Investigating the potential neuroprotective effects of statins on DNA damage in mice striatum

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To **God,** our loving Father, who has created all things, all grace and glory be.

For ever since the creation of the world His invisible
nature and attributes, that is, His eternal power and divinity,
have been made intelligible and clearly discernible
in and through the things that have been made.
(Romans 1:20)

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Abstract

Parkinson's disease occurs after loss of the nigrostriatal neurons responsible for regulating normal motor function (Chase, *et al.*, 1998).

Oxidative damage to DNA is caused by endogenous cellular sources (Marnett, 2000) such as hydrogen peroxide, which is extremely reactive and can add bases to or abstract hydrogen atoms from DNA (Cooke, *et al.*, 2006). There therefore exists a baseline level of DNA damage (Marnett, 2000) which is continually being repaired. This process is critical to the survival of all cells and a failure to protect the genome would result in the induction of mutations leading to cell death (Cooke, *et al.*, 2006).

Current treatment of Parkinson's disease focuses on symptomatic management with dopaminergic drugs such as L-DOPA. This approach is only highly effective in the early stages of the disorder and long-term treatment often loses its efficacy (Jenner, 2003) and leads to the occurrence of side-effects. The challenge is to find methods to conserve and protect the nigrostriatal neurons, thereby preventing the onset of Parkinson's disease.

The widening role of the statin drugs, used in the treatment of dyslipidaemias (Hamelin and Turgeon, 1998), has been the subject of recent studies and they have as such been shown to reduce LDL oxidation, preserve endogenous superoxide dismutase, increase α-tocopherol (an antioxidant), reduce lipoprotein oxidation in a number of oxidative systems, protect against DNA damage caused by antineoplastic agents, and to reduce DNA damage in hypercholesterolemic patients (Shin, *et al.*, 2005).

We therefore investigated the potential neuroprotective effect of selected statins drugs (pravastatin, simvastatin and atorvastatin) on the striatum. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) treated C57Bl/J6 mice were used as an animal model to replicate Parkinson's disease. MPTP is a neurotoxin which causes selective neuronal death in the striatum through the inhibition of mitochondrial complex I.

Groups of ten mice were treated with 70 mg / kg of pravastatin, simvastatin, atorvastatin or no drug (control group) for five consecutive days. Five mice from each group received an "immediate onset PD model for rapid degeneration with necrotic cell death" dose of MPTP (50 mg / kg) intraperitonially. After decapitation the striatum was isolated and analysed.

The immediate state of DNA damage in the tissue (baseline damage) was determined using the microgel electrophoresis (comet) assay. Further DNA damage was induced by treating the sample with H_2O_2 for thirty minutes after which the process was stopped and the DNA damage determined. Two more comet assays were performed at twenty minute intervals to determine the amount of repair that took place.

MPTP treatment increased the level of DNA damage in the striatum. Treatment with statins also increased levels of DNA damage, but left the repair processes intact, increasing the amount of repair that took place as well. The DNA repair of mice treated with MPTP and statins, however was decreased.

The results obtained do not substantiate the hypothesis that the beneficial effects of statins in PD patients could be ascribed to their capacity to reduce DNA damage. The protective mechanism of the statins in PD patients may be attributed to mechanisms other than protection against DNA damage, such as its antioxidative or anti-inflammatory properties.

Opsomming

Parkinson se siekte word veroorsaak deur die verlies van nigrostriatale neurone wat verantwoordelik is vir die regulering van normale bewegingsfunksies (Chase, *et al.*, 1998).

Oksidatiewe skade aan DNA word veroorsaak deur endogene sellulêre bronne (Marnett, 2000) soos waterstofperoksied wat hoogs reaktief is, en basisse kan byvoeg of waterstofatome kan verwyder vanaf DNA (Cooke, *et al.*, 2006). Daar bestaan dus 'n basislyn van DNA-skade (Marnett, 2000) wat gedurige herstel ondergaan. Hierdie proses is noodsaaklik vir die oorlewing van selle en indien die herstelprosesse in gebreke bly, sal dit lei tot mutasies en seldood (Cooke, *et al.*, 2006).

Behandeling van Parkinson se siekte fokus tans op die bestuur van simptome deur middel van dopaminergiese middels soos L-DOPA. Hierdie benadering is slegs in die vroeë stadium van die siekte hoogs effektief, en langtermynbehandeling lei tot verminderde effektiwiteit en die ontwikkeling van newe-effekte (Jenner, 2003). Die groot uitdaging is om maniere te vind om die nigrostriatale neurone te behou en te beskerm, en dus die aanvang van Parkinson se siekte te voorkom.

Huidige navorsing toon dat die rol van statiene, wat gebruik word in die behandeling van dislipidemie (Hamelin and Turgeon, 1998), besig is om te verbreed. Statiene verminder onder andere LDL-oksidasie, behou endogene superoksieddismutase, verhoog α-tokoferol ('n anti-oksidant), verminder lipoproteïenoksidasie in verskeie oksidatiewe sisteme, beskerm teen DNA-skade veroorsaak deur antineoplastiese middels, en verminder DNA-skade in hipercholesterolemiese pasiënte (Shin, *et al.*, 2005).

Daar is dus in hierdie studie ondersoek ingestel na die potensiële neurobeskermende effek van geselekteerde statiene (pravastatien, simvastatien en atorvastatien) in die striatum. MPTP(1-metiel-4-feniel-1,2,3,6-tetrahidropiridien)-behandelde C57Bl/J6 muise is as proefdiermodel gebruik om Parkinson se siekte na te boots. MPTP is 'n neurotoksien wat selektiewe skade in die striatum aanrig deur die inhibisie van mitochondriale kompleks I.

Groepe van tien muise is met 70 mg / kg pravastatien, simvastatien, atorvastatien, of geen geneesmiddel (kontrolegroep) vir vyf opeenvolgende dae behandel. Vyf muise vanuit elke groep het 'n "onmiddellike aanvang Parkinson se siektemodel vir vinnige degenerasie met

nekrotiese seldood" dosis MPTP (50 mg / kg) intraperitoniaal ontvang. Na die muise gedekapiteer is, is die striatum geïsoleer en ontleed.

Die onmiddellike vlak van DNA-skade in die weefsel (basislyn-skade) is bepaal deur gebruik te maak van mikrojelelektroforese (komeet) analise. Verdere DNA skade is aangerig deur behandeling met H_2O_2 vir dertig minute waarna die proses gestop is en die skade weer bepaal is. Nóg twee komeetanalises is uitgevoer op twintig-minuut-intervalle om die hoeveelheid herstel wat plaasgevind het te bepaal.

MPTP-behandeling het die vlak van DNA-skade in die striatum verhoog. Behandeling met statiene het ook die vlakke van DNA-skade verhoog, maar die herstelprosesse het behoue gebly en die herstel het dus ook toegeneem. Die DNA-herstelvermoë van muise wat met MPTP en statiene behandel is, is egter verlaag.

Die resultate wat verkry is in hierdie studie ondersteun nie die hipotese dat die voordelige effek van statiene in Parkinson-pasiënte toegeskryf kan word aan hul kapasiteit om DNA-skade te verminder nie. Die beskermende meganisme van statiene in Parkinsons pasiënte mag dus vanweë ander meganismes as beskerming teen DNA-skade wees, byvoorbeeld as gevolg van hul antioksidatiewe of anti-inflammatoriese eienskappe.

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Abbreviations

8-OH-dG	8-hydroxy-deoxyguanine
8-OH-dGMP	8-hydroxy-deoxyguanine monophosphate
8-OH-dGTP	8-hydroxy-deoxyguanine triphosphate
8-OH-Gua	8-hydroxy-guanine
A	adenine
AP	apurinic-apyrimidinic
ATP	adenosine triphosphate
C	cytosine
CASP	Comet Assay Software Project
COMT	catechol-O-methyl transferase
DAT	dopamine transporter
dATP	deoxy-adenosine triphosphate
ddH_2O	double distilled water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DOPAC	3,4-dihydroxy-β-phenylacetic acid
EDTA	ethylenediaminetetraacetic acid
Eph-BS	electrophoresis buffer solution
ESCODD	European Standards Committee on Oxidative DNA Damage
EtBr	ethidium bromide

G	guanine
H_2O_2	hydrogen peroxide
HMPA	high melting point agarose
hMTH1	human Mut T homologue
hMYH	human Mut Y homologue
hOGG1	human 8-OH-Gua glycosylase 1
hOGG2	human 8-OH-Gua glycosylase 2
KCl	potassium chloride
L-DOPA	3,4-dihydroxyphenyl-L-alanine
LMPA	low melting point agarose
LS	lysing solution
MAO	monoamine oxidase
MPP^+	1-methyl-4-phenyl-2,3-dihydropyridium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	mincing solution
NaCl	sodium chloride
NaOH	sodium hydroxide
NMDA	N-methyl d-aspartate
•OH	hydroxyl radical
PARP	poly(ADP-ribose)polymerase
PBS	phosphate buffer solution

PD	Parkinson's disease
RC	repair capacity
RNA	ribonucleic acid
ROS	reactive oxygen species
SD	standard deviation
SCGE	single cell gel electrophoresis
SNpc	substantia nigra pars compacta
T	thymine
Tris	tris-(hydroxymethyl)aminomethane buffer
Tris HCl	tris-(hydroxymethyl)aminomethane hydrochloride

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Chapter 1 - Introduction

Parkinson's disease (PD) is a neurological disorder occurring after damage to the nigrostriatal neurons which might be either age-related or caused by subclinical levels of environmental toxins (Riess and Kruger, 1999). The neurons of the nigrostriatal pathway control normal motor activity through the synthesis and release of dopamine (Von Bohlen und Halbach, *et al.*, 2004).

Excessive hydrogen peroxide (H_2O_2) levels have been found in the post mortem frontal cortex of PD patients (Kienzl, *et al.*, 1995) and attack by reactive oxygen species to deoxyribonucleic acid (DNA) and other cellular components is one of the main mechanisms for nigrostriatal cell death. Endogenous DNA damage arises from reactive oxygen species, formed on a continual basis inside the cell (Marnett, 2000), which attack the DNA bases (pyrimidines and purines) and sugars (Evans, *et al.*, 2004).

The substantia nigra pars compacta is exposed to higher levels of oxidative stress because of the higher levels iron and dopamine (Coyle and Puttfarcken, 1993) and lower levels of glutathione peroxidase (Sian, *et al.*, 1994) responsible for defence against oxidative stress.

Both the autoxidation and the monoamine oxidase (MAO) mediated metabolism of dopamine involve the formation of H_2O_2 which, when reduced to the extremely reactive hydroxyl radical (•OH), increases oxidative stress (Graham, 1978; Graham, *et al.*, 1978). Dopamine biosynthesis and the turnover in surviving neurons are increased with a loss of dopaminergic neurons (Fornstedt, *et al.*, 1990). The subsequent excessive autoxidation and metabolism of dopamine in these cells increase the oxidative stress, contributing to the progressive loss of dopaminergic neurons observed in PD (Hermida-Ameijeiras, *et al.*, 2004).

Repair to oxidative damage to DNA is an ongoing process and critical to the survival of all cells. The failure to protect or repair the genome would consequently result in the induction of mutations and arrest of cellular growth and multiplication. Examples of enzymes that form this repair mechanism in mammalian cells include human Mut T homologue (hMTH1), specific glycosylases that initiate base excision repair and human Mut Y homologue (hMYH) (Cooke, *et al.*, 2006).

Current therapy is dominated by symptomatic management of PD with L-DOPA and other dopaminergic drugs (Jenner, 2003) but current research is shifting towards preventative measures against DNA damage and cell death.

An animal model reflecting many of the features of human PD can be achieved through the treatment of primates and rodents with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Speciale, 2002). The toxic effect of MPTP are induced through its conversion to the 1-methyl-4-phenyl-2,3-dihydropyridium ion (MPP^+) in astrocytes in the brain (Nicklas, *et al.*, 1985). This leads to severe damage of the nigrostriatal dopaminergic system and a dramatic loss of neurons (Sedelis, *et al.*, 2001). MPP^+ induces DNA fragmentation, the production of reactive oxygen species (Brill and Bennet, 2003) and apoptotic cell death (Fall and Bennett, 1999).

The drugs known as the statins are widely used in the treatment of hypercholesterolaemia (Hamelin and Turgeon, 1998). One of the statins, simvastatin, has been shown to significantly reduce DNA damage of leucocytes in hypercholesterolemic patients and has a beneficial effect on the repair of DNA damage (Shin, *et al.*, 2005).

1.1 Aim of Study

The aim of this study is to determine whether three of the commercially available statins (pravastatin, simvastatin, and atorvastatin) has any protective effects on the effect of MPTP on DNA integrity.

Control mice and MPTP treated mice were treated with pravastatin, simvastatin and atorvastatin before decapitation and analysis of striatal DNA. The baseline level of DNA damage was measured before DNA damage was induced *in vitro*, after 30 min of damage, after 20 min of repair and after 40 min of repair.

Chapter 2 - Literature Review

2.1 Parkinson's Disease

Parkinson's disease (PD) was first described by James Parkinson in 1817 (Parkinson, 1817). It is a neurodegenerative disorder defined as a syndrome associated with specific neuropathological lesions.

2.1.1 Symptoms

The syndrome is characterised by three main symptoms namely bradykinesia, resting tremor, and rigidity. *Bradykinesia* is defined as the slowing of normal movement. *Resting tremor* is the involuntary unsteady movements in cycles of about four to six per second of the involved limbs when in a state of rest. These tremors are often enhanced by stress and are less severe during voluntary activity. *Rigidity* is a resistance to perform passive movement and is responsible for the characteristic flexed posture seen in many patients (Von Bohlen und Halbach, *et al.*, 2004).

Other symptoms include postural instability, decline in intellectual function, immobility of the face, infrequent blinking and a tremor about the mouth and lips (Aminoff, 2004).

2.1.2 Pathology

PD occurs after a loss of 70 to 80 % (Bernheimer, *et al.*, 1973) of the neurons regulating normal motor function through the synthesis and release of dopamine, in the nigrostriatal pathway (Figure 2-1) (Chase, *et al.*, 1998). The neurons of the nigrostriatal pathway project from the basal ganglia to the striatum and their cell bodies are located inside the substantia nigra pars compacta (Linert and Jameson, 2000).

The pathological lesions characteristic to PD are known as Lewy bodies (Figure 2-1) and consist of eosinophilic inclusions (Forno, 1996). To reveal Lewy bodies, tissue samples can be stained with anti-α-synuclein antibodies. The Lewy bodies have intensely immunoreactive central zones surrounded by faintly immunoreactive peripheral zones (Linert and Jameson, 2000).

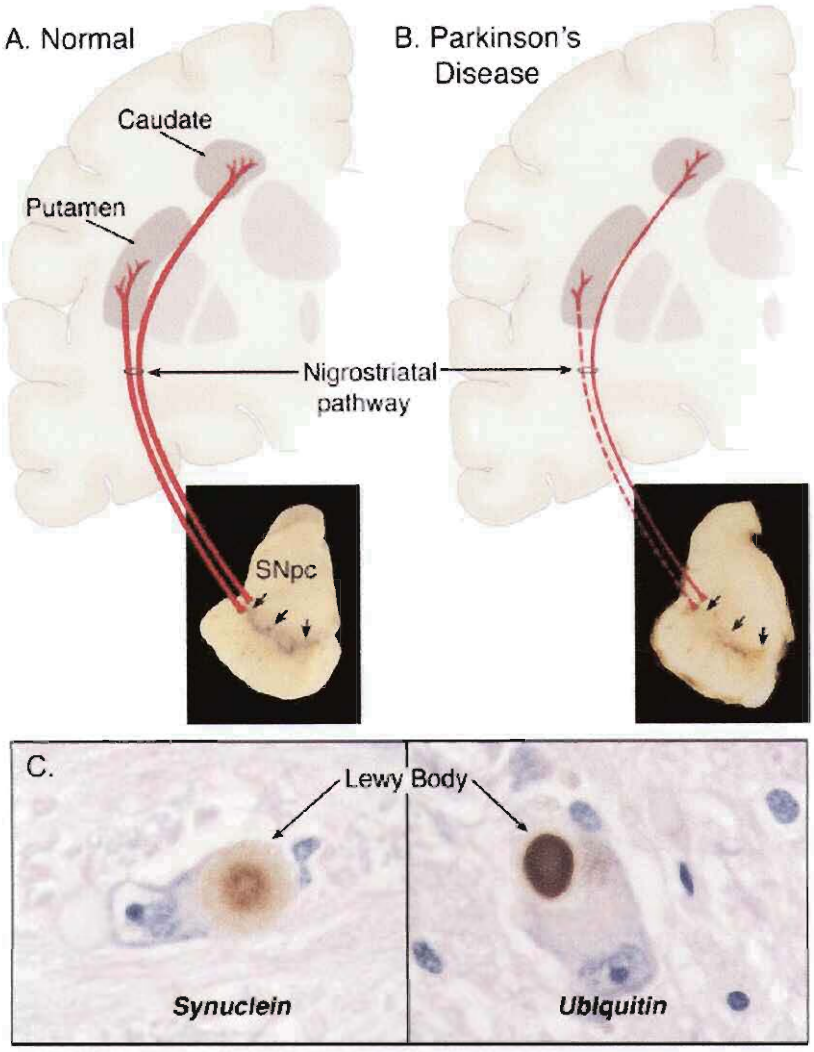

Figure 2-1 **Neuropathy of PD. Illustration of (A) normal nigrostriatal pathway (in red); (B) diseased nigrostriatal pathway (in red), and (C) Lewy Body formation (Linert and Jameson, 2000)**

The link between the movement disturbances associated with PD and the pathological lesions to the nerve terminals in the striatum (Chase, *et al.*, 1998; Nagatsu, *et al.*, 2000) and substantia nigra pars compacta (Jenner, 2003) has been established.

Lewy bodies have been found in many other regions of the nervous system of PD patients, including the substantia nigra, locus coeruleus, cortex, limbic areas, hypothalamus, nucleus basalis, cranial nerve motor nuclei, and central and peripheral divisions of the autonomic nervous system (Takahashi and Wakabayashi, 2001).

The presence of Lewy Bodies in association with nerve cell loss in the substantia nigra and various other regions of the nervous system is a diagnostic hallmark of PD (Forno, 1996).

2.1.3 Aetiology

A number of exogenous toxic substances have been identified as causative factors in the development of PD. No single toxin has been found in the brain of PD patients and the condition induced by toxins is not that of typical Lewy body PD. The toxin (see Section 2.3) used in this study, does however cause PD-like symptoms (Olanow and Tatton, 1999).

The pathogenesis of normal PD is thought to be multifactorial, deriving from environmental factors acting on genetically predisposed individuals with aging (Riess and Kruger, 1999). It has therefore been hypothesised that this disorder may be secondary to subclinical, environmentally (toxin) induced damage to the substantia nigra, followed by the continued age-related attrition of nigral neurons (Langston, 1990).

Recently, specific genetic defects have been identified but the relationship between genetic and environmental factors is poorly understood and most models of PD focus on single genes or toxins.

In particular, members of the c-fos and c-jun families of genes have been implicated in changes associated with neuronal damage or chronic adaptive responses in the nervous system. C57Bl6/J6 mice, treated with MPTP, had an elevation of these mRNAs in the striatum (Péreaz-Otaño, *et al.*, 1998).

It has also been postulated that the loss of dopaminergic neurons, which takes place in both normal aging and PD, are closely related to the particular capacity of dopaminergic neurons to generate oxidative stress (Cohen, 1990; Fornstedt, *et al.*, 1990).

The accumulation of reactive oxygen species has been recognized to attribute to cell damage and death in many diseases. The nervous system is particularly vulnerable (Figure 2-2) because of its high metabolic rate, high lipid content, iron and copper content of certain areas and low rate of cell division (Sagara, *et al.*, 1997). The high metabolic rate of the nervous system contributes to an increase in the formation of reactive oxygen species, a lower rate of cell division and a decrease in the replacement of damaged cells and DNA. The lipids that make up the neuronal content are highly prone to oxidative attack and lipid peroxidation produces more peroxyl radicals (Burcham, 1999).

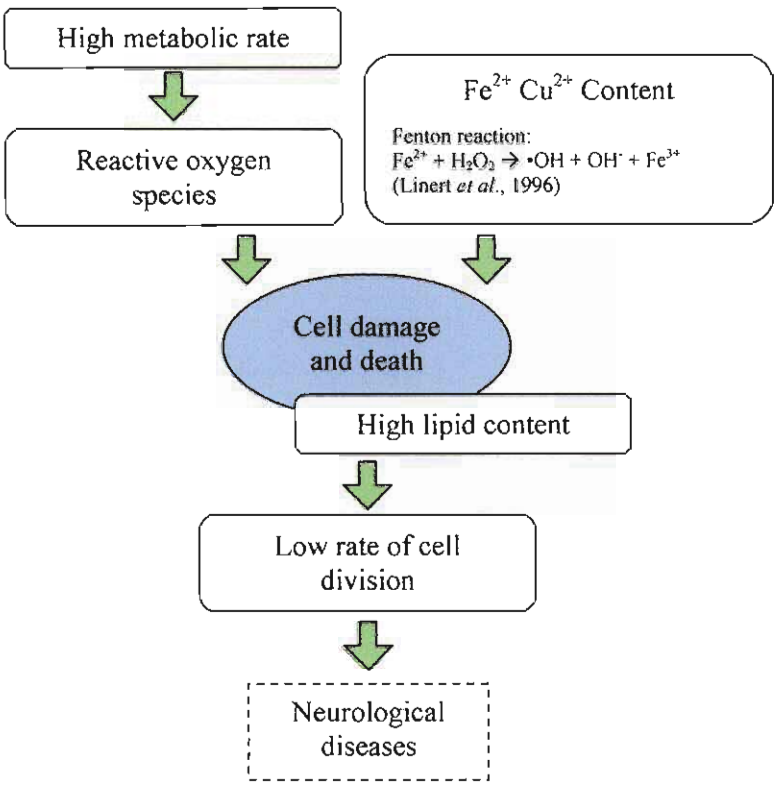

Figure 2-2 Factors relating to oxidative damage in the nervous system

The DNA-damaging properties of peroxyl radicals are reputed to catalyse the propagation phase of membrane autoxidation and to react with macromolecules. They differ from other endogenous oxygen radicals because of a comparatively long lifetime (approx. 5 to 10 sec). Furthermore, since they form in nuclear membranes, DNA seems a likely target for peroxyl radicals (Burcham, 1999).

