*., 1987). GR expression of 3T3 fibroblast incubated in metal extracts for the first two weeks was slightly greater than those in control. Then this was decreased in week 3 and 4, for those controls expressed higher levels of GR expression.

(Cartana *et al*., 1992) have observed that, in rat (*Rattus sp*) liver and kidney cells Ni (3.3 ± 0.4 μg/g) administration causes a transient drop in hepatic GSH levels, followed by a notable increase. It was also observed that effects of this metal on GSH metabolism showed conflicting results, as for Cu^{2+} (5.37 ± 0.45 μg/g) toxicity induces GSH depletion and inhibition of several enzymes associated with GSH metabolism.

It was shown that depletion of glutathione impairs cell proliferation in 3T3 fibroblasts. The latter also decreases with increased levels of oxidation stress studying the oxidative stress was performed by measuring the activity and expression levels antioxidant enzymes in the 3T3 cell. GR levels were increased after 6 days to 0.112 mU/mg compared to 0.093 mU/mg in control (Markovic *et al.*, 2009). This is similar to my findings in this study; GR activity levels in metal treated medium were slightly higher than those in control.

Observations from former studies have shown that metals, including Cu, Fe and Cr undergo redox cycling, while Ni and Hg lead to diminished glutathione and protein-bond Sulphydryl (SH) group (Stohs and Bagchi, 1995). In this study Cu^{2+} (18-25.5 µM) was measured leaching from metal alloys (NPG and NPG^{+2}), 100 µM Cu^{2+} was found to develop hydroquinone (HQ) induced toxicity in stromal bone cells as shown in (Li and Trush, 1993) study, which also revealed Cu was shown to significantly accelerate the oxidation of hydroquinone to benzoquinone in a concentration-dependent manner.

The incubation of Ni^{2+} (2mM) with cultured 3T3 cells results in a dose-dependent decrease in cytoskeletal protein sulphhydryls as well as cellular glutathione contents. This study has shown that Ni^{2+} (0.02 – 0.04 mM) transiently induced a reduction in GR expression at week 3-4, and, in activity at in all time points, and this is possibly explained by the findings of (Li *et al.*, 1993). One antioxidative role of CAT is to lower the risk of hydroxyl radical formation from H_2O_2 via the fentoreaction catalysed by Cu^{2+} and Fe^{2+} (Halliwell, 1999).

It was observed in this study that catalase has undergone up-regulation in terms of expression, similarly the activity was increased. This would therefore act as an important cytoprotective mechanism against metal-induced hydroxyl radical formation.

CAT enzyme expression revealed that 3T3 fibroblasts were up-regulated when exposed to metal stress. Compared to control, cells cultured in metal containing medium have shown significant difference in week 1 and 2, while highest level of CAT expression in week 3. Results suggest that fibroblast were under oxidation stress that CAT reacted to ROS generated H_2O_2 in progressive pattern. Previous study by (Simon *et al.*, 1981) have shown that CAT but not GPx or SOD was able to protect the human fibroblasts

against free radicals generated by the acetaldehyde-xanthine oxidase system, (Michiels *et al*., 1994). Findings from (Kim *et al*., 2009) had shown that the mRNA level of catalase increased by 11-fold in HepG2 cells after a 24 h treatment with metals (AgNPs) and that $AgNO_3$-induced catalase mRNA expression increases of 26- and 44-fold at 8 and 24 h after treatment.

Variations of the antioxidant enzyme level under environmental stresses are suggested as indicators of biotic and abiotic stress. This was studied in (Lijun *et al*., 2005) which revealed that CAT played an important role in the protection mechanisms of reactive oxygen species. It was suggested in the study of (Michiels *et al*., 1994) that during exposure of cells to oxidative stresses, the cellular defensive mechanism responds with an induction of catalase enzyme. SOD enzyme was used as an antioxidant stress biomarker in rat tissue exposed to H.M.M (heavy metal mixture), and changes in the levels of antioxidants enzymes were noticed when toxicants given in combination (Tabrez and Ahmad, 2011).