Recent evidences suggest that there is a significant increase in iron content in the pigmented nigrostriatal dopaminergic neurons which undergo degeneration in idiopathic PD. Iron is known to drive the Fenton reaction in the presence of H_2O_2 to produce cytotoxic •OH in a biological system, causing tissue injury (Mohanakumar, *et al.*, 1998).

It can also be demonstrated histochemically that excessive H_2O_2 accumulates in the post mortem frontal cortex of a PD patients (Kienzl, *et al.*, 1995). •OH formed by monoamine oxidases during neurotransmitter catabolism is another likely cause of oxidative cell damage (Duffy, *et al.*, 1998).

For these reasons, oxidative damage may be a causative factor in PD.

2.1.4 Treatment

Symptomatic management of PD with L-DOPA and other dopaminergic drugs dominates current therapy and is highly effective in managing early stages of the disorder. Long-term treatment often goes along with a loss of drug efficacy, the onset of dyskinesias and the occurrence of psychosis (Jenner, 2003).

It has also been suggested that treatments targeted at mitochondrial function hold promise to slow the progression of PD (Shults, 2004).

Current research and treatment strategies, however, are gradually shifting from symptomatic management towards preventative measures against DNA damage and cell death.

2.2 Oxidative DNA Damage

Over the past few years the focus in research has shifted from exogenous sources of DNA damage to damage caused by endogenous cellular sources. Improvements in analytical chemistry have made it possible to detect endogenous DNA damage both in quantity and quality. Application of these techniques to analysis of nuclear DNA from human tissues has debunked the notion that the human genome is pristine in the absence of exposure to environmental carcinogens and that a certain baseline level of DNA damage does exist (Marnett, 2000).

Oxidation is considered to be the major contributor to baseline DNA damage and it is estimated that an average of 11,500 adducts $cell^{-1}$ day^{-1} are excreted by humans (Burcham, 1999). Endogenous DNA damage arises from the intermediates and the products of oxygen reduction that interact with either the bases or the deoxyribosyl backbone of the DNA. Alternatively, other cellular components such as lipids can interact with oxygen radicals and couple to DNA bases (Marnett, 2000).

Various external events, such as exposure to ionising and ultraviolet radiation, can lead to an increase in the generation of reactive oxygen species. The resulting shift of the pro-oxidant/antioxidant balance towards the former, leads to a condition of oxidative stress. Cellular components are subsequently prone to oxidation and DNA polymerase activity is altered (Cooke, *et al.*, 2006).

2.2.1 Reactive oxygen species

Free radicals are defined as any chemical moiety capable of existing with a lone electron in an orbital, i.e. an unpaired electron (denoted as •). Free radicals are more reactive than non-radicals because orbital pairing of electrons increases stability. Reactive oxygen species (ROS) are oxygen containing molecules which may be radical, for example, superoxide ($\bullet O_2^-$) and •OH or non-radical, for example H_2O_2 and singlet oxygen ($^1O_2$) (Cooke, *et al.*, 2006). The different reactive species responsible for oxidative damage are represented in Figure 2-3.

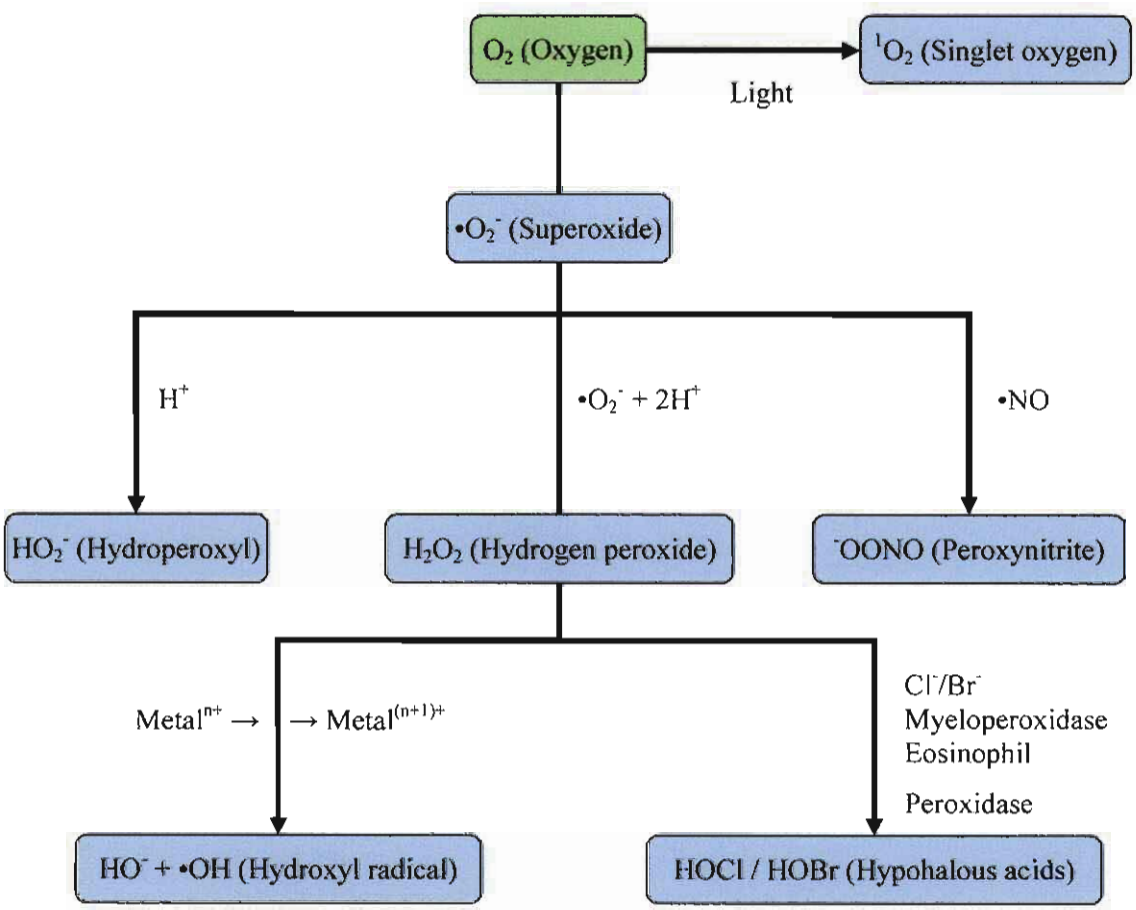

Figure 2-3 Reactive species responsible for oxidative damage (adapted from Cooke, *et al.*, 2006)

•OH is extremely reactive, adding bases to or abstracting hydrogen atoms from DNA, producing over twenty different products that occur in the genome (Cooke, *et al.*, 2006; Marnett, 2000).

•OH does not diffuse more than one or two molecular diameters before reacting with another molecule or cellular component. It therefore has to be generated immediately adjacent to the DNA molecule to be able to oxidise it. H_2O_2 is likely to act as a diffusible, latent form of •OH that reacts with a metal ion in the vicinity of DNA to generate the oxidant (Marnett, 2000).

In most tissues, mitochondrial leakage of the superoxide anion radical ($O_2^{\cdot}$) and H_2O_2 are probably major culprits in oxidative DNA damage, although peroxisomal and microsomal oxidases may also contribute. In neuronal tissues •OH is also formed by monoamine oxidases during neurotransmitter catabolism (Burcham, 1999). The latter aspect will be discussed in more detail under Section 2.2.4.

2.2.2 Damage to DNA bases and sugars

Reactive oxygen species attack the DNA bases (pyrimidines and purines), as well as the sugars associated with them to cause oxidative damage to the genome.

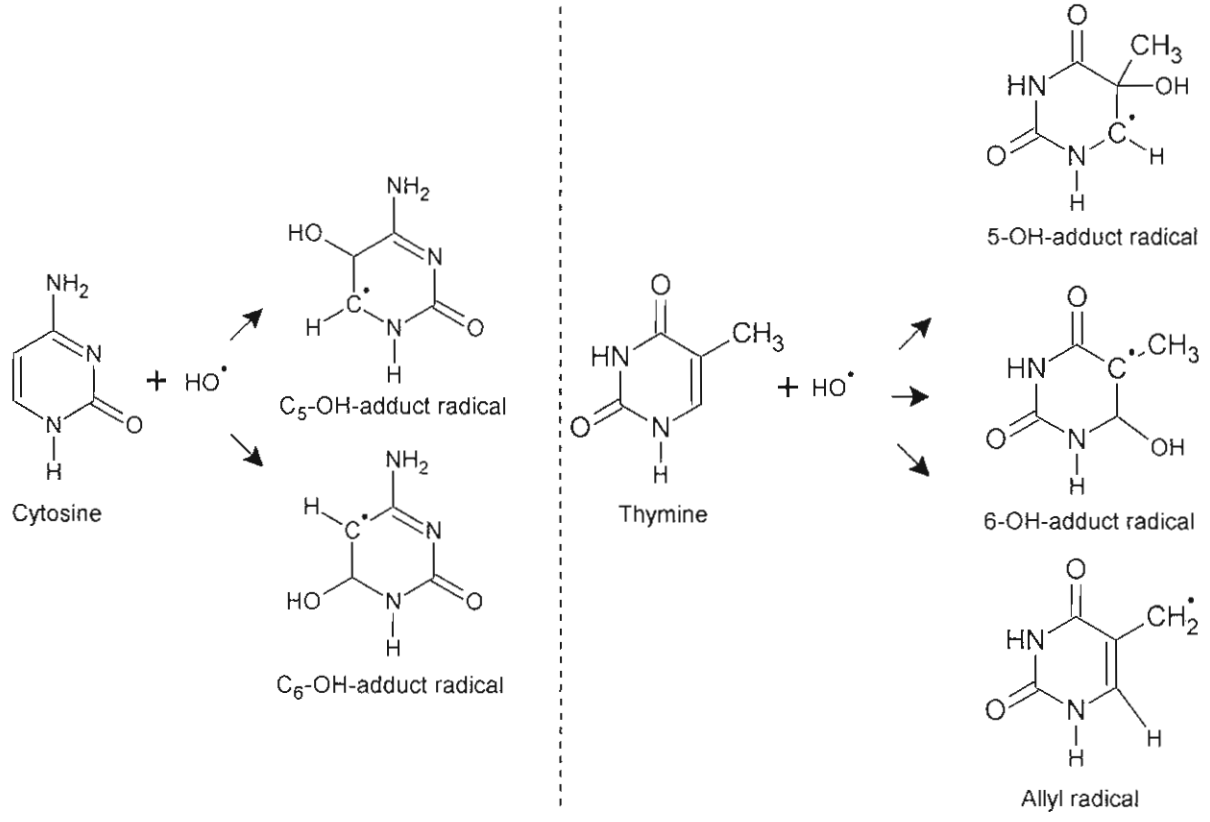

Figure 2-4 Reactions of •OH with pyrimidines (Evans, *et al.*, 2004)

•OH adds to the double bond of pyrimidines and purines as indicated in Figure 2-4 and Figure 2-5. The area of attack depends on the electron density of the site of the attacked molecule.

•OH is electrophilic by nature, therefore it preferentially adds to the site with the highest electron density. Pyrimidine OH-adduct radicals and the allyl radical are oxidised or reduced depending on their redox properties, redox environment and reaction partners, yielding a variety of products as shown in Figure 2-4 (Evans, *et al.*, 2004).

Guanine (a purine) forms OH-adduct radicals by addition of •OH to the C4-, C5- and C8-positions generating C4-OH-, C5-OH- and C8-OH-adduct radicals (Candeias and Steenken, 2000; O'Neill, 1983; Steenken, 1989) as shown in Figure 2-5. The 6-substituted purines such as adenine undergo analogous reactions, yielding C4-OH- and C8-OH-adduct radicals (Steenken, 1989; Candeias and Steenken, 2000). The OH-adduct radicals of purines differ in their redox properties, with C4-OH-adduct radicals being oxidising, and C5-OH- and C8-OH-adduct radicals being primarily reducing (Evans, *et al.*, 2004).

•OH abstracts an H^+ atom from each of the carbon atoms of the sugar moiety in DNA. The resulting C-centred radicals undergo further reactions to yield a variety of sugar modifications, the mechanism of which has been elucidated. Some sugar products are released

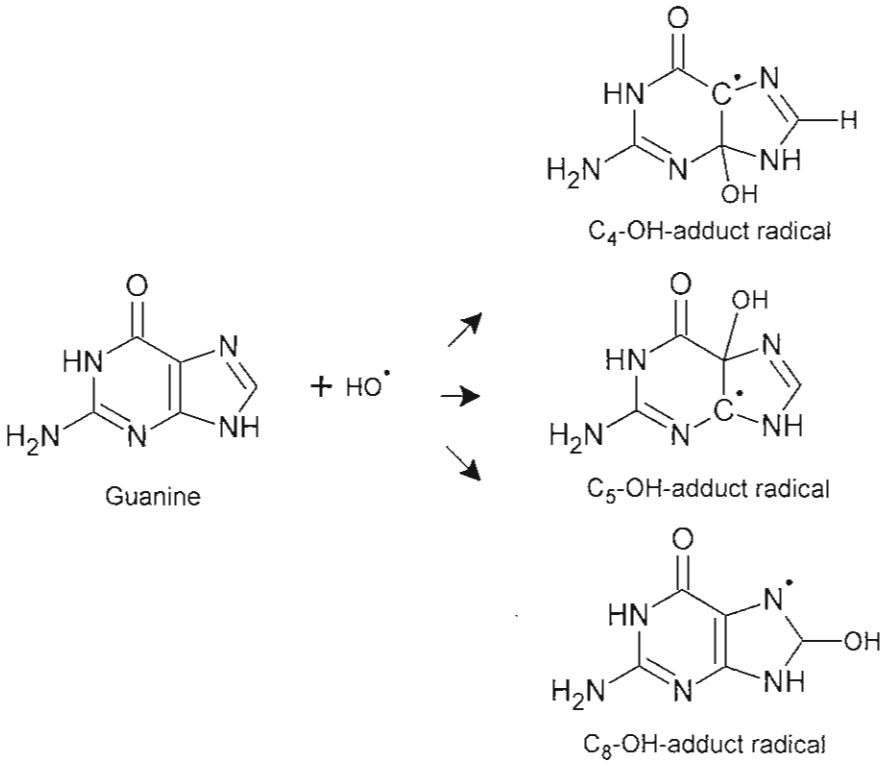

Figure 2-5 Reactions of •OH with guanine as an example of a purine (Evans, *et al.*, 2004)

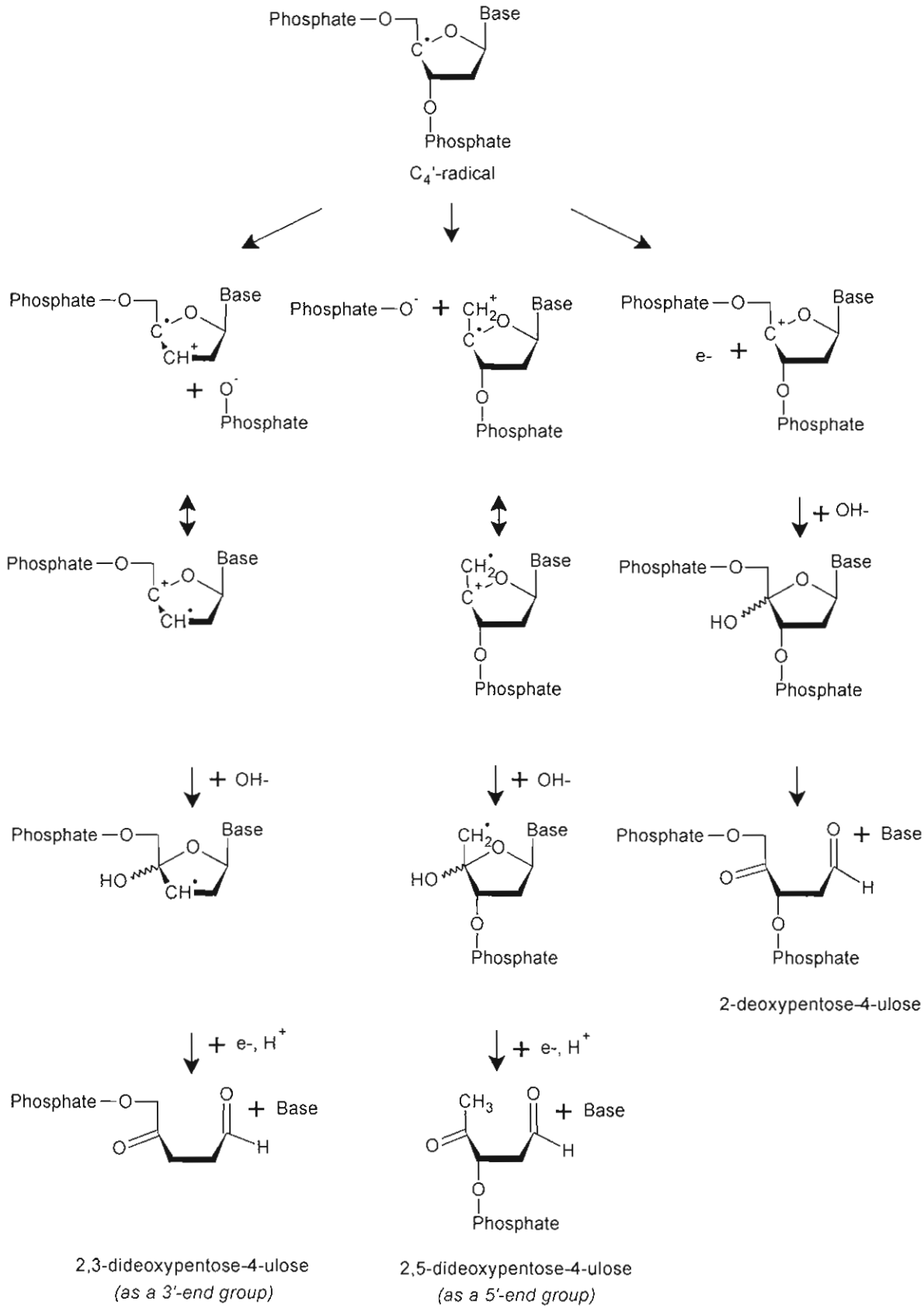

Figure 2-6 **Reactions of the C4'-radical of the sugar moeity of DNA in the absence of oxygen (Evans, *et al.*, 2004)**

from DNA as free modified sugars, whereas others remain within DNA or constitute end groups of broken DNA strands. The C4-radical of the sugar moiety in DNA undergoes oxidation and reaction with water (addition of OH^-) followed by elimination of the unaltered base. This C4-radical reacts, in the absence of oxygen, as shown in Figure 2-6 to form an alkoxyalkyl radical with a phosphate group in the β-position. It can readily lose the phosphate group on either side of the DNA chain resulting in strand breaks and two different radical cations as end groups (Evans, *et al.*, 2004).

2.2.3 Mitochondrial DNA damage

There is an intimate link between oxidative stress generation, mitochondrial DNA damage, and defects in electron transport function (Andreassi, 2004).

The mitochondrial genome is more vulnerable to oxidative damage than nuclear DNA because it is not protected by histones and reveals only limited capability for DNA repair (Yakes and Van Houten, 1997). Mitochondrial DNA mutations and alterations in mitochondrial genomic function have been associated with tumor formation (Penta, *et al.*, 2001; Tan, *et al.*, 2002) and ischemic heart disease (Andreassi, 2004; Ferrari, 1996; Ide, *et al.*, 2001).

Mitochondria are intracellular organelles that provide energy for cell functions through the process of oxidative phosporylation. The human mitochondrial genome is a circular, double-stranded DNA molecule composed of 16,569 base pairs, which encodes 13 polypeptides involved in oxidative phosphorylation, as well as 2 ribosomal ribonucleic acids (RNAs) and 22 transfer RNAs that are required for protein synthesis in mitochondria (Zeviani, *et al.*, 1998). Oxygen is reduced to water by four enzyme complex activities: complex I (NADH-ubiquinone oxidoreductase), complex II (succinate-ubiquinone oxidoreductase), complex III (ubiquinol-cytocrome c reductase) and complex IV (cytochrome c oxidase) (Andreassi, 2004).