(Kim *et al*., 1996) have examined *Streptomyces coelicolor* for the effects of metals and chelating agents on the expression of SOD I and II which were detected on native PAGE. Media containing Ni at micromolecular concentration induced the expression of SODI (NiSOD); however, the expression of SODII (FeZnSOD) was suppressed, suggesting that metals do not modulate the activity per se but the amount of each protein. Those findings are consistent with this study results on SODI and SODII expression, where SODI showed increased levels in week 2, 3 and 4, while SODII levels were higher in week 1, 2 and 3.

(Brown *et al*., 2004) have demonstrated that oxidative stress induces SODI to undergo upregulation via transcriptional mechanisms. In human HepG2 hepatoma cells, it was observed that the activity of a chloramphenicol acetyltransferase reporter gene was found to increase 3 to 4 fold in the presence of Cu, Zn and Cd, this resulted in increased SODI expression (Yoo *et al*., 1999).

In my study Cu^{2+} was the most abundant metal ion leached from both alloy. SODI showed transient increase expression levels in week 2, 3 and 4. SODII levels were higher in week 1, 2 and 3. Antioxidant enzymes work in synergy to provide global

cytoprotection for the cells. This suggests that although the copper ions leached considerably. This might be related to the compensating cellular mechanism of expressing another enzyme to adapt to the oxidative stress (Michiels *et al*., 1994).

Cell growth and division is directly connected to oxidative stress and SOD levels (McCord, 2000), 3T3 cells incubated in metal containing media, expressed lower proliferation rate with the progress of this experiment with transient decrease in Superoxide dismutase. This suggests that with progression in oxidative stress for 4 weeks, cell division decreased in conjunction with fall in SOD activity.

In Pancreatic islets and RINm5F cells (Tiedge *et al*., 1997) have shown that increasing glutathione peroxidase and catalase enzyme expression through stable transfection induced 10-fold increase in catalase enzyme activity, while GPx was less effective. Pancreatic islets have responded to H_2O_2 oxidative stress by over expressing the catalase enzyme which seems to be critical importance for cytoprotection mechanism. In this study GPx expression in 3T3 fibroblast cells was increased at each time point, however, the enzyme activity was decreased in all 4 weeks, with the lowest observation in week 1 (0.33 ±0.04 umol/mg/min, mean ± SEM). My results appear to have shown that metal ions do not modulate antioxidant enzyme activity per se but the amount of each enzyme protein, possibly through regulating transcription mechanisms.

CONCLUSION

NPG and NPG^{+2} dental alloys undergo corrosion when at both pH 7 and pH4. Regardless of metal proportions in each dental alloy, different metals have diverse leaching behaviour. In terms of leaching $Cu^{2+}>Ni^{2+}>Mn^{2+}>Zn^{2+}>Al^{3+}$. Acidic pH4 promotes metal corrosion. 3T3 fibroblast cells growth was inhibited when cultured in metal containing medium. Adding 2% gold to dental alloys have no significant effect on controlling metal corrosion.

It was not clear which metal ion triggers which enzyme, however, metal ions affect the activities of enzymes; those were decreased to some extent apart from the catalase. Antioxidant enzymes work in synergy to provide global cellular cytoprotection. This favours the expression of certain enzymes over others. To cope with oxidative stress resulting from exposure of 3T3 fibroblasts to metal containing media catalase enzyme was significantly expressed at the first two time points; after 1 and 2 weeks exposure to metals. The increased catalase improves cytoprotection against metal induced hydroxyl radicals.

In this *vitro* study, I have focused on certain type of metal ions to initiate the oxidation stress and targeting 3T3 cells. Further factors like temperature, nutrient depletion and incorporating different metals, may contribute to potentiate an existing oxidative stress on cells. Those already stressed cells may respond differently to metal ions. This would be worth investigating in future studies.

Using these alloys for healthy compromised patients, i.e. those taking immunosuppressants, or experiencing chronic disorders and diseases, may present potential health risks. My research may provide better understanding of the metabolic response of fibroblasts to ions released from dental alloys. NPG dental alloys are still imported and used in the UK. The NHS is encouraging the use of alloy containing 30% gold. This is important to develop more reliable dental alloys for casting dental restorations for patients, whether in NHS or in private dentistry.