The mitochondrial respiratory chain normally releases small amounts of $\bullet O_2^-$ and H_2O_2. The sensitivity of mitochondrial DNA predisposes it to injury when cells are exposed to genotoxins or oxidative stress. Alteration or deletion of mitochondrial gene products also increases the intermediates of reactive oxygen species in the respiratory chain and observations that the inhibition of the respiratory chain results in an increase in free radical

generation (Ide, *et al.*, 1999; Kowaltowski and Vercesi, 1999), support this hypothesis (Andreassi, 2004).

This mitochondrial dysfunction is characterized by an increase in mitochondrial lipid peroxidation, a decrease in mitochondrial DNA copies and mitochondrial RNA transcripts, and a reduction in activity of complexes I, III, and IV (Ide, *et al.*, 2001). In contrast, complex II and citrate synthase are encoded only by nuclear DNA and their activities are therefore unchanged (Andreassi, 2004).

Damage to mitochondrial DNA would therefore affect the respiratory function, leading to an increased production of oxygen free radicals. This, in turn, will lead to additional mitochondrial DNA damage, worsening the defects of electron transport and the occurrence of a vicious cycle of mitochondrial function decline (Andreassi, 2004).

2.2.4 Dopamine autoxidation

Dopamine (3-hydroxytyramine) is a catechol neurotransmitter widely distributed throughout the brain with higher concentrations in the striatum (Palkovits and Brownstein, 1989). The biosynthesis of dopamine occurs preferentially in the nerve terminals from tyrosine and through the sequential action of tyrosine hydroxylase and aromatic l-amino acid decarboxylase (Hermida-Ameijeiras, *et al.*, 2004).

Under physiological conditions, dopamine is non-enzymatically oxidized by molecular oxygen to form H_2O_2 and the corresponding o-quinone. Then, the o-quinone undergoes an intramolecular cyclization which is immediately followed by a cascade of oxidative reactions resulting in the final formation of a black, insoluble polymeric pigment known as neuromelanin (Graham, 1978; Graham, *et al.*, 1978; Hermida-Ameijeiras, *et al.*, 2004).

Dopamine is also enzymatically deaminated by MAO to form H_2O_2 and 3,4-dihydroxy-β-phenylacetaldehyde. This latter compound is then oxidized by aldehyde dehydrogenase to give 3,4-dihydroxy-β-phenylacetic acid (DOPAC), which subsequently is methylated by catechol-O-methyl transferase (COMT) to form 3-methoxy-4-hydroxy-phenylacetic acid (homovanillic acid, HVA) as shown in Figure 2-7.

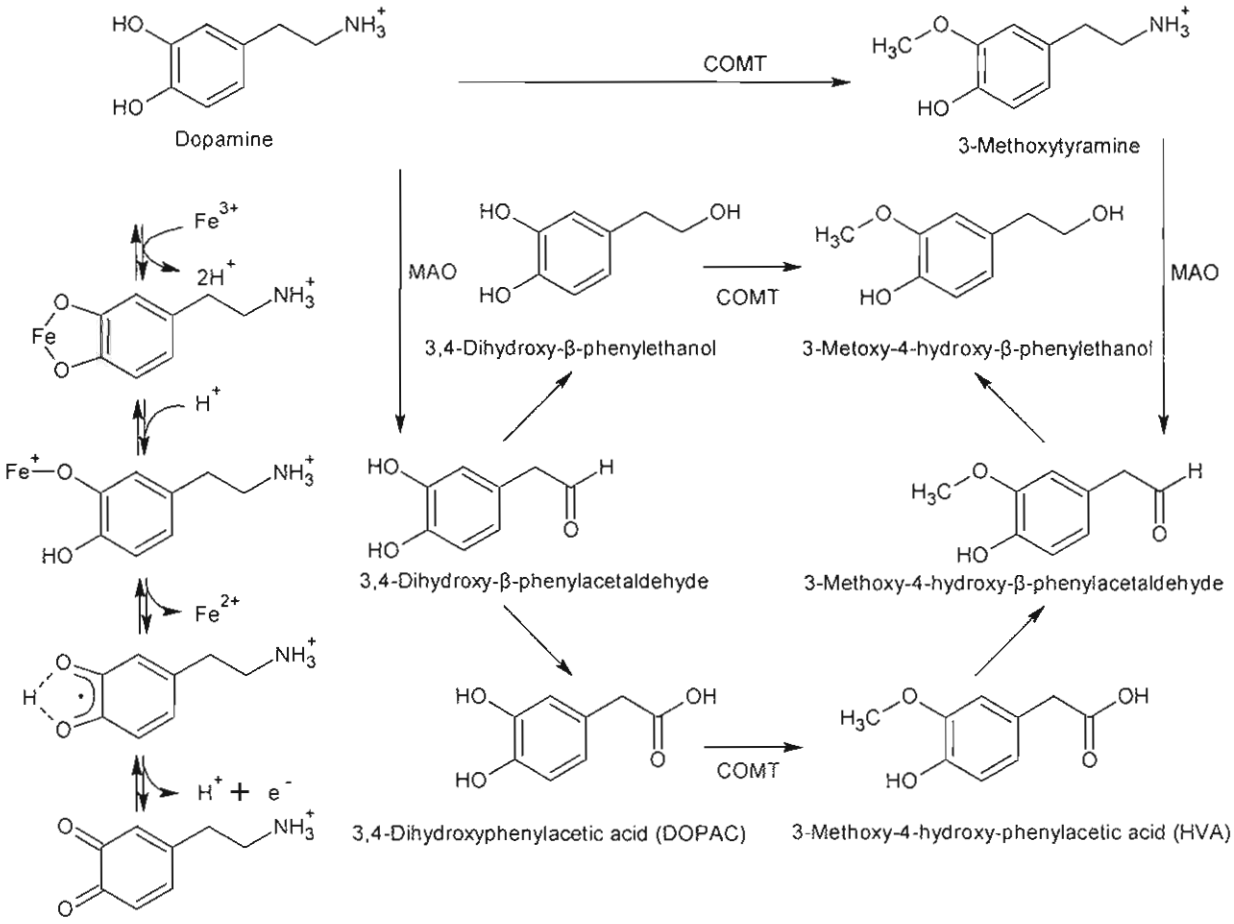

Figure 2-7 **The autoxidation of dopamine by Fe^{3+} (Hermida-Ameijeiras, 2004) and its enzymatic deamination, oxidation and methylation (Fornstedt, *et al.*, 1990)**

Therefore, both the autoxidation and the MAO-mediated metabolism of DA involve the formation of H_2O_2 that can be reduced to the •OH. Dopamine biosynthesis and the turnover in surviving neurons are increased with a loss of dopaminergic neurons (Fornstedt, *et al.*, 1990). The subsequent excessive autoxidation and metabolism of dopamine in these cells increase the oxidative stress, contributing to the progressive loss of dopaminergic neurons observed in PD (Hermida-Ameijeiras, *et al.*, 2004).

2.2.5 Repair of DNA damage

Repair to oxidative damage to DNA is an ongoing process and critical to the survival of all cells. The failure to protect or repair the genome would consequently result in the induction of mutations, microsatellite instability, loss of heterozygosity, chromosomal aberrations, altered gene expression, and eventually cytostasis (arrest of cellular growth and multiplication), cytotoxicity, or neoplastic growth (Cooke, *et al.*, 2006).

The best understood repair process is that which repairs 8-hydroxy-guanine (8-OH-Gua) and may be regarded as a template for the processes that repair other lesions. To combat the deleterious biological effect of the presence of 8-OH-Gua, cells have developed specific mechanisms to remove this lesion from cellular DNA. In mammalian cells three enzymes form this repair mechanism. The first is hMTH1 which hydrolyses 8-hydroxy-deoxyguanine triphosphate (8-OH-dGTP) (a potential substrate for DNA polymerase), thereby eliminating it from the nucleotide pool. The second is specific glycosylases that initiate base excision repair. The final enzyme is hMYH which removes mis-paired adenine associated with 8-OH-Gua. Nucleotide excision repair, which involves the removal of a lesion-containing oligonucleotide, may complement this system (Cooke, *et al.*, 2000), based upon evidence that repair to oxidative DNA damage may be by this route (Brooks, *et al.*, 2000; Cooke, *et al.*, 2006; Kuraoka, *et al.*, 2000; Reardon, *et al.*, 1997).

2.2.5.1 Base excision repair

The glycosylase, considered to have the primary responsibility for the removal of 8-OH-Gua in human cells, is the human 8-OH-Gua glycosylase (hOGG1) (Arai, *et al.*, 1997; Rosenquist, *et al.*, 1997). This enzyme acts via short patch repair, and has a specificity for 8-OH-Gua:Cytosine pairs present in double stranded DNA (Figure 2-8). Characterisation studies of hOGG1 have revealed two isoforms, designated a-hOGG1 and h-hOGG1, which exhibit specificity for sub-cellular localisation to the nucleus and mitochondrial inner membrane, respectively (Cooke, *et al.*, 2006; Croteau, *et al.*, 1997; Nishioka, *et al.*, 1999).

Base excision repair is complemented by at least two other processes, mis-match repair, involving hMYH, and prevention of mis-incorporation, involving hMTH1 (Cooke, *et al.*, 2006).

2.2.5.2 Mismatch repair and prevention of incorporation

Another glycosylase, hMYH, removes adenine paired opposite 8-OH-Gua. This pair may arise either from incorporation of 8-OH-dGTP opposite adenine in the template strand, or incorporation of deoxy-adenosine triphosphate (dATP) opposite unrepaired 8-OH-Gua in the template strand. The removal of incorporated adenine, by hMYH, allows a more likely replacement by cytosine, offering hOGG1 a further chance to repair the lesion. Conversely,

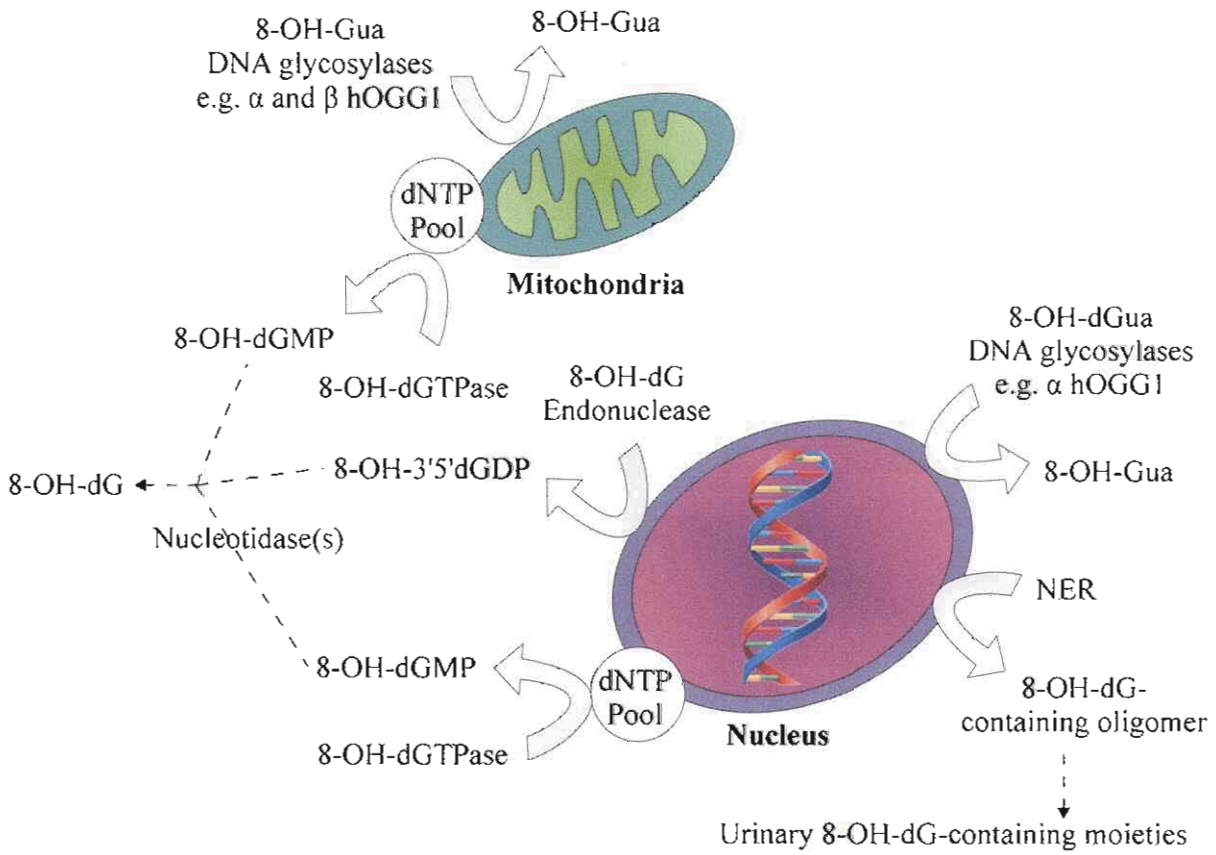

Figure 2-8 **Overview of the pathways responsible for the maintenance of genome integrity with respect to 8-OH-Gua (Cooke, *et al.*, 2000)**

removal of an incorporated 8-OH-Gua, opposite adenine in the template strand, can be accomplished by hOGG2, a glycosylase whose activity is directed towards the nascent strand. This strand specificity is important as removal of adenine in the template strand would cause increased mutation (Cooke, *et al.*, 2006).

In contrast, hMTH1 acts at an earlier stage to prevent the erroneous incorporation of 8-OH-Gua into DNA. hMTH1 degrades 8-OH-dGTP to 8-hydroxy-deoxyguanine monophosphate (8-OH-dGMP) which in turn is ultimately degraded to 8-hydroxyguanine (8-OH-dG) and excreted (Hayakawa, *et al.*, 1995). Very recently, two new DNA-glycosylases have been discovered. Nei-like glycosylase 1 preferentially removes 8-OH-Gua from pairs with guanine and adenine (Cooke, *et al.*, 2006; Hazra, *et al.*, 2002).

2.2.6 The Brain and DNA damage

Numerous neurodegenerative conditions including Alzheimer's disease, Huntington's disease and PD have oxidative stress implicated in their pathophysiology (Alam, *et al.*, 1997; Lovell, *et al.*, 1999; Zhang, *et al.*, 1999). The role of oxidative stress and oxidative damage to

biomolecules other than DNA, in the pathogenesis of neurodegenerative disease, and Alzheimer's disease specifically, has been supported in several recent reviews of the subject (Smith, *et al.*, 2000), although the greatest significance for the pathogenesis of the disease has been placed upon lipid and protein oxidation (Cooke, *et al.*, 2006).

The substantia nigra pars compacta is exposed to higher levels of oxidative stress, namely catabolism of dopamine via MAO-B-mediated deamination, dopamine autoxidation and high levels of iron combine to increase the number of reactive oxygen species formed (Coyle and Puttfarcken, 1993). The region also contains lower glutathion peroxidase levels, diminishing its ability to cope with oxidative stress (Sian, *et al.*, 1994).

PD patients show an increase in the levels of 8-OH-dG in the serum, cerebrospinal fluid (Kikuchi, *et al.*, 2002) and substantia nigra (Alam, *et al.*, 1997) compared with matched controls. Levels of 8-OH-dG and 8-OH-Gua in cytoplasmic DNA and RNA respectively are also elevated (Zhang, *et al.*, 1999) in the substantia nigra (Evans, *et al.*, 2004).

2.3 MPTP

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a bypass product in the chemical synthesis of a meperidine analogue with potent heroin-like properties. Drug addicts who took MPTP accidentally, developed a syndrome that clinically and pathologically resembled PD (Langston, *et al.*, 1983).

2.3.1 Toxicity

MPTP is highly lipophilic and readily crosses the blood-brain barrier. The toxic effects of MPTP are induced through its conversion to MPP^+ in astrocytes (Figure 2-9) in the brain by the enzyme monoamine oxidase B (Nicklas, *et al.*, 1985).

MPP^+ has a high affinity for the dopamine transporter (DAT) (Javitch and Snyder, 1984) and is therefore selectively toxic to dopaminergic neurons. Consequently, mice lacking this transporter are protected from MPTP toxicity (Bezard, *et al.*, 1999).

Several gene products are also involved in MPTP neurotoxicity. Knockout of neuronal (Przedborski, *et al.*, 1996) or inducible (Dehmer, *et al.*, 2000) nitric oxide synthase,

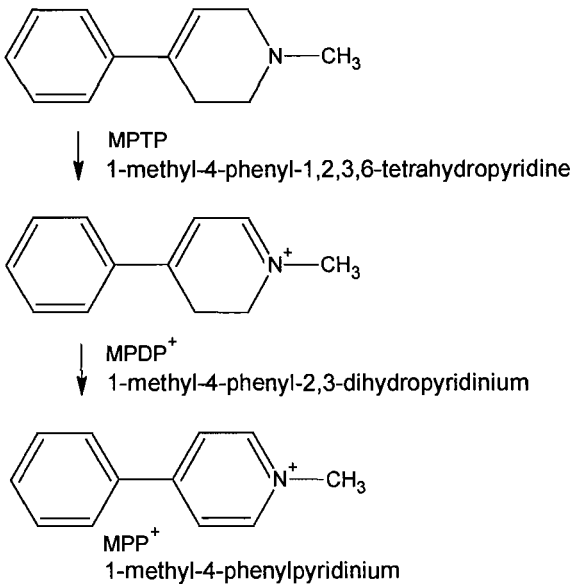

Figure 2-9 Chemical conversion of MPTP to MPP^+ in the brain

poly(ADP-ribose)polymerase (PARP; Mandir, *et al.*, 1999), or the growth regulatory gene p53 (Trimmer, *et al.*, 1996), reduces MPTP-induced loss of nigral dopamine neurons. These nigral dopamine neurons are protected against MPTP toxicity if the anti-apoptotic protein, bcl-2, is overexpressed (Offen, *et al.*, 1998; Yang, *et al.*, 1998), or if the pro-apoptotic protein, bax, is knocked out (Brill and Bennet, 2003; Vila, *et al.*, 2001).

MPP^+ induces apoptotic death (Fall and Bennett, 1999) and brings about many responses that likely regulate this process. These responses include an increase in oxidative stress (Cassarino, *et al.*, 1997), activation of pro- and antiapoptotic signaling pathways (Cassarino, *et al.*, 2000; Halvorsen, *et al.*, 2002), and increasing levels of the antiapoptotic proteins bcl-2 and bcl-XL (Dennis and Bennett, 2002; Veech, *et al.*, 2000) and the proapoptotic protein Bax (Dennis and Bennett, 2002). MPP^+ also induces DNA fragmentation measured with flow cytometry, and increases ROS production and anaerobic metabolism (Brill and Bennet, 2003).

An animal model reflecting many of the features of human PD (Speciale, 2002) can be achieved through the treatment of primates and rodents with MPTP. In mice, MPTP can be administered systemically or intracranially. This leads to severe damage of the nigrostriatal dopaminergic system, including symptoms of motor control disturbances such as akinesia, rigidity, tremor, gait and posture abnormalities (Sedelis, *et al.*, 2001) and a dramatic loss of dopaminergic neurons.

2.3.2 Mechanism

MPP^+ is transported into dopaminergic neurons by dopaminergic transporters and once inside the neuron, it acts by inhibiting the electron transport system of the mitochondrial complex I, resulting in cellular energy failure (Nicklas, *et al.*, 1987) and the formation of superoxide anions (Dawson, 2000).

Following a schedule of MPTP treatment, nigrostriatal production of •OH and the nigrostriatal activities of the major ROS-scavenging enzymes, such as mitochondrial superoxide dismutase, increase. Oxidative stress induced by complex I inhibition increase nuclear-encoded antioxidant enzymes due to complex I inhibition. Other factors related to complex I dysfunction such as decreased adenosine triphosphate (ATP) production (Ali, *et al.*, 1994; Bowling and Beal, 1995), excitotoxicity (Bowling and Beal, 1995), or impaired calcium-homeostasis (Sheehan, *et al.*, 1997) are also involved (Cassarino, *et al.*, 1997).

2.3.2.1 Mitochondrial impairment

MPP^+ accumulates inside the mitochondria of dopaminergic neurons leading to impairment in mitochondrial function. MPP^+ binds to complex I of the respiratory chain (Ramsay, *et al.*, 1991), which blocks the electron transport, and thus leads to an energy failure. Consequently ATP is depleted and the level of free radicals increases. The time course of this rapid ATP loss and restoration has been shown to correlate with MPP^+ brain levels (Chan, *et al.*, 1991, 1992).

Selective ablation of the mitochondrial genome of SY5Y cells through long-term exposure to low concentrations of ethidium bromide (EtBr) gives rise to a mitochondrial DNA depleted cell known as a ρ^0 (Cassarino, *et al.*, 1997, 2000; Swerdlow, *et al.*, 1996). Exposure of ρ^0 cells to 5 mM MPP^+ does not induce apoptotic cell death and does not show increases in ROS or lactate production. The electron transport chain therefore needs to be intact for MPP^+ to increase the oxidative stress (Brill and Bennet, 2003; Fall and Bennett, 1999).

Mitochondria are central not only to the bioenergetics of the cell but also to the process of cell death. PD patients show mitochondrial dysfunction, particularly of complex I, and it appears likely that the mitochondria contribute to the pathogenic processes that occur. Treatments targeted at mitochondrial function hold promise to slow the progression of PD (Shults, 2004).

2.3.2.2 Energy failure

Mitochondrial function is essential for cellular energy supply. The inhibition of complex I and impaired ATP formation disables energy-dependent processes such as maintenance of the calcium homeostasis and of the cellular membrane potential as well as ion and transmitter transport in general (Di Monte, 1991). Energy consuming repair processes further deplete available ATP and energy within the cell. In particular, enzymes such as PARP, responsible for DNA repair and requiring ATP, are critically involved in MPTP toxicity. MPP^+ is also thought to inhibit the α-ketoglutarate dehydrogenase of the tricarboxyclic acid cycle (complex II of the respiratory chain) (Mizuno, *et al.*, 1987) and these mechanisms act synergistically to enhance the MPTP-induced disruption of cellular energy metabolism.

2.3.2.3 Calcium homeostasis

An apparent consequence of severe energy impairment is the decreased activity of the energy dependent calcium-ATPase which leads to intraneuronal calcium-overload. Elevated intracellular calcium levels activate degradative enzymes like phosphatases and proteases. Degradation of cell membranes or the cytoskeleton in turn results in disrupted cell function, loss of cell membrane potential and finally neuronal death. This mechanism of toxicity is supported by the fact that binding of excess calcium, and exogenously applied calcium channel blockers, reduce MPTP induced nigral degeneration (German, *et al.*, 1992; Kupsch, *et al.*, 1995, 1996).

2.3.2.4 Glutamate release

Local administration of MPP^+ via reverse microdialysis enhanced release of glutamate, an exitatory amino acid with neurotoxic properties (Carboni, *et al.*, 1990). The neurotoxic properties of glutamate are due to its activation of glutamate (N-methyl d-aspartate, NMDA) receptors and a massive influx of calcium leading to the formation of reactive oxygen species as well as a reduced intracellular glutathione synthesis (Murphy, *et al.*, 1989). It is also indirectly toxic through a cascade of events which enable normally non-toxic levels of glutamate to become cytotoxic (Beal, *et al.*, 1993).

2.3.2.5 Reactive oxygen and nitrogen species

Reactive oxygen species are generally formed as by-products of biochemical reactions. •OH is the most reactive ROS and reacts at a high rate with almost every biomolecule. Oxidative damage of cell membrane lipids, proteins and nucleic acids are characteristic predictors of •OH mediated toxicity and tissue damage.

MPP^+ is known to cause dopamine release in the striatum (Santiago, *et al.*, 1991) and MPTP administration therefore produces an increased dopamine turnover (Teismann and Ferger, 2001) which resembles the enhanced dopamine turnover in PD. It has also been found that in the later stage of PD, after more than 80 % of the dopaminergic neurons underwent neurodegeneration, the remaining dopaminergic neurons try to compensate by producing more dopamine (Fahn and Cohen, 1992).

When dopamine is oxidized, one molecule H_2O_2 is formed for each molecule of dopamine. H_2O_2 reacts with Fe^{2+} ions to form reactive oxygen species such as $\bullet O_2^-$ and •OH. $\bullet O_2^-$ is less deleterious than •OH. Transgenic mice, which over express the enzyme superoxide dismutase responsible for the elimination of $\bullet O_2^-$, showed a significant protection against MPTP toxicity (Przedborski, *et al.*, 1992).

$\bullet O_2^-$ can also react with nitric oxide (NO) to form peroxinitrite, another neurotoxin. Peroxynitrite inhibits complex I, II and III of the mitochondrial respiratory chain and irreversibly depletes energy production (Radi, *et al.*, 1991).

Nitric oxide also seems to be involved in the toxicity of MPTP (Castagnoli, *et al.*, 1997; Di Monte, *et al.*, 1997). Mice with a deficiency in nitric oxide synthase or when treated with nitric oxide synthase inhibitors have been shown to be protected against MPTP toxicity (Hantraye, *et al.*, 1996; Przedborski, *et al.*, 1996; Schulz, *et al.*, 1995).

Complex I of the mitochondria is also highly vulnerable to oxidative damage (Allen, *et al.*, 1995). Reactive oxygen species can destroy the integrity of the mitochondrial membrane, disturbing the calcium homeostasis. Inhibition of complex I and increased calcium levels, again enhance the formation of reactive oxygen species leading to an ultimately destructive cycle and cell death (Cleeter, *et al.*, 1992).

2.3.3 Model for Parkinson's Disease

Animal models are a very important approach to study the pathogenesis and therapeutic intervention strategies of human diseases. Since many human disorders do not arise spontaneously in animals, characteristic functional changes have to be mimicked by neurotoxic agents (Schmidt and Ferger, 2001).

MPTP is administered to the C57Bl/J6 strain of mice, which is more sensitive to a systemic injection and more selective in terms of targeting the nigrostriatal dopaminergic neurons than other mice strains. This model is thought to be the most practicable choice to study neuroanatomical and neurochemical alterations (Schmidt and Ferger, 2001).

The regimen of MPTP treatment has been shown to determine the mode of neuronal death in the substantia nigra. Chronic administration leads to apoptotic cell death of dopaminergic neurons (Tatton and Kish, 1997). Acute administration leads to necrotic cell death and, at least during the first four days, to a loss of dopaminergic phenotype without destroying the neuron (Jackson-Lewis, *et al.*, 1995).

2.4 The Statins

The enzyme, hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, catalyzes the conversion of hydroxymethylglutaryl (HMG) to mevalonate in an early and rate limiting step in cholesterol synthesis (Figure 2-10). Inhibition of HMG-CoA reductase causes a decrease in cholesterol synthesis. This leads to up-regulation of low-density lipoprotein (LDL) receptors and increase in the removal of LDL from plasma. HMG-CoA reductase is the target for a class of highly effective inhibitors of cholesterol synthesis, also known as the *statins* (Hamelin and Turgeon, 1998). Previous studies have shown one of these drugs (simvastatin)

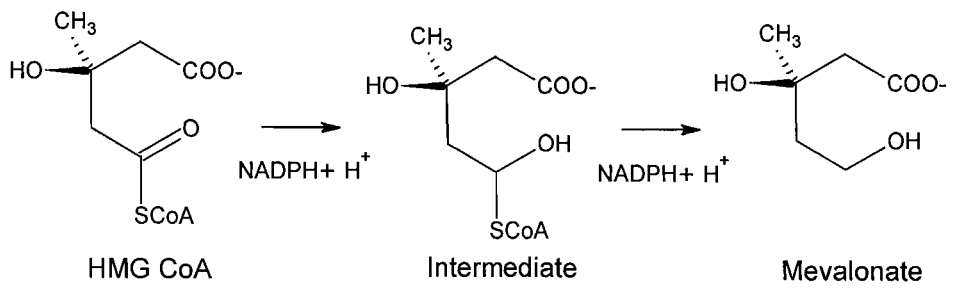

Figure 2-10 Reaction catalysed by HMG-CoA reductase (Witztum, 1996)

to reduce DNA damage of hypercholesterolemic patients and to have a beneficial effect on the repair of DNA damage (Min-Jeong, *et al.*, 2005; Shin, *et al.*, 2005).

2.4.1 Chemistry

Examples of statin drugs include lovastatin, simvastatin, pravastatin, fluvastatin and atorvastatin. The chemical structure of the first three drugs are closely related (Figure 2-11). Pravastatin's physico-chemical properties are however, different from that of lovastatin and simvastatin. Atorvastatin is the Ca^{2+} salt of a pentasubstituted pyrrole and presents a distinct chemical structure (Hamelin and Turgeon, 1998).

Lovastatin is derived from a fungal source, and simvastatin and pravastatin are chemical derivatives thereof. Lovastatin and simvastatin possess a methyl at position 6 whereas pravastatin possesses a hydroxyl. Simvastatin also possesses a methyl group at position 2 on the butanoate lateral chain (Hamelin and Turgeon, 1998).

While pravastatin (Hamelin and Turgeon, 1998) and atorvastatin (Ertürk, *et* al., 2003) are administered as the readily active open hydroxy-acid form, lovastatin and simvastatin are administered as inactive lactones which must be metabolized to their corresponding open hydroxy-acid forms in order to inhibit HMG-CoA reductase (Hamelin and Turgeon, 1998).

2.4.2 Absorption, distribution and metabolism

In humans, 34 % of pravastatin (Singhvi, *et al.*, 1990) and 80 to 85 % of the simvastatin lactone (Duggan and Vickers, 1990) are absorbed when given orally. The absolute average bioavailability of pravastatin is 18 %, simvastatin is < 5 %, and atorvastatin is 12 % (Christians, *et al.*, 1998). HMG-CoA reductase inhibitors are metabolized to active metabolites that appear in the systemic circulation. This is being regarded as an index of potential untoward effects in peripheral tissues because endogenous cholesterol synthesis takes place in the liver (Hamelin and Turgeon, 1998).

Pravastatin is hydrophilic, whilst simvastatin and atorvastatin are lipophilic drugs (Christians, *et al.*, 1998). Simvastatin is three times more lipophilic than the open hydroxy-acid forms which in turn is approximately hundred times more lipophilic than pravastatin. These drugs therefore cross cellular membranes by passive diffusion to different degrees and this explains

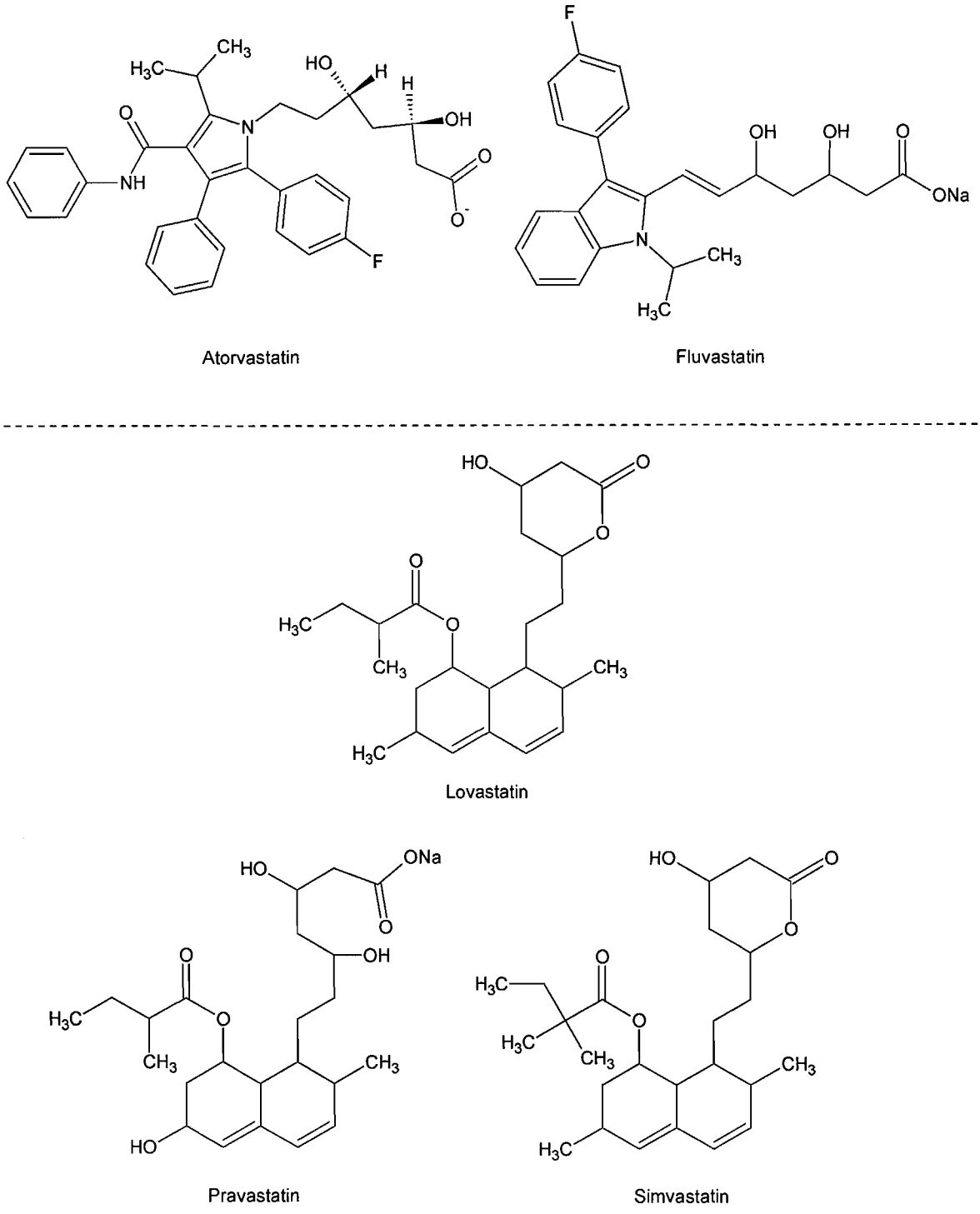

Figure 2-11 Chemical structures of the HMG-CoA reductase inhibitors (Hamelin and Turgeon, 1998; Ertürk, *et al.*, 2003)

why pravastatin does not easily cross cellular membranes whereas simvastatin does (Hamelin and Turgeon, 1998). Simvastatin in its lactone form, but not in its acid form, penetrates the central nervous system while the more hydrophilic pravastatin does not (Christian, *et al.*, 1998; Hamelin and Turgeon, 1998).

Treatment with hydrophilic statins has however been shown to be relevant in disorders of the central nervous system. Epidemiological studies suggest that statins are effective in lowering the prevalence of Alzheimer's disease which has been linked to brain cholesterol homeostasis (Wolozin, *et al.*, 2000). Whether this effect is due to a local inhibition of cholesterol synthesis in the brain or whether it is mediated by the reduced levels of cholesterol in the circulation is not known (Lütjohann, *et al.*, 2004). Also, in the brains of guinea pigs cholesterol levels are greatly reduced when treated with simvastatin and pravastatin (Lütjohann, *et al.*, 2004).

Pravastatin is the only HMG-CoA reductase inhibitor that is mainly eliminated unchanged. Its main metabolite is inactive and it has a terminal plasma half-life slightly shorter than that of pravastatin. Simvastatin and atorvastatin are eliminated mostly as metabolites that significantly contribute to their lipid-lowering effect. There is indirect evidence that active metabolites of simvastatin and atorvastatin with longer terminal half-lives than the parent compound exist, but none of these have been characterized. For this reason, pharmacokinetic studies using non-specific analytical methods yielded markedly longer terminal plasma half-lives for lovastatin and simvastatin than studies using specific methods. Long terminal half-lives of the parent drug or its metabolites lead to accumulation in plasma and tissues (Christians, *et al.*, 1998).

2.5 The Widening Role of Statins

Statins have a multitude of effects other than its inhibition of HMG-CoA reductase and cholesterol lowering properties. For example, they have been shown to:

- influence gene expression in the aorta, with pravastatin up-regulating 30, and down-regulated 42 known genes involved in cytoskeleton organization and G-protein signal transduction within the cell (Liu, *et al.*, 2006).

- modulate precerebral atherothrombosis, improve endothelial homeostasis and prevent dementia (Vaughan, 2003). They have been shown to have anticancer effects and to attenuate the duration and intensity of the response to DNA damage in hepatocytes (Päärjärvi, *et al.*, 2004).

- exhibit a positive effect on proliferation and osteoblastic differentiation of human PDL cells (Yazawa, *et al.*, 2005) and pravastatin treatment has been shown to reduce stroke risk by 19 % (Vaughan, 2003).

- inhibit DNA damage-dependant stress responses when stimulated with radiation, making it prospective useful in the clinic for preventing endothelial cell damage associated with radiation therapy (Nubel, *et al.*, 2006).

Recent research has revealed that the role of statins is progressively widening. However, it is mainly their anti-oxidant and anti-inflammatory properties that play a part in their potential neuroprotective properties.

2.5.1 Antioxidative Properties

Statins possess antioxidative properties that may be of value in the fight against oxidative DNA damage. Statins have been shown to reduce lipoprotein oxidation and ameliorate free radical injury and simvastatin, pravastatin and atorvastatin possess significant antioxidant activity against •OH and peroxyl radicals. In particular, simvastatin is the most active statin against •OH (Franzoni, *et al.*, 2003).

The simvastatin and pravastatin predecessor, lovastatin, reduces LDL oxidation and preserves the endogenous antioxidant enzyme superoxide dismutase (Chen, *et al.*, 1997), and simvastatin increases α-tocopherol, an antioxidant, in hypercholesterolemic patients (Human, *et al.*, 1997).

Atorvastatin metabolites reduce lipoprotein oxidation in a number of oxidative systems (Aviram, *et al.*, 1998).

2.5.2 Anti-inflammatory Properties

Statins have been shown to possess a wide range of vascular benefits linked to attenuation of chronic vascular inflammation. It is thought that they inhibit NF-κB, a transcription factor involved in immune and inflammatory responses (Hölschermann, *et al.*, 2006).

2.5.3 Other Mechanisms for Neuroprotection

Statins also seem to offer protection against injury to brain tissues before, during and after cerebral ischemia (Vaughan and Delanty, 1999).

Cytokines such as interleukin-1, tumour necrosis factor, and interleukin-6 are produced by neurons, glia, and endothelium, and appear to be important mediators of inflammatory and immunological responses in the brain (Vaughan and Delanty, 1999).

The inducible form of nitric oxide synthase (iNOS) contributes to neuronal death through oxidation of structural neuronal proteins during ischemia (Vaughan and Delanty, 1999) and has been implicated in cerebral ischemia, Alzheimer's disease and PD (Vodovotz, *et al.*, 1996). It is produced in response to cytokines (Hu, *et al.*, 1995) such as interleukin and tumour necrosis factor and statins reduce the elaboration of these cytokines from macrophages (Vaughan and Delanty, 1999).

Furthermore, lovastatin has been shown to reduce iNOS induction and NO production in rat astrocytes, microglia and macrophages (Pahan, *et al.*, 1997), supporting the possibility that statins may suppress the inflammatory response involved in central nervous system disease.

Statins, therefore, seem to have the potential to reduce neurological injury by also modulating inflammatory receptor activity and/or expression (Cucchiara and Kasner, 2001).

Chapter 3 - Methods

3.1 Single Cell Gel Electrophoresis Assay

The single cell gel electrophoresis (SCGE), comet or microgel electrophoresis assay was developed from the original concepts of Rydberg and Johanson (Singh, 2000). The comet assay (since the final appearance of the cells following electrophoresis can be likened to a comet with a nucleus and tail) was developed as a method to examine DNA integrity at a single cell level (Rojas, *et al.*, 1999; Singh, *et al.*, 1988). Because this technique uses small numbers of cells, it has proven useful for the analysis of DNA damage when sample availability is limited, e.g. with small animals or in biomarker studies using pin-prick samples of blood (Cooke, *et al.*, 2006).

3.1.1 History

In the 1970s, Peter Cook and colleagues (Cook, *et al.*, 1976) developed an approach to investigate nuclear structure based on the lysis of cells with non-ionic detergent and high-molarity sodium chloride. This treatment removes membranes, cytoplasm, nucleoplasm, disrupts nucleosomes and dissolves almost all histones (Collins, 2004).

What is left is the nucleoid, consisting of a nuclear matrix composed of DNA, ribonucleic acid (RNA) and proteins. The DNA is negatively supercoiled as a consequence of the turns made by the double helix around the histones of the nucleosome. Free rotation of the DNA is not possible because of the survival of these supercoils (Collins, 2004).

A model was proposed with the DNA attached at intervals to the matrix so that it is effectively arranged as a series of loops, rather than as a linear molecule. When the negative supercoiling was unwound by adding an intercalating agent (EtBr) the loops expanded out from the nucleoid core to form a halo. A similar effect was seen when ionizing radiation was used to relax the loops - one single-strand break being sufficient to relax the supercoiling in that loop (Collins, 2004).

In 1978, Rydberg and Johanson developed a technique to estimate DNA damage in individual cells by lysing cells and unwinding DNA with sodium hydroxide and measuring the number

of single stranded DNA. This gave an approximation of the number of breaks in the double stranded DNA (Rydberg and Johanson, 1978).

The first demonstration of "comets" (though they did not use the word) was by Östling and Johanson in 1984, who described the tails in terms of DNA with relaxed supercoiling and referred to the nucleoid model of Cook, *et al.* (1976). They electrophoresed the microgel microscope slides after lysis causing the DNA fragments to migrate through the agarose gel in the direction of the cathode. DNA damage was estimated by using the ratio of fluorescence intensity at two points along the migrated DNA. Because of the significant amounts of RNA that was still present, the amount of DNA could not be estimated correctly. The sensitivity was also limited because the DNA maintained its tertiary and quaternary structures, causing it to move unpredictably through the agarose gel (Ostling and Johanson, 1984).

Singh, *et al.* (1988) electrophoresed lysed cells under alkaline conditions, effectively degrading RNA, disrupting secondary structure and removing tertiary and quaternary structure of the DNA. This allowed a more predictable movement of DNA in the agarose. The electrophoresed slides were stained using EtBr to enable visualization of the DNA.

In 1998 a tray was developed to simultaneously process eight slides from electrophoresis through the neutralization to DNA precipitation (Singh, 1998) and in 1999 Singh, *et al.* (1999) made use of the newly designed clear window frosted slides.