PRESENTATION FROM THIS WORK

- BTS Annual Congress 2011 “Release of Soluble Metal ions from Copper based Dental Alloys measured by ICPMS” P027, the 32nd Spring meeting of the British Society joint with the Dutch Society for Toxicology (NVTS). 27-30 March 2011, Durham University, UK.

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5. APPENDIX A: Solutions and reagents used in this project

1. Versene/EDTA in PBS, pH 7.2

Compound	Weight
NaCl	12g
KCl	0.3g
Na_2HPO_4	1.73g (anhydrous)
KH_2PO_4	0.3g
EDTA	0.3g
Phenol Red	1% (w/v) 2.25ml

2. TRIS Buffered Saline (TBS) for trypsin stock

Compound	Weight
NaCl	4g
Na2HPO4	0.05g
Glucose	1.5g
KCl	0.19g
Phenol Red	1.5ml of 5% solution
Penicillin	0.03g
Streptomycin	0.05g

3. Freezing Medium

Material	Volume
Sterile medium	20ml
Sterile Foetal Calf FCS	25ml
Dimethyl Sulfoxide DMSO	5ml

4. LDH assay

Buffer pH 7.0	Solution 1	Solution 2
1M NaPi	14.2g of 0.2M Na_2HPO_4	2.4g of 0.2M Na_2HPO_4
Distilled water	500ml	100ml
13ml of 0.2M NaH_2PO_4 were mixed with 87ml of 0.2M Na_2HPO_4, to obtain buffer of pH 7.6. The pH was checked then, and adjusted with either solution as required. 100ml deionised water was used to dilute the buffer. Freshly made of day of experiment, 3mg NADH + 3mg pyruvic acid in 1ml of buffer.		

5. Setting up Lowry assay reagents and standard curve

Stock	Reaction A	Reaction B
0.5M NaOH	1ml 1% $CuSO_4$	4-fold dilution of Folin's reagent + distilled water
1% (w/v) $CuSO_4$	1ml 2% Na-K tartrate	
2% (w/v) Na – K tartrate	98ml 2% Na_2CO_3	
2% (w/v) Na_2CO_3		
Folin's (Ciocalteau) reagent		
0.02% (w/v) BSA Solution was stored at -20°C	solutions A and B were prepared on the day of the assay	

Lowry assay standard curve

0.5M NaOH (ml)	1.0	0.875	0.75	0.50	0.25	0
BSA (ml)	0	0.125	0.25	0.50	0.75	1.0
µg/ml protein concentration	0	25	50	100	150	200

6. **Setting up GR assay procedure**

0.2 potassium phosphate buffer, pH 7.0, containing 2nM EDTA	**NADPH 2mM in 10mM [pH 7.0, Tris-HCl (121.14mg Tris/100ml)**	**GSSG, 20 mMin water (65.6mg/5ml)**
KH2PO4 (6.81 g/500ml distilled water) and K2HPO4 (17.4g/500ml distilled water) were prepared as indicated. 50 ml of both solutions were mixed to give 100 ml, and 74.4 mg EDTA was added. Using the two phosphate solutions, the pH was adjusted.	To obtain a final concentration of 0.1mM,10 mg NADPH was dissolved in 5.9ml Tris	1mM was the final concentration used for this assay.

7. **Setting up CAT assay procedure**

Reagents used in CAT assay

	Reference	**Sample**
Cell homogenate	250µl	250µl
0.08% Triton X	100µl	100µl
50mM phosphate buffer	2.65ml	1.65ml
30mM H_2O_2	---------	1ml

Solutions used in CAT assay

	Solution A	Solution B
Phosphate buffer	50mM, pH 7.0	
KH_2PO_4	1.36g in 200ml DW	
$Na_2HPO_4.2H_2O$	1.78g in 200ml DW	
	100ml of KH_2PO_4 solution was mixed with $Na_2HPO_4.2H_2O$ solution at ratio of [1:1.5 (v/v)]	
H_2O_2		**30mM (0.34ml 30% H_2O_2 in 100ml buffer)**