3.1.2 Sensitivity, Reproducibility and Optimization

Sensitivity and reproducibility are critical in the determination of DNA damage, and even when a method is thoroughly standardised it is necessary to test the ability of laboratories to produce reproducible results. The sensitivity of the method depends most importantly on how the assay was performed, for example the use of enzymes, more intensive fluorescent dye, changes in electrophoresis conditions, etc. (Singh, 2000).

The comet assay has been compared to other methods during the studies co-ordinated by the European Standards Committee on Oxidative DNA Damage (ESCODD), and generally the comet assay produces the lowest cellular DNA levels of 8-OH-dG (ESCODD, 2002) compared to the chromatographic methods. Underestimation of the levels of lesion using this assay has been considered a potential confounding factor because of the inability to distinguish between closely spaced DNA strand breaks. Despite the various issues concerning

absolute quantification of DNA lesions, the comet assay has advantages in terms of sample size and processing and has found use in *in vitro* mechanistic studies and also human intervention/dietary studies (Cooke, *et al.*, 2006).

3.1.3 Apoptosis and necrosis

Apoptosis or "programmed cell death" is not associated with inflammation or scarring, unlike necrosis. Apoptosis is a normal event that occurs both during and after development and can be induced through mild to moderate genotoxic and cytotoxic insults. With an increase in insults the cell death mode shifts to necrosis (Singh, 2000).

Apoptosis is characterised by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation and formation of apoptotic bodies (Carnevali, *et al.*, 2003). Apoptosis is generally considered an energy-dependent process requiring active participation of many

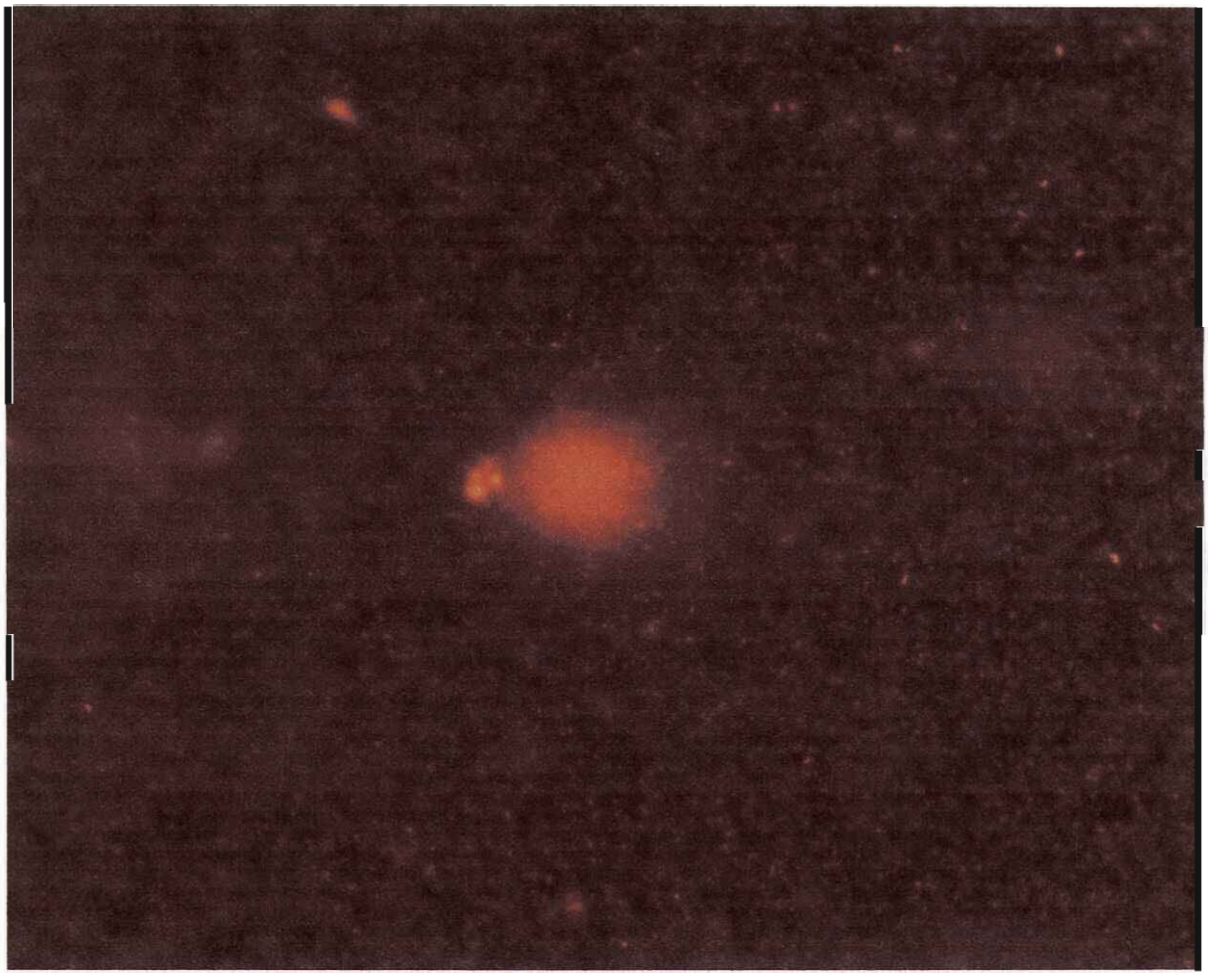

Figure 3-1 An example of a hedgehog comet

proteins and other cellular macromolecules resulting in the extensive formation of double-strand breaks. Similarly, the DNA of necrotic cells also undergoes extensive degradation. Such cells can be detected using either neutral or alkaline electrophoresis conditions. Apoptotic cells were thought to be readily distinguishable from necrotic cells in the alkaline SCGE assay and were said to form comets with large fan-like tails and small heads (so-called hedgehogs; Tice, *et al.*, 2000).

Collins (2004) argues that although relatively severely damaged cells will go through programmed cell death, they cannot be described as apoptotic because apoptosis is irreversible. Cells, treated with H_2O_2 to form hedgehog comets can repair their damage so that hedgehogs are no longer seen (Collins, *et al.*, 1995).

Apoptosis is further characterized by fragmentation of DNA to the size of nucleosome oligomers and, because such small pieces of DNA should disappear during lysis or electrophoresis, the high molecular weight DNA in apoptotic cells will rather present as the "ghosts" of comets that are sometimes seen (Collins, 2004).

3.1.4 Considerations

- DNA break frequency is linearly related to the percentage of tail DNA up to a certain level. Saturation occurs once the entire DNA is in the tail, and a deviation from linearity is seen. Particular care is needed when the damage induced with H_2O_2 during the assay is superimposed on already high levels of strand breakage; there is then a tendency for the damaged bases to be underestimated (Collins, 2004).
- DNA is denatured and unwound at a pH above 12,0 because of the disruption of hydrogen bonds between double-stranded DNA. At pH conditions of 12,6 or higher, alkali labile sites (e.g., apurinic sites) are readily transformed to single-strand breaks. A pH of > 13 would be expected to maximize the expression of alkali labile sites as single-strand breaks (Tice, *et al.*, 2000).
- Singh, *et al.* (1994) reported that electrophoresis time is one of the most important factors that increase the sensitivity of SCGE. The voltage and time of electrophoresis is related to the levels of damage to be detected.
- Although most of the DNA moves towards the anode during electrophoresis, it also diffuses in all dimensions during lysis, electrophoresis and neutralization. The degree of

diffusion depends upon a number of factors, such as concentration of agarose, size of DNA, composition of LS, temperature, etc. (Singh, 2000).

3.2 Experimental Design

3.2.1 Hypothesis

In this study the neuroprotective effect of three commercially available statins (simvastatin, pravastatin and atorvastatin) against striatal damage induced by MPTP is investigated and it is hypothesised that these drugs will have a positive effect on the levels of DNA damage and the DNA repair capacity when compared to control groups.

3.2.2 Statin selection

The statin drugs were selected to be used in this study because of its antioxidative (Aviram, *et al.*, 1998; Chen, *et al.*, 1997; Franzoni, *et al.*, 2003; Human, *et al.*, 1997), anti-inflammatory (Cucchiara and Kasner, 2001) and neuroprotective (Vaughan and Delanty, 1999) properties. Previous studies have also shown simvastatin to reduce DNA damage and to have a beneficial effect on DNA repair (Shin, *et al.*, 2005).

Statins were selected to include active hydroxyl-acid forms (pravastatin and atorvastatin; Ertürk, *et* al., 2003; Hamelin and Turgeon, 1998) and a lipophilic prodrug (simvastatin; Hamelin and Turgeon, 1998). One of the hydroxyl-acid statins is hydrophilic (pravastatin) and the other lipophilic (atorvastatin). Pravastatin, although hydrophilic and unable to enter the central nervous system (Christians, *et al.*, 1998), does reduce cholesterol levels in the brains of guinea pigs (Lütjohann, *et al.*, 2004), and was therefore selected.

3.2.3 Design

Forty male, C57Bl/J6 mice were divided into eight groups of five mice each. Groups A1 and B1 were treated with saline and served as control groups, groups A2 and B2 were treated with pravastatin, groups A3 and B3 were treated with simvastatin and groups A4 and B4 were treated with atorvastatin. Group A was injected with saline while group B was injected with MPTP (Figure 3-3).

The level of DNA damage in the eight groups were compared at four stages during the comet assay (Figure 3-2, [A] to [D]).

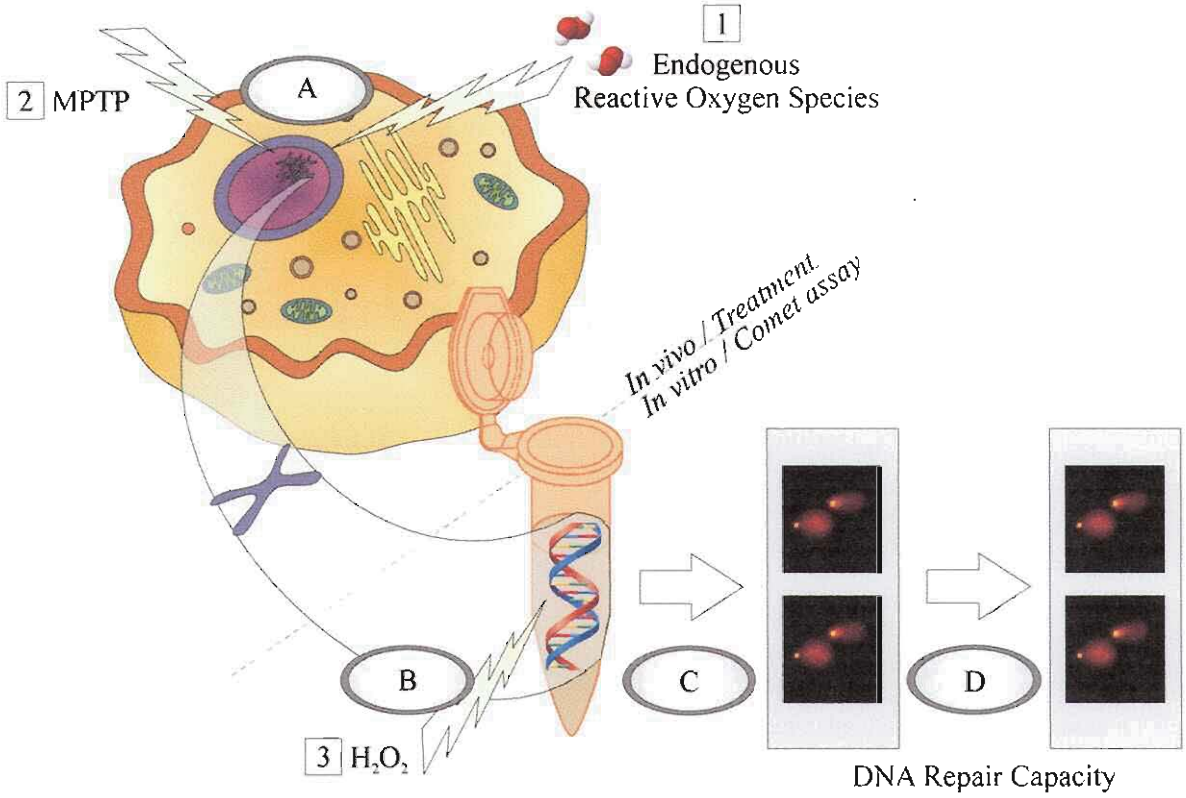

Figure 3-2 **Experimental design – The neuroprotective effect of statins against DNA damage from 1 - endogenous ROS; 2 - MPTP; and 3 - H_2O_2 is measured at A - the start of the assay; B - after 30 min of H_2O_2 induced DNA damage; C - after 20 min repair; and D - after 40 minutes of repair**

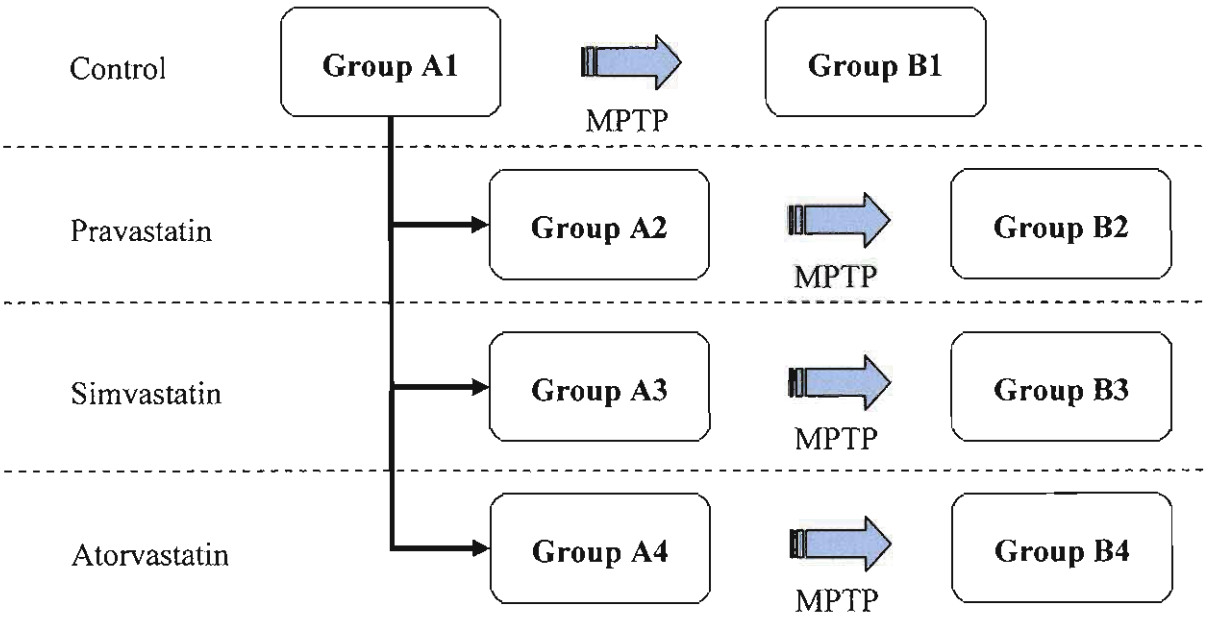

Figure 3-3 **Schematic representation of the study design**

The baseline level DNA damage was determined at the start of the assay and this measurement included the effects of endogenous ROS (Figure 3-2 [1]), MPTP (Figure 3-2 [2]) and statin treatment. DNA damage was induced *in vitro* with H_2O_2 (Figure 3-2 [3]), and another measurement was taken. The cells were then allowed to repair their DNA and two more measurements were taken at 20 min intervals.

This allowed us to compare the effects of statin treatment on both the level of DNA damage and the capacity of the cells to repair their DNA.

The effect of MPTP on the baseline DNA damage and the DNA repair capacity can be determined by comparing the results from groups A1 and B1, horizontally (Figure 3-3).

The effect of statin treatment on the baseline level of DNA damage (caused by endogenous ROS) and the DNA repair capacity can be determined by comparing the results from groups A2, A3, and A4 with group A1, vertically (Figure 3-3).

The effect of statin treatment on baseline DNA damage (caused by endogenous ROS and MPTP) and the DNA repair capacity (after MPTP treatment) can be determined by comparing groups B2, B3, and B4 with group B1, vertically (Figure 3-3).

3.3 Experimental Procedures

The design and execution of the SCGE assay were done according to the guidelines set out by Hartmann, *et al.* (2003).

3.3.1 Experimental animals

The use of experimental animals in this study was approved by the Ethics Committee of the North-West University (approval number 05D03). Forty male, C57Bl/J6 mice were provided by the Animal Testing Centre, Potchefstroom Campus of the North-West University and were housed under constant conditions of temperature (25 °C) and humidity (55 ± 5 %) and a 12 h day / night cycle.

Mice receiving MPTP (group B) were injected with 30 mg / kg MPTP at 08:00 on day three of treatment while the control mice (group A) were injected with saline. This dosage of MPTP correlates to an "immediate onset model for rapid degeneration with necrotic cell death" as proposed by Schmidt and Ferger (2001).

3.3.2 Dosages

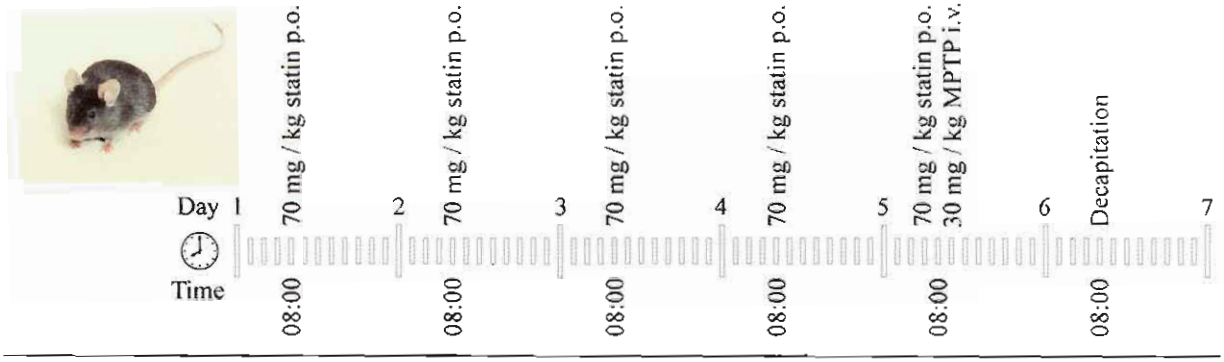

Figure 3-4 Treatment regime for experimental animals

The statins (obtained commercially) were triturated in a mortar and pestle (Díaz-Zagoya, *et al.*, 1999). The powder was suspended in a 1,5 % aqueous carboxymethylcellulose suspension, 10 min before administration. An oral dose of 0,5 ml (70 mg / kg) (Johnston, *et al.*, 2001) was administered daily at 08:00 from days one to five of treatment while control groups receive the suspension vehicle (Figure 3-4).

3.3.3 Decapitation, dissection and storage

All mice were decapitated at 08:00 on day six of the treatment, their brains removed and placed on ice. The striatum (also called the neostriatum, caudate putamen) was dissected out and transported on ice at 4 °C for single cell gel electrophoresis which was conducted without delay.

3.3.4 Single cell gel electrophoresis assay

The procedure for the single cell microgel electrophoresis employed in this study is outlined in Figure 3-5.

3.3.4.1 Slide Preparation

A master cell suspension is prepared by homogenizing the isolated striatum in 500 µl of mincing solution (MS) and leaving it to incubate for 15 min at room temperature. The sample was then centrifuged (5 500 g for 5 min), the supernatant removed and the pellet suspended with 300 µl phosphate buffer solution (PBS).

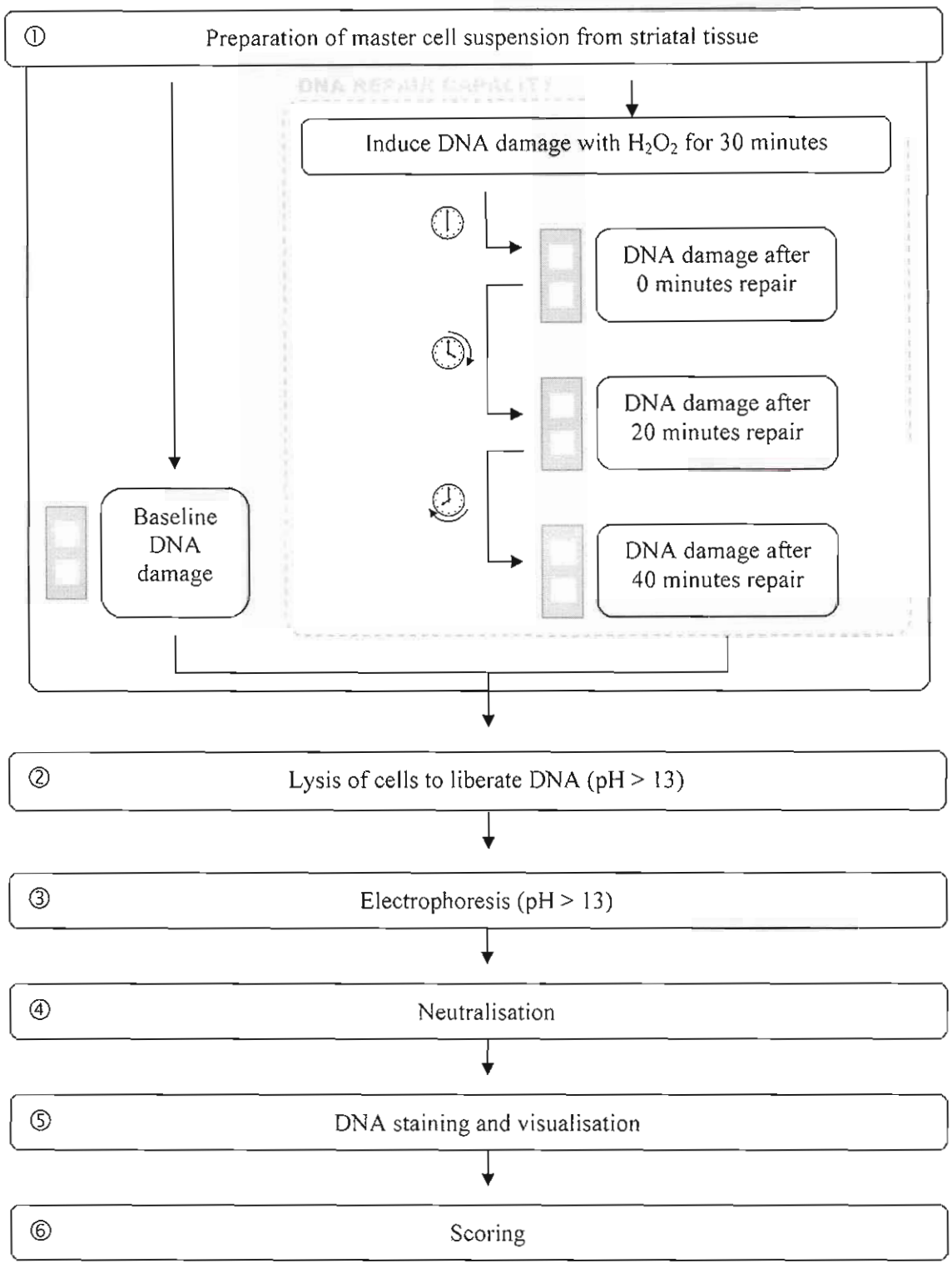

Figure 3-5 **Procedure for single cell microgel electrophoresis employed in this study (Tice, *et al.*, 2000)**

The first slide window (Figure 3-2 [A]) was coated with 40 µl of this solution mixed with 150 µl low melting point agarose (LMPA) at 37 °C.

To induce DNA damage, 17.2 µl of a 3520 µM H_2O_2 solution was added to the remaining solution, and left to incubate at 37 °C. After 30 min the solution was washed twice with 400 µl PBS and centrifuged (5 500 g for 5 min), and the pellet suspended in Ham's cell culture solution.

The second (Figure 3-2 [B]), third (Figure 3-2 [C]) and fourth (Figure 3-2 [D]) slide windows were coated with 40 µl of the solution mixed with 150 µl LMPA at 37 °C after 0 min, 20 min and 40 min of DNA repair.

The cells were lysed overnight at 4 °C in lysing solution (LS), incubated in the electrophoresis buffer solution (Eph-BS) for 30 min and then electrophoresed (pH > 13) at 4 °C for 20 min at 30 V and 300 mA using a computerised power supply.

Neutralisation was brought about through incubation in tris-(hydroxymethyl)aminomethane buffer (Tris) solution for 15 min, after which the DNA was stained with EtBr for 15 min, and pictures were taken.

Agarose and the Eph-BS were prepared fresh for each analysis while the other solutions were used from stock solutions prepared in advance.

3.3.4.2 Slide Analysis

a) Comet Selection

Analysis of a minimum of 50 comets per slide is recommended (Collins, 2004). Comets were selected without bias and the gel scanned in a systematic way as to be representative of the whole gel. The edges and areas around air bubbles were avoided as unusually high levels of damage are displayed in these areas (Collins, 2004).

b) Comet Scoring

The Comet Assay Software Project (CASP) software was used in the scoring of the comets. CASP can analyse either colour or gray-scale images of fluorescence-stained comets saved in

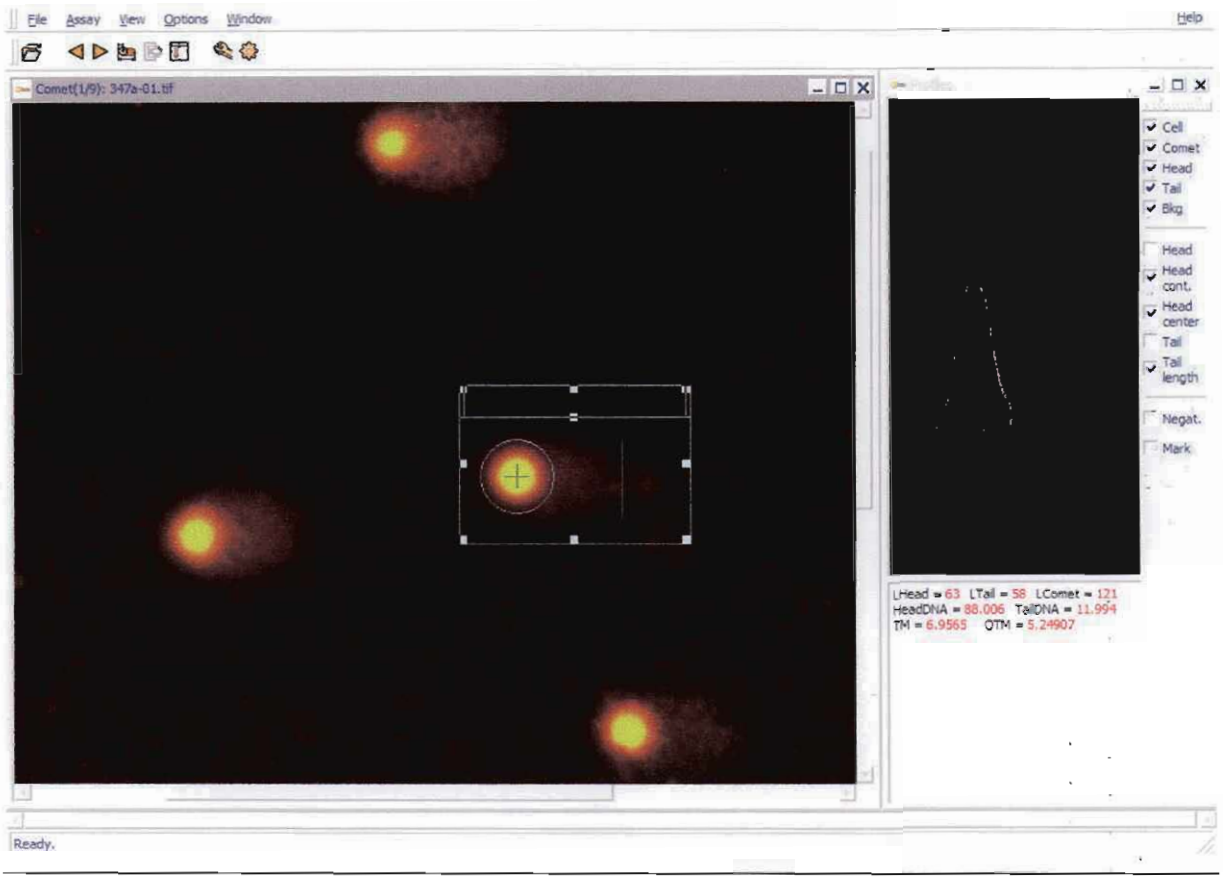

Figure 3-6 Comet measurement

tagged image format (TIF). Only comets oriented from left to right can be analysed correctly (Konca, 2005).

Five to ten images were taken per slide, marked for analysis and scored by drawing a measurement frame on the screen and locking it into place. The frame was moved onto a comet and a measurement taken. An intensity profile shows up on a "profile" window together with selected result values (Figure 3-6). This process was repeated until all comets on the slide were measured, after which the results were exported into a text file and imported into Microsoft Excel® 2003.

Several parameters for each comet are available for comparison (Table 3-1). Generally, cells with a high level of DNA damage exhibit increased comet parameters (Faust, *et al.*, 2004).

The most commonly used parameters are tail length, relative fluorescence intensity of head and tail (normally expressed as a percentage of DNA in tail), and tail moment (Collins, 2004). There are however certain advantages and disadvantages when measuring comets using these three parameters:

1. Mean tail length increases only at relatively low damage levels while tails are first becoming established after which the tail increases in intensity but not in length. Tail length is also sensitive to the background or threshold settings of the image analysis program (see Section 3.3.4.3m)), as the end of the tail is defined by a certain excess of fluorescence over background (Collins, 2004).

2. Relative tail intensity or percentage DNA in tail (Tail DNA %) is the most useful parameter, as it bears a linear relationship to break frequency and is relatively unaffected by threshold settings. It allows discrimination of damage over the widest possible range (in theory, from 0 to 100 % DNA in tail) and also gives a very clear indication of what the comets actually looked like (Collins, 2004).

3. In contrast, the third parameter, tail moment (essentially the product of tail length and tail intensity) is not linear with respect to dose and does not give any impression of the comet's appearance (Collins, 2004).

Table 3-1 Parameters measured by CASP (Konca, 2005)

Parameter	Description
Name	Name of image file
Head Area	Area of the comet head in pixels (sum of pixels in the head)
Tail Area	Area of the comet tail in pixels (sum of pixels in the tail)
Head DNA	Amount of DNA in the comet head (sum of intensities of pixels in the head)
Tail DNA	Amount of DNA in the comet tail (sum of intensities of pixels in the tail)
Head DNA %	Percent of DNA in the comet head
Tail DNA %	Percent of DNA in the comet tail
Head Radius	Radius of the comet head (in pixels)
Tail Length	Length of the comet tail measured from right border of head area to end of tail (in pixels)
Comet Length	Length of the entire comet from left border of head area to end of tail (in pixels)
Head Mean X	Center of gravity of DNA in the head (x coordinate)
Tail Mean X	Center of gravity of DNA in the tail (x coordinate)
Tail Moment	Tail DNA % × Tail Length
Olive Tail Moment	Tail DNA % × (Tail Mean X - Head Mean X)

The percentage of DNA in tail is the preferred way to present DNA damage results (García, *et al.*, 2004). Three parameters (percentage of DNA in tail, tail length, and tail moment) were however used by Shin, *et al.* (2005) to study the effect of simvastatin on DNA damage (Shin, *et al.*, 2005). The results from this study are expressed using these same parameters.

3.3.4.3 Technical Specifications and Protocols

a) Instrumentation

Table 3-2 Instrumentation used in conducting the comet assay

System	Specifications
Sandblasted microscope slides (76 x 26 mm)	RESY
Power supply unit (30 V, 300 mA)	Bio-Rad Model 200/2.0 Power supply
Fluorescent microscope	Olympus IX-70
Camera	SIS, D-48153 Muenster
Imaging Software	AnalySIS 3.0

b) Chemicals and reagents

Table 3-3 Chemicals and reagents used for comet assay and the suppliers of the chemicals

Reagents	Supplier
Dimethylsulphoxide (DMSO)	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Sigma
EtBr	Sigma
Potassium chloride (KCl)	Sigma
$K(H_2)PO_4$	Merck
NaCl	Sigma
$Na_2(HPO_4)$	Merck
NaOH	Sigma
High melting point agarose (HMPA)	Sigma
LMPA	Sigma
Triton X-100	Sigma
Tris HCl	Sigma

c) Preparation of EDTA

EDTA was prepared according to the following protocol:

- 37,20 g EDTA disodium salt (Mr: 372,2)
- Double distilled water (ddH_2O) to a volume of 1 000 ml

d) Preparation of PBS

PBS was prepared according to the following protocol:

- 8,00 g NaCl
- 0,20 g KCl
- 1,15 g Na_2HPO_4
- 0,20 g KH_2PO_4
- ddH_2O to a volume of 1 000 ml

The pH was adjusted to be between 7 and 8 and the solution kept in the refrigerator.

e) Preparation of LS

LS was prepared according to the following protocol:

- 500 ml NaCl (5 M)
- 250 ml EDTA (0,4 M) titrated to a pH of 7 to 8 with NaOH (0,1 M) before use
- 10 ml Triton X-100
- 100 ml DMSO (10 %)
- 140 ml ddH_2O to a volume of 1 000 ml

f) Preparation of Eph-BS

Eph-BS was prepared fresh for each analysis according to the following protocol:

- 20 ml EDTA (0,05 M)
- 500 ml NaOH (0,6 M)
- 480 ml ddH_2O to a volume of 1 000 ml

g) Preparation of Tris solution

Tris solution was prepared according to the following protocol:

- 63,04 g Tris HCl
- ddH_2O to a volume of 500 ml

The solution was stored at 4 °C for at least an hour before the pH was adjusted to 7.5 with 0.6 M NaOH after which the volume was adjusted to 1 000 ml with ddH_2O.

h) Preparation of H_2O_2

3520 µM H_2O_2 was prepared according to the following protocol:

- 20 µl 30 % (8,8 M) H_2O_2
- 980 µl PBS } Solution ①
- 20 µl of Solution ①
- 980 µl PBS

i) Preparation of EtBr dye solution

EtBr dye solution was prepared according to the following protocol:

- 0,005 g EtBr
- ddH_2O to a volume of 1 000 ml

The container was covered with foil and the solution was kept at 4 °C for at least an hour before use. Ethidiumbromide dye solution is carcinogenic and should only be handled with the necessary protective gear.

j) Preparation of HMPA

1,0 % HMPA was prepared according to the following protocol:

- 0,5 g HMPA
- 50 ml EDTA (0,1 M)

The solution was heated in a microwave oven for 15 second intervals until all the HMPA is dissolved.

k) Preparation of LMPA

0,5 % LMPA was prepared according to the following protocol:

- 0.25 g LMPA
- 50 ml EDTA (0,1 M)

The solution was heated in a microwave oven for 15 second intervals until all the LMPA was dissolved and then kept in a water bath at 42 °C.

l) Image Specifications

Analysis of 50 comets per slide is recommended (Collins, 2004) and to obtain sufficient comets five to ten images of each slide were taken in the format specified below.

Table 3-4 Specifications of slide images

Dimensions	1280 × 1024 pixels
Resolution	200 × 200 dpi
Colour Depth	24 bits
Format	TIF

m) CASP Specifications

Table 3-5 Specifications of CASP (Konca, 2005)

Program Version	1.2.2
Release Date	29 February 2004
Download URL	http://sourceforge.net/project/showfiles.php?group_id=91972
Download date	5 October 2004 11:40 am

Table 3-6 Settings used when scoring the comets

Paramater	**Setting**
Head Center Threshold	0,8
Comet Threshold	0,05
Use Comet Threshold	Checked
Counting (quantification) of DNA	Profile 1 (Tail Cluster)

The head center threshold regulates the detection of the head center and determines points to detection of the head center which is the center of gravity of found points. Reducing the threshold moves the head center towards the tail. Setting the value to 0,8 has the effect that all points of intensity greater then 80 % of maximal intensity are used to calculate the head center (Konca, 2005).

The comet threshold was used as the tail and head threshold because the "Use Comet Threshold" checkbox is checked. The comet threshold is the intensity of points found that is still considered to be part of the comet. A value of 0 gives the background intensity and 1 gives the maximum intensity. The optimal value is considered to be 0,05. Reducing the tail threshold will reduce the tail size (Konca, 2005).

In the detection of the tail, two profiles are available. Profile 1, used in this study, is based on the method of percolation. Only those pixels that lie to the right of the head center and form an uninterrupted "carpet" of pixels touching the head region are considered as belonging to the tail. This process is considered to be the optimal profile setting as it eliminates pixel

debris not belonging to the tail. In profile 2, all adequately intensive pixels outside the head region, located between the head center and end of the tail are considered as belonging to the tail (Konca, 2005).

Chapter 4 - Results

The SCGE results are expressed as % DNA in Tail, Tail Length and Tail Moment, and indicate the level of DNA damage at a particular time:

- baseline — level of DNA damage at the start of the assay
- t0 — level of DNA damage after 30 min incubation in H_2O_2
- t20 — level of DNA damage after 20 minutes of repair, and
- t40 — level of DNA damage after 40 minutes of repair

The above parameters are collectively referred to, in the text, as *levels of DNA damage.*

Since the % DNA in Tail, Tail Length and Tail Moment are descriptive of the size of the tail, an increase in these parameters indicate an increase in DNA damage.

The change (amount of damage or repair) in DNA damage that took place inside each cell was determined by calculating the difference between the measurement at a particular time and the preceding measurement. The following parameters are therefore introduced to give an indication of the rate of DNA damage per time interval:

- $\Delta_{\text{damage}} = t0 - \text{baseline}$ — change in DNA damage over 30 min (H_2O_2 incubation)
- $\Delta_{\text{repair 1}} = t20 - t0$ — amount of DNA repair over the first 20 min period
- $\Delta_{\text{repair 2}} = t40 - t20$ — amount of DNA repair over the second 20 min period
- $\Delta_{\text{total repair}} = t40 - t0$ — total amount of DNA repair

The above parameters are collectively referred to, in the text, as *changes in DNA damage.*

Another parameter, the repair capacity (RC), was also calculated:

- $RC = 1 - \left[\frac{t40}{t0}\right]$ where larger RC values indicate better repair:

$RC = 0$	no DNA repair took place
$1 \geq RC > 0$	DNA repair took place
$RC \geq 1$	DNA was restored to baseline levels or greater

4.1 Statistical Analysis

Statistical analysis was carried out at the Department of Statistical Services at the North-West University (Potchefstroom Campus) using Statistica® software (STATSOFT, 2005) and followed the guidelines, set out by Lang and Secic in *How to Report Statistics in Medicine* (1997).

The baseline levels of DNA damage, Δ_{damage}, $\Delta_{\text{repair 1}}$, $\Delta_{\text{repair 2}}$, $\Delta_{\text{total repair}}$ and RC of the different groups were compared in triplicate (data expressed as % DNA in Tail, Tail Length and Tail Moment). All values, including outliers, were included.

The statistical significance (p-level) of a result represents a decreasing index of the reliability of a result. The lower the p-level, the less doubt exists whether the observed relation between levels of DNA damage in our sample represents the relation between levels of DNA damage in the population. Specifically, the p-level represents the probability of error that is involved in accepting our observed result as valid.

A $p \leq 0.05$ (1 in 20) was treated as an acceptable level of error to indicate statistically significant differences. There is therefore a 5 % probability that the relation between the levels of DNA damage found in our sample is a coincidence. In other words, assuming that there was no relation between the levels of DNA damage in the population, and we repeated our experiments, we could expect that approximately one in every 20 replications would have a relation equal or stronger than our relation.

4.1.1 ANOVA and Student's t-test

An analysis of variance test (ANOVA or F-test) was used to test for significant differences between the means of the groups, except when only two groups were compared, as in § 4.2.1, Student's t-test for independent samples was used. Results from statistical analysis are expressed using the following syntax:

- f (df) = result, p = p-level where *f* indicates the statistical test used, e.g. F or t
  df indicates the degrees of freedom
  (group, interval values for the F-test)
  result is the test statistic, and
  p-level is the statistical significance detected

The degrees of freedom is calculated on the basis of sample size and is a mathematical concept that helps determine which probability distribution is used. Statistical testing results in the calculation of the test statistic. The test statistic is then compared to the appropriate probability distribution and the p-level associated with that statistic is then calculated. The p-level is the probability value associated with the test statistic: The probability of getting a result as extreme or more extreme than the one observed, assuming there is no difference between the groups (Lang and Secic, 1997).

4.1.2 Post-Hoc Comparisons

A statistically significant F-test from the ANOVA is indicative of a statistically significant difference between the groups under investigation. Further analysis of such findings was done using Dunnett's post-hoc test. This test compares a control group mean and the remaining treatment group means in an analysis of variance setting, i.e. which groups are particularly different from each other. Post-hoc comparison techniques specifically take into account that more than two samples were taken and eliminate overestimating the statistical significance of mean differences when performing a series of t-tests to compare all possible pairs of means.

The results of all statistical analyses are attached in APPENDIX B – Statistical Analysis.

4.2 Results

The levels of DNA damage as measured at a particular time are presented in Figure 4-1 and Figure 4-2. Data, as presented in the figures, are expressed as group means ± standard deviation (SD) between individual mice.

The Control Group values are indicated in purple, pravastatin treated groups in maroon, simvastatin treated groups in yellow, and atorvastatin treated groups in cyan. Groups treated with MPTP are indicated with diagonal stripes.

The data for levels of DNA damage and changes in DNA damage are attached in APPENDIX A – Data.