8. **Setting SOD assay and standard curve:**

Solutions				Reaction buffer solution
A	**B**	**C**	**D**	
216 mM Potassium phosphate buffer, pH 7.8 at 25°C temperature. (50 ml was made of 1.47g KH2PO4 in 30 ml distilled water; pH was adjusted with 1M KOH).	10.7mM EDTA	1.1 mM Cytochrome c, Xanthine oxidase (XO: 0.05 U/ml) and SOD (10 U/ml in buffer) were freshly made and kept on ice	0.108 mM Xanthine, this was made as 8.21mg in 500 ml distilled water, titrated into solution with 1M KOH	**5.75 distilled water** **6.25ml Phosphate buffer (A)** **0.5ml EDTA (B)** **0.5ml Cyt c (C)** **25 ml Xanthine (D)**

	SOD Standards (U/ml)				
	1	2.5	5	7.5	10
Reaction buffer (ml)	2.8	2.8	2.8	2.8	2.8
Distilled water dH_2O (µl)	90	75	50	25	0
XO (µl)	100	100	100	100	100
SOD (µl)	10	25	50	75	100

9. Stock Solutions used to prepare GPx assay:

H_2O_2	**5mM**	**5mM= 1ml 8.8M stock /200ml dH_2O**
GSH	100mM	30.7 mg/ml
Glutathione Reducatase	1.4U/sample	1.4U/30µl
Cumene peroxidase	24mM	100µl in 23.3ml dH_2O
Aminotriazole	100mM	16.82mg/2ml
NADPH	28.6mM	47.7mg/2ml
KCN	75mM	48.8mg/10ml
0.1M Phosphate buffer, pH 7.0		

10. Reagents and solutions used in preparation of immunoblotting assay

Solutions	Volumes and concentration
Gelatin	
Tween 20	
Acrylamide – Bisacrylamide	40% (w/v) sol
Tetramethylethylenediamine TEMED	
Ammonium persulphate (APS)	0.075mg 1.5%
Stacking gel buffer	0.5M Tris buffer pH 6.8
Resolving gel buffer	1.5M 0.5M Tris buffer pH 8.8
Primary antibodies (Abcam Ltd., UK)	Rabbit polyclonal to GR (1:1000 dilution) Rabbit polyclonal to GPx (1:1000 dilution) Rabbit polyclonal to SOD (1:1000 dilution) Rabbit polyclonal to Catalase (1:1000 dilution
Secondary antibodies were conjugated with alkaline phosphatase	Anti-rabbit (1:3000 dilution)
TTBS	Tween tris-buffered saline
Electrophoresis buffer 10x	10x Tris/Glycine sodiumdodecylsulphate (SDS) buffer: This contained 0.25M Tris/ pH 8.3 1.92M Glycine 1% SDS
BioRad	Alkaline phosphatase detection system
TBS Tris-buffered saline 10x	5M NaCl: 292.2g/l 200mM Tris: 24.22g/l
Laemmli buffer	It contained 1ml 0.5M Tris at pH 6.8; 1.6ml 10% w/v) SDS; 0.8ml glycerol; 0.4 ml β-mercaptoethanol; 0.4ml dH2O and 0.2ml 0.05% (w/v) bromophenol blue
Tris –Glycine buffer 10x, pH 8.3	0.25M Tris : 30g/l 1.92M Glycine: 144g/l
Towbin's buffer -Transfer buffer	2100 ml dH2O 300ml / Glycine buffer 10x 600ml methanol

Gels solutions

Solutions	Stacking gel	10% Resolving gel
Distilled water	3ml	20.25ml
Acrylamide/bis acrylamide (40% (w/v) solution)	0.5ml	11.25ml
Stacking gel buffer	1.25ml	-
Resolving gel buffer	-	11.25ml
1.5 % APS (75mg/5ml dH_2O)	0.25ml	2.25ml
TEMED	5µl	34.5µl
***Ammonium persulphate (APS) was freshly made, and before casting each gel TEMED was added.**		

APS was freshly made; TEMED was added before casting each gel.

Detection system preparation system

Solutions	Volumes
24 ml dH2O + 1ml development buffer	25 ml
Removal of 0.5ml of this	- 0.5ml
	24.5ml
Addition of 0.25ml A-solution	+ 0.25ml
Addition of 0.25ml B-solution	+ 0.25ml
Total volume	25ml