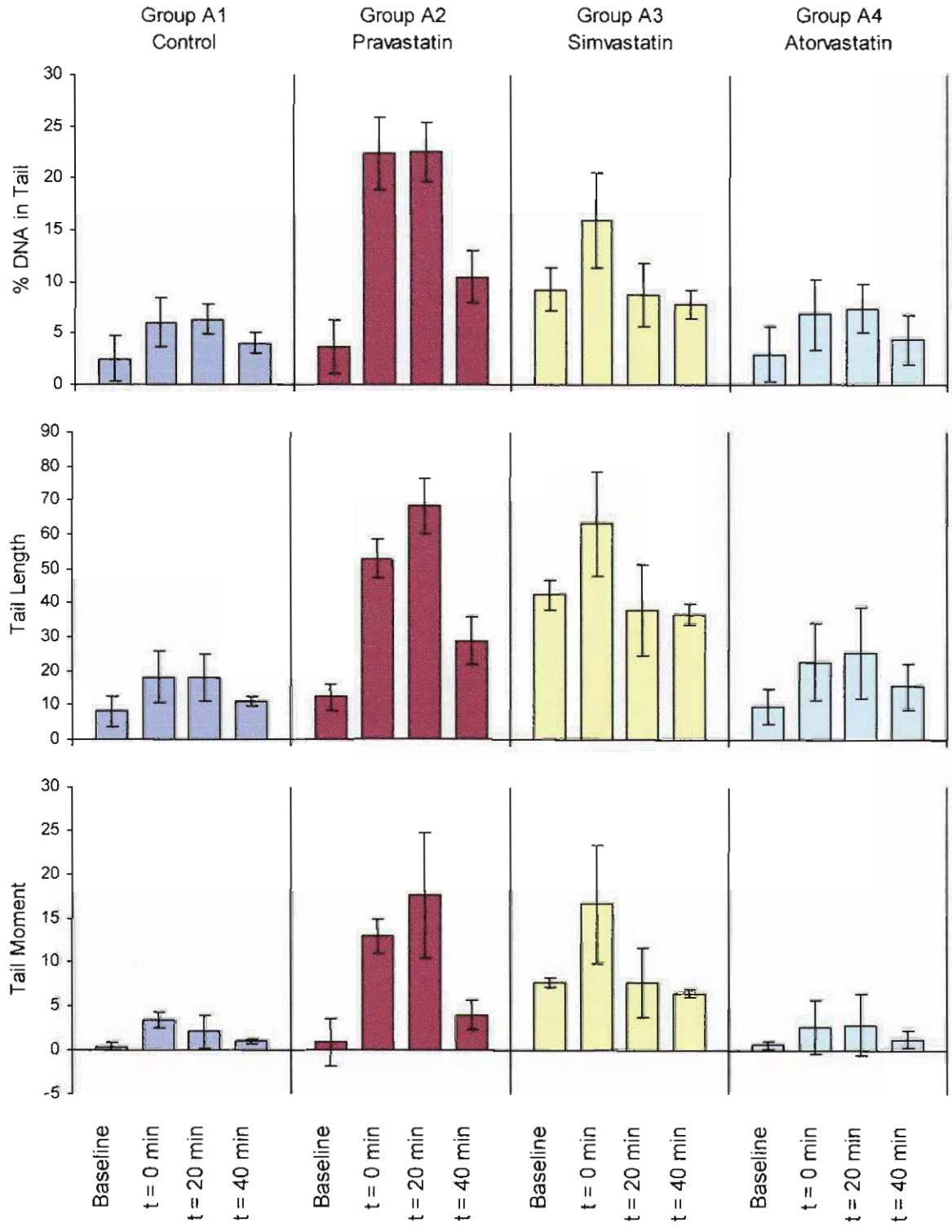

Figure 4-1 **Mean levels of DNA damage in group A measured at the start of the assay (baseline), at t = 0 min, t = 20 min and t = 40 min, expressed as % DNA in Tail, Tail Length and Tail Moment, with SD between mice in each group (n = 5)**

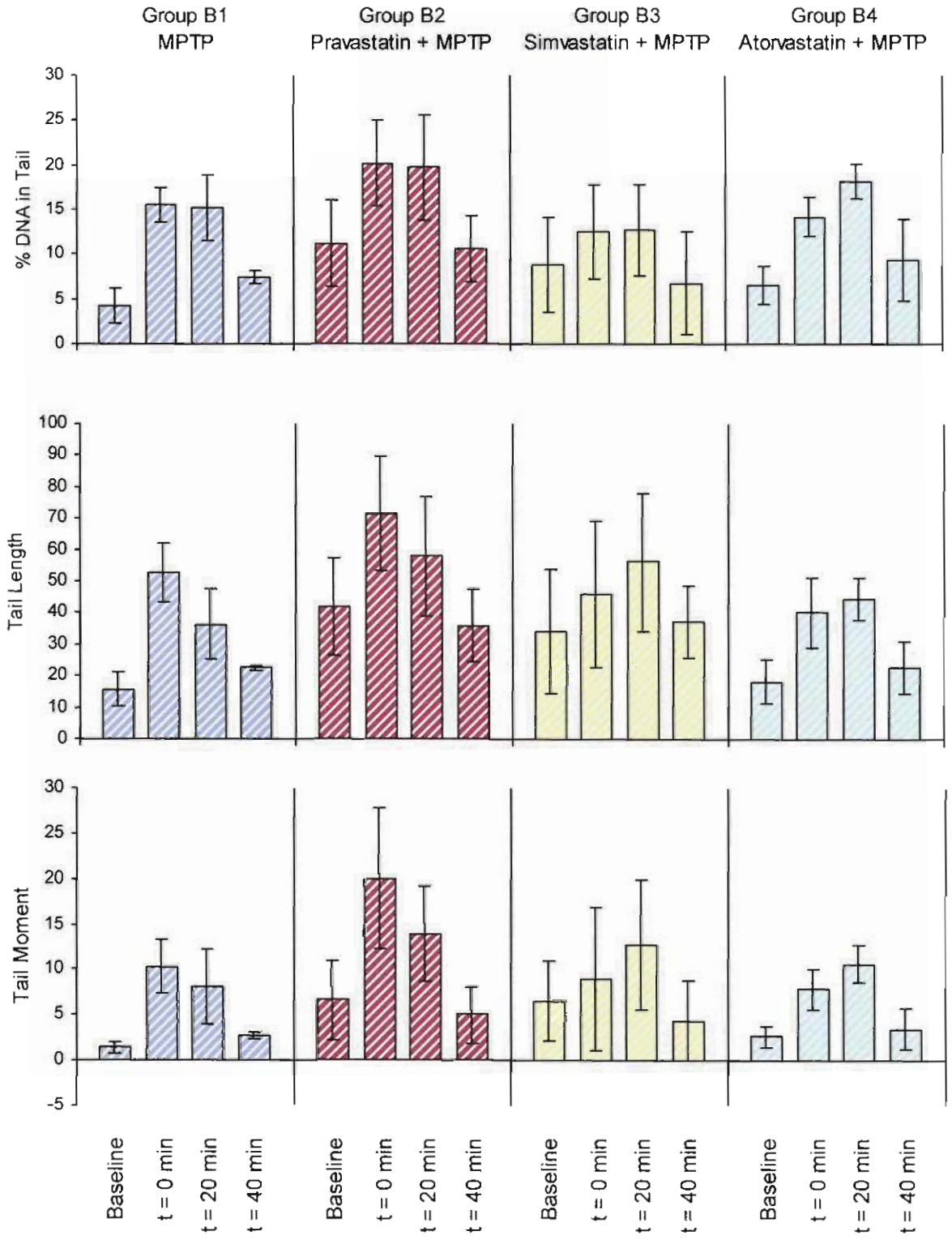

Figure 4-2 **Mean levels of DNA damage in group B measured at the start of the assay (baseline), at t = 0 min, t = 20 min and t = 40 min, expressed as % DNA in Tail, Tail Length and Tail Moment, with SD between mice in each group (n = 5)**

4.2.1 DNA damage by MPTP

To determine the DNA damage caused by MPTP, mice receiving MPTP (group B1) were compared to mice receiving saline (group A1). The baseline level of DNA damage, changes in DNA damage, and RC values for these two groups were compared.

The alternative hypothesis that a difference exists between the two groups was tested using Student's t-test for independent samples by group. If the difference between the groups is in the predicted direction, only one half (or tail) of the probability distribution could be considered and the standard p-level divided by two (STATSOFT, 2005). Since we did not predict the change in level of DNA damage to be in either direction, a two-tailed t-test was used.

Data expressed as % DNA in Tail, Tail Length and Tail Moment are presented and compared separately, and then discussed:

When the data is expressed as % DNA in Tail (Figure 4-3) there is a statistically insignificant trend towards greater baseline DNA damage, Δ_{damage}, $\Delta_{\text{repair 1}}$, $\Delta_{\text{repair 2}}$ and $\Delta_{\text{total repair}}$ in group B1 when compared to group A1.

Data expressed as Tail Length (Figure 4-4) reported a trend towards greater baseline DNA damage, Δ_{damage}, $\Delta_{\text{repair 1}}$ and $\Delta_{\text{repair 2}}$ in group B1 compared to group A1. The increase in Δ_{damage} was statistically significant ($p = 0{,}025$).

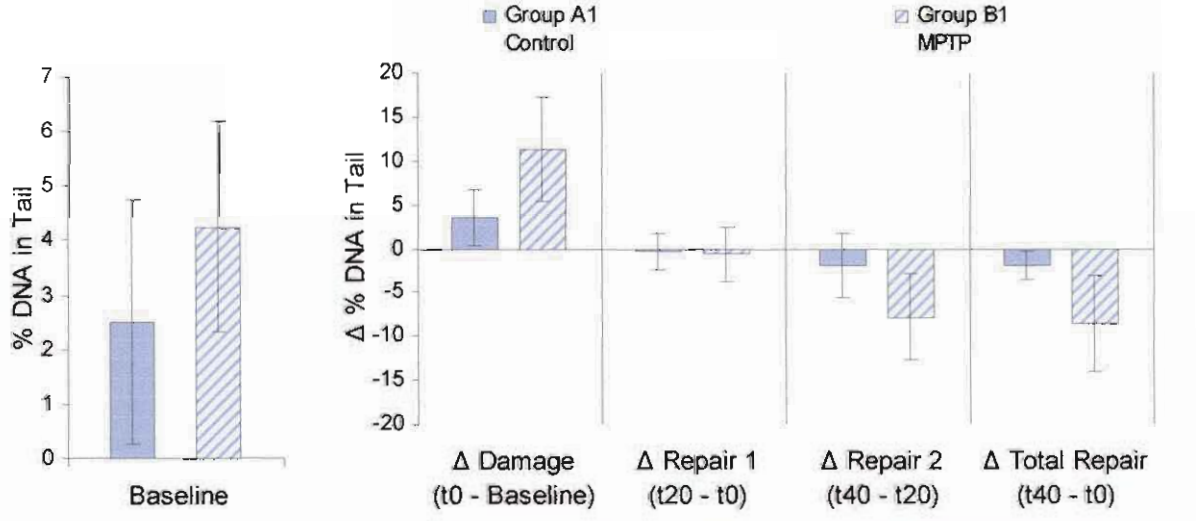

Figure 4-3 **Mean levels of baseline DNA damage and change in DNA damage in groups A1 and B1 expressed as % DNA in Tail, with SD between mice in each group (n = 5)**

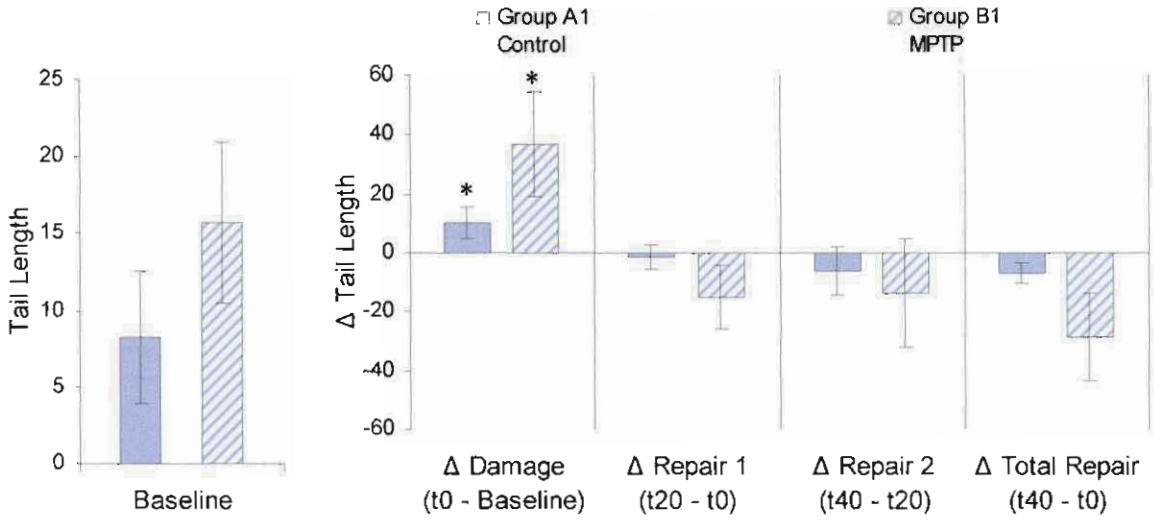

Figure 4-4 **Mean levels of baseline DNA damage and change in DNA damage in groups A1 and B1 expressed as Tail Length, with SD between mice in each group (n = 5) | Student's t-test: * t(7) = -2.88 (p = 0,025) |**

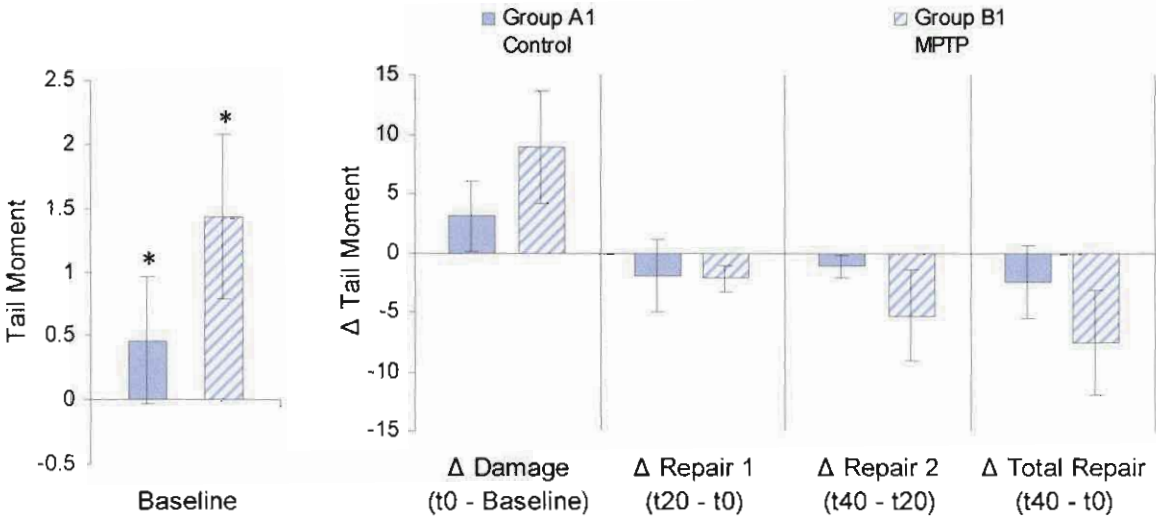

Figure 4-5 **Mean levels of baseline DNA damage and change in DNA damage in groups A1 and B1 expressed as Tail Moment, with SD between mice in each group (n = 5) | Student's t-test: * t(7) = -2,45 (p = 0,039) |**

Data expressed as Tail Moment (Figure 4-5) reported a trend towards greater baseline DNA damage, Δ_{damage}, $\Delta_{repair\ 1}$ and $\Delta_{repair\ 2}$ in group B1 compared to group A1. The increase in the baseline level of damage was statistically significant (p = 0,039).

The RC for group B1 was increased compared to that of group A1 (Figure 4-6), although not to a statistically significant degree.

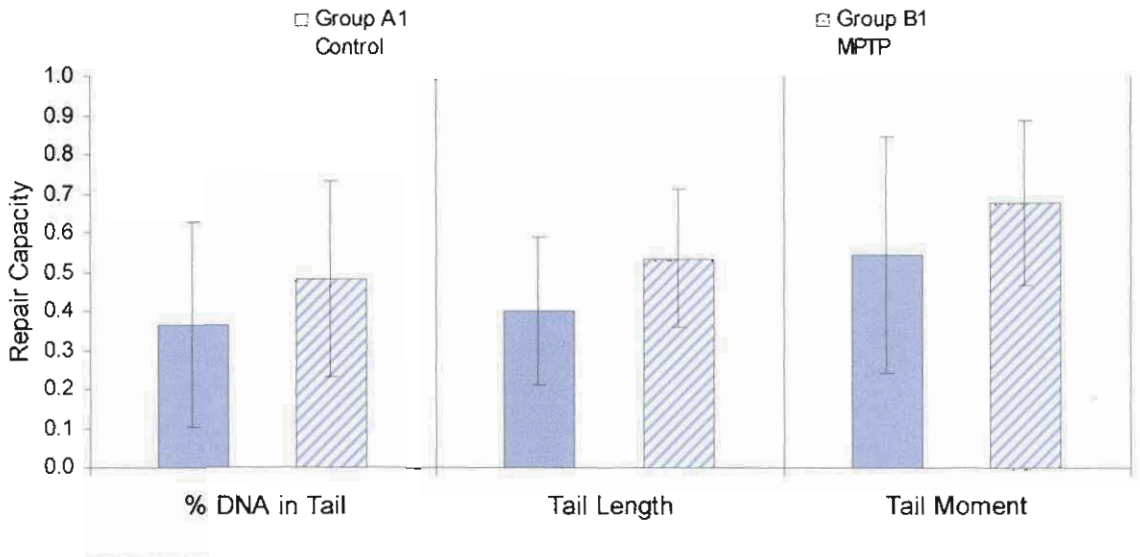

Figure 4-6 **Mean RC for groups A1 and B1 with SD between mice in each group (n = 5), computed using % DNA in Tail, Tail Length and Tail Moment**

Treatment with MPTP (group B1) therefore had the effect of increasing the baseline DNA damage in the striatum of mice. MPTP not only increased the susceptibility of DNA to damage induced with H_2O_2, but also increased the amount of repair that took place during both 20 min intervals, and the total repair. The RC, or ability of the striatal cells to recover from the damage induced by H_2O_2, therefore increased as well in groups treated with MPTP.

4.2.2 Effect of statin treatment on DNA damage

To determine the effect of statins on DNA damage, mice receiving pravastatin (groups A2), simvastatin (group A3) and atorvastatin (group A4) were compared to mice injected with saline (group A1). A one-way analysis of variance (ANOVA; F-test) was used to investigate whether between-group variability existed. Further analysis was carried out using Dunnett's post-hoc test to compare group A2, group A3 and group A4 to the control group A1, to reveal which within-group means contributed to the possible variability.

Data is presented and discussed separately expressed as % DNA in Tail, Tail Length and Tail Moment:

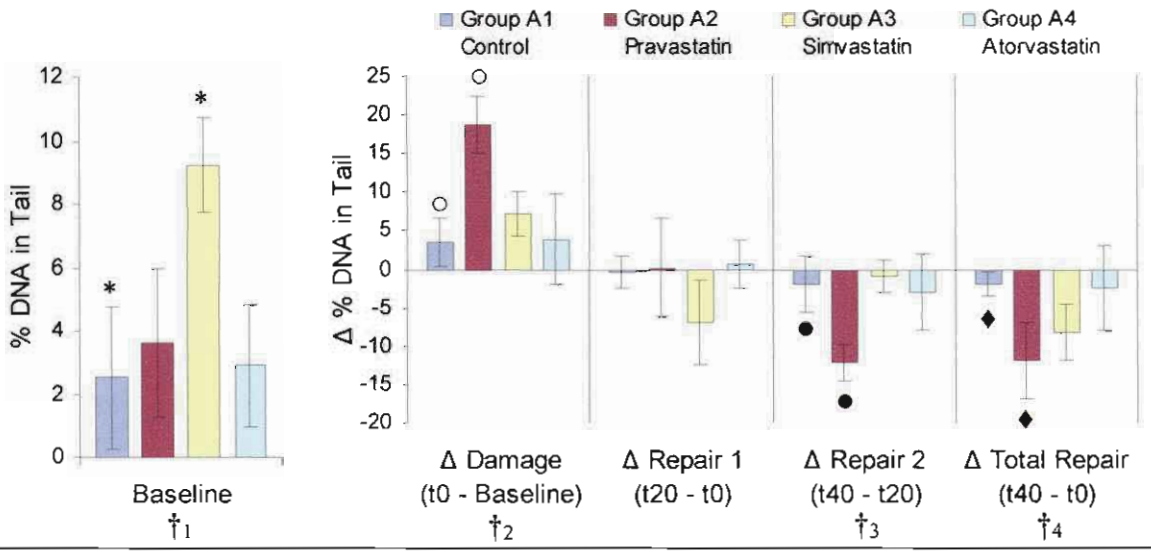

Figure 4-7 **Mean levels of baseline DNA damage and change in DNA damage in group A expressed as % DNA in Tail, with SD between mice in each group (n = 5) | $\dagger_1$ F(3;13) = 9,73 (p = 0,0012), $\dagger_2$ F(3;13) = 23,38 (p < 0,0001), $\dagger_3$ F(3;13) = 14,71 (p = 0,0001), $\dagger_4$ F(3;13) = 6,9 (p = 0,0049), Dunnett's post-hoc test: * p = 0,0018, ○ p < 0,0001, ● p = 0,0007, ♦ p = 0,0093 |**

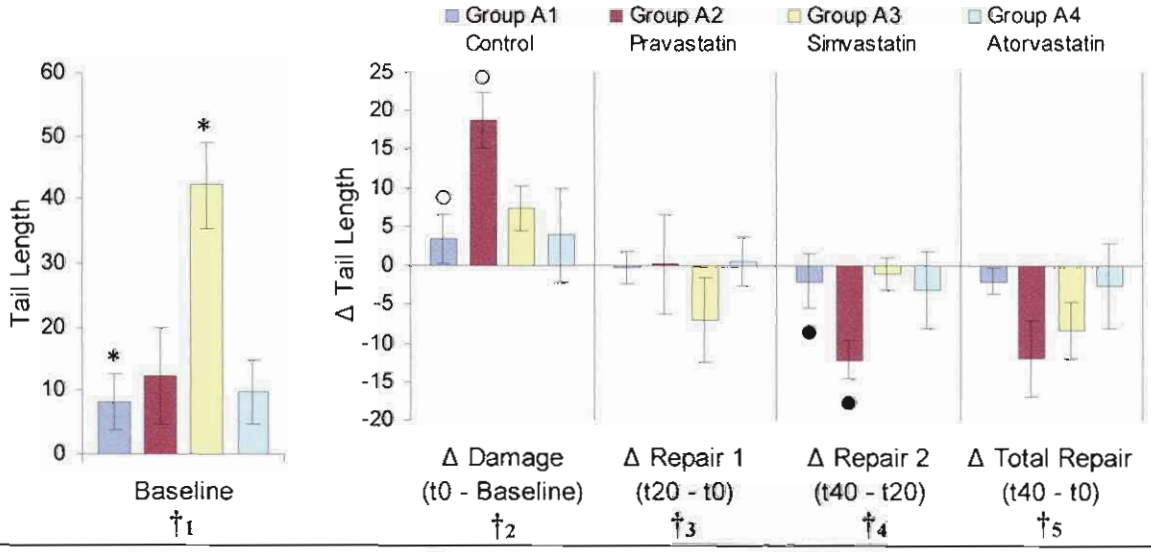

Figure 4-8 **Mean levels of baseline DNA damage and change in DNA damage in group A expressed as Tail Length, with SD between mice in each group (n = 5) [$\dagger_1$ F(3;13) = 31,96 (p < 0,0001), $\dagger_2$ F(3;13) = 16,60 (p < 0,0001), $\dagger_3$ F(3;13) = 7,33 (p = 0,0040), $\dagger_4$ F(3;13) = 13,53 (p = 0,0003), Dunnett's post-hoc test: * p < 0,0001, ○ p = 0,0002, no statistically significant difference for $\Delta_{\text{repair 1}}$, ● p = 0,0012, no statistically significant difference for $\Delta_{\text{total repair}}$ |**

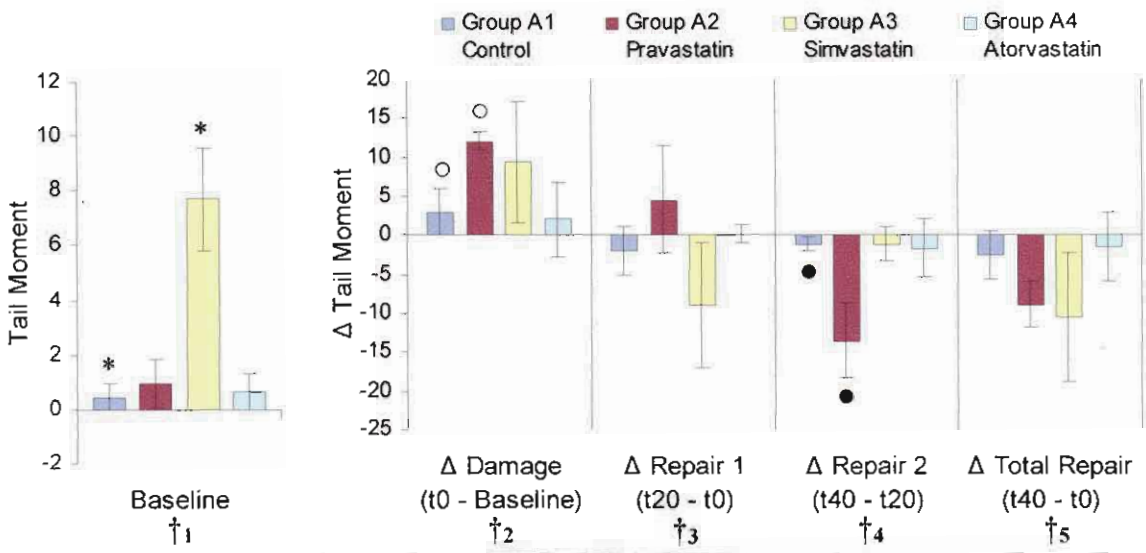

Figure 4-9 **Mean levels of baseline DNA damage and change in DNA damage in group A expressed as Tail Moment, with SD between mice in each group (n = 5) [$\dagger_1$ F(3;13) = 43,87 (p < 0,0001), $\dagger_2$ F(3;13) = 6,06 (p = 0,0082), $\dagger_3$ F(3;13) = 4,46 (p = 0,0230), $\dagger_3$ F(3;13) = 18,89 (p < 0,0001), $\dagger_3$ F(3;13) = 3,92 (p = 0,0339), Dunnett's post-hoc test: * p < 0,0001, ○ p = 0,0351, no statistically significant difference for $\Delta_{\text{repair 1}}$, • p = 0,0002, no statistically significant difference for $\Delta_{\text{total repair}}$]**

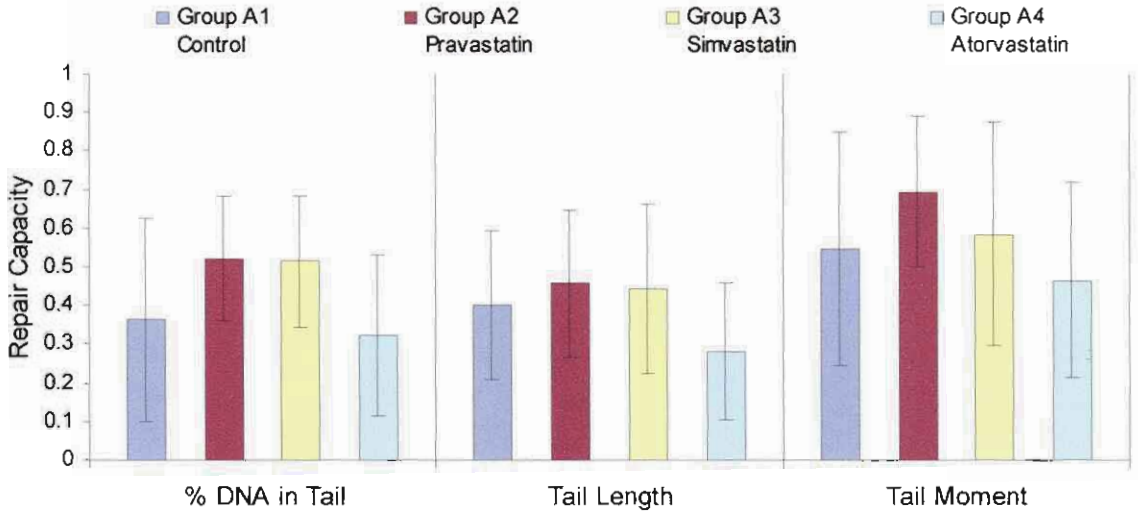

Figure 4-10 **Mean RC for group A with SD between mice in each group (n = 5), computed using % DNA in Tail, Tail Length and Tail Moment [no statistically significant difference]**

When data is expressed as % DNA in Tail (Figure 4-7), Tail Length (Figure 4-8) or Tail Moment (Figure 4-9), the following were observed:

- Mice treated with pravastatin (group A2; indicated in red) showed a statistically insignificant trend towards increased baseline levels of DNA damage. DNA damage

induced *in vitro* with H_2O_2, however, increased the damage to the DNA significantly ($p \leq 0,05$ when expressed as any of the parameters) and more so than any other statin treatment. The DNA repair did not take place in the first 20 minute interval, possibly because of increased double strand breaks which takes longer to repair. The total repair and RC of mice treated with pravastatin, however, increased considerably compared to that of the control group, although not to a statistically significant degree.

- Mice treated with simvastatin (group A3; indicated in yellow) showed a marked increase in baseline DNA damage ($p \leq 0,05$ for % DNA in Tail and Tail Length). DNA damage induced *in vitro* with H_2O_2 was not increased to a significant degree, and the repair was increased significantly ($p \leq 0,05$ only when expressed as Tail Length). The total repair and RC increased compared to control mice, but not to a statistically significant degree.
- Mice treated with atorvastatin (group A4; indicated in cyan) showed baseline levels of DNA damage similar to that of the control group. The extent of the DNA damage and repair that took place after H_2O_2 treatment was also comparable to that of the control group. DNA repair was comparable to that of the control. Atorvastatin treatment lowered the RC, but not to a statistically significant degree.

Statin treatment therefore increased the baseline level of DNA damage, with simvastatin treatment showing a marked increase. Statin treatment also increased the level of DNA damage caused by H_2O_2, but slightly increased the DNA repair and RC of treated mice.

4.2.3 Effect of statin treatment on DNA damage in MPTP treated mice

To determine the effect of statins on DNA damage in MPTP treated mice, these mice receiving pravastatin (group B2), simvastatin (group B3) and atorvastatin (group B4) were compared to the control group (group B1). A one-way analysis of variance (ANOVA; F-test) was used to investigate whether between-group variability existed. Further analysis was carried out using Dunnett's post-hoc test to compare group B2, group B3 and group B4 to the control group B1, to reveal which within-group means contributed to the possible variability.

Data is presented and discussed separately expressed as % DNA in Tail, Tail Length and Tail Moment, then discussed:

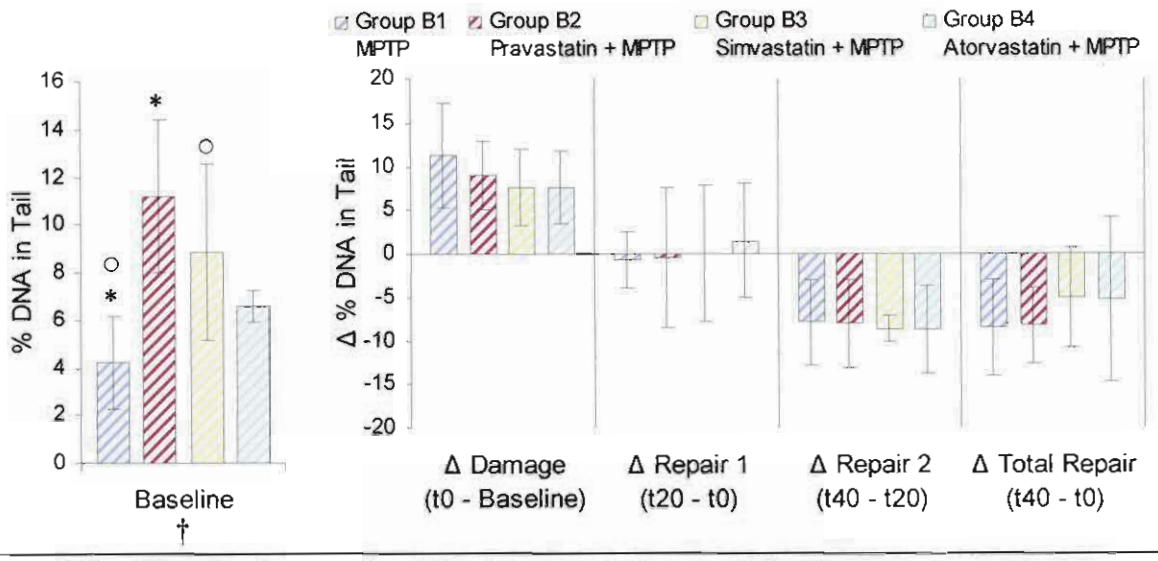

Figure 4-11 **Mean levels of baseline DNA damage and change in DNA damage in group B expressed as % DNA in Tail, with SD between mice in each group [† $F(3;10) = 7,19$ ($p = 0,0074$), Dunnett's post-hoc test: * $p < 0,0036$, ○ $p = 0,021$]**

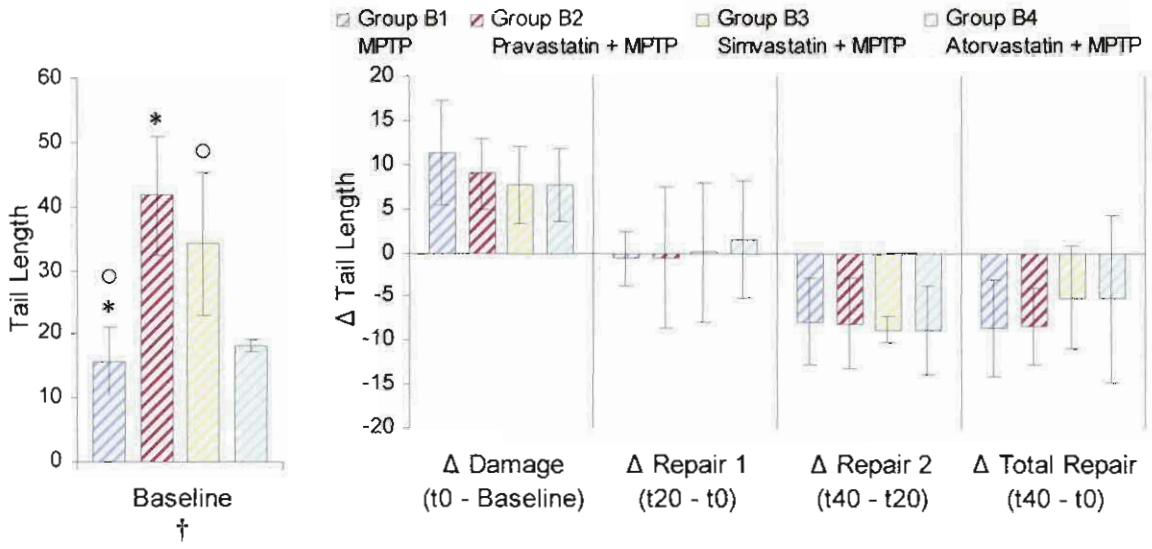

Figure 4-12 **Mean levels of baseline DNA damage and change in DNA damage in group B expressed as Tail Length, with SD between mice in each group [† $F(3;10) = 22.28$ ($p < 0,0001$), Dunnett's post-hoc test: * $p < 0,0001$, ○ $p = 0,0015$]**

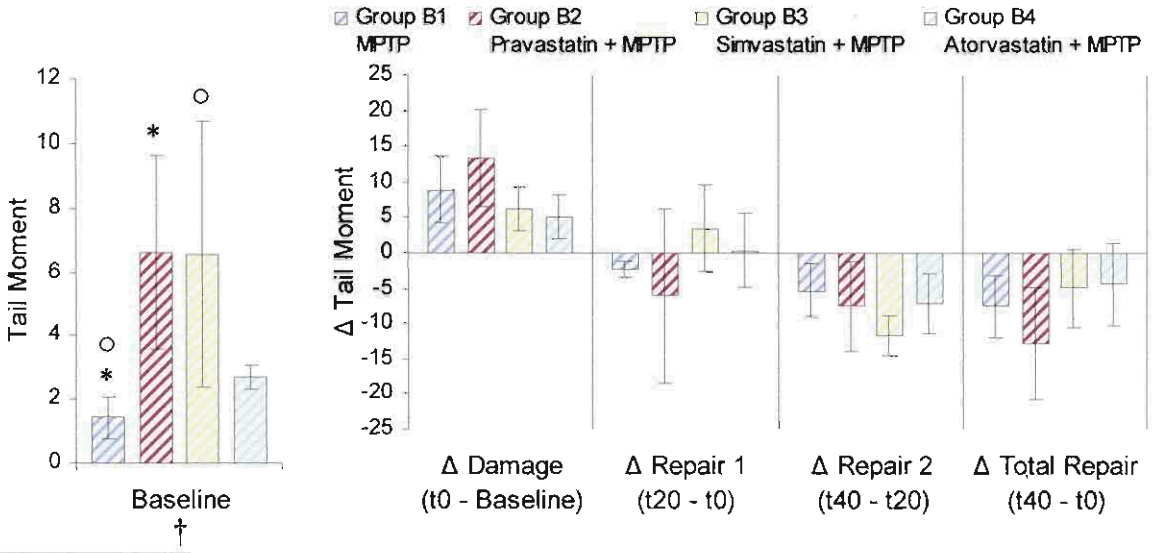

Figure 4-13 **Mean levels of baseline DNA damage and change in DNA damage in group B expressed as Tail Moment, with SD between mice in each group [† F(3;10) = 12,13 (p = 0,0013), Dunnett's post-hoc test: * p = 0,0009, ○ p = 0,0027]**

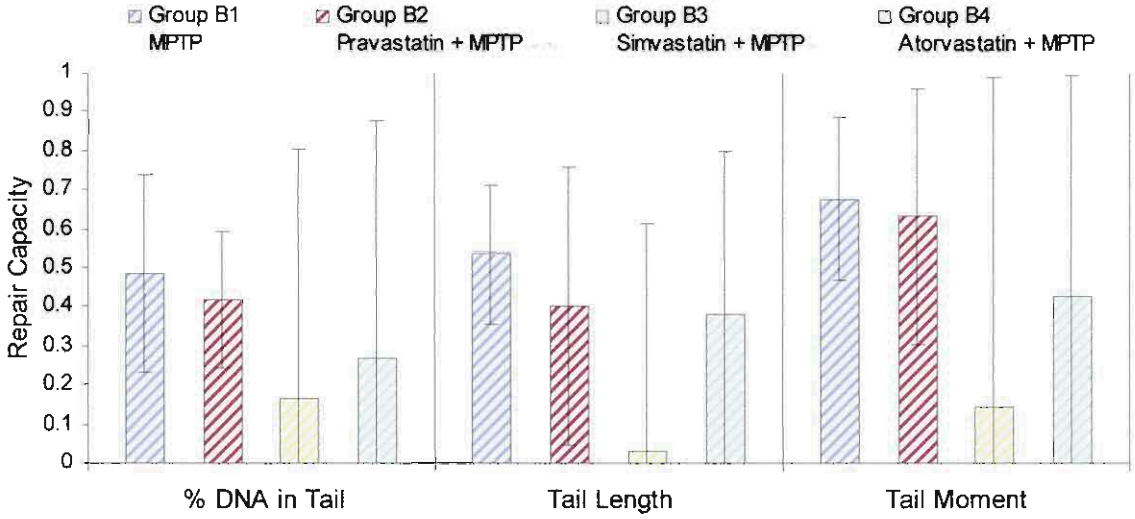

Figure 4-14 **Mean RC for group B with SD between mice in each group, computed using % DNA in Tail, Tail Length and Tail Moment [no statistically significant difference]**

When data is expressed as % DNA in Tail (Figure 4-11), Tail Length (Figure 4-12) or Tail Moment (Figure 4-13), the following were observed:

- Mice treated with pravastatin (group B2; indicated in striped red) showed significantly increased baseline levels of DNA damage ($p \leq 0,05$ when expressed as any of the parameters). DNA damage induced *in vitro* with H_2O_2, however, was lower (when expressed as % DNA in Tail or Tail Length). DNA repair did not take place in the first

20 min interval, and repair during the second 20 min interval, and the total repair was comparable to that of the control group (Group B1). The RC for mice treated with pravastatin decreased very slightly.

- Mice treated with simvastatin (group B3; indicated in striped yellow) showed significantly increased baseline levels of DNA damage ($p \leq 0,05$ when expressed as any of the parameters). DNA damage induced *in vitro* with H_2O_2, however, was lower (when expressed as any of the parameters). DNA repair did not take place in the first 20 min interval, and repair during the second 20 min interval was comparable to that of the control group (Group B1). The total repair and the RC for mice treated with simvastatin decreased compared with the control, but with a greater variance between the means for the RC.
- Mice treated with atorvastatin (group B4; indicated in striped cyan) had slightly increased baseline levels of DNA damage, but more comparable to the control than the other statin treated groups. The DNA damage induced *in vitro* with H_2O_2 showed a statistically insignificant decrease. DNA repair did not take place in the first 20 min interval, and repair during the second 20 min interval was comparable to that of the control group (Group B1). The total repair and RC for mice treated with atorvastatin decreased slightly.

Pravastatin and simvastatin therefore increased the level of DNA damage when administered with MPTP treatment. Statin treatment offered slight protection against H_2O_2 induced damage and simvastatin and atorvastatin decreased the DNA repair and RC of the DNA, although very slightly.

4.3 Discussion

MPTP is highly lipophilic and readily crosses the blood-brain barrier where it exerts its toxic effects (Nicklas, *et al.*, 1985). In this study we found that mice treated with MPTP tended to have higher levels of DNA damage, which is in agreement with previous studies (Brill and Bennet, 2003). When damage is induced *in vitro* the DNA of these mice was also damaged and repaired to a greater extent. These increases, however, were generally not of statistical significance.

Despite their antioxidative properties (Franzoni, *et al.*, 2003), this study found that none of the statins offered any protection against oxidative DNA damage. Statin treatment rather increased the baseline striatal DNA damage. The baseline DNA damage of mice treated with

atorvastatin was increased to a lesser extent and more comparable to that of the control groups.

Statin treatment increased the damage caused by H_2O_2 but the repair processes seemed to remain intact, because in these groups, the repair also increased. In the groups receiving MPTP and statin treatment the repair and RC decreased, possibly because the repair processes are then overwhelmed by the oxidative damage.

4.4 Suggestions for further research

During the course of this study, certain factors were identified which require further investigation:

- The human absorption (Singhvi, *et al.*, 1990) and bioavailability (Christians, *et al.*, 1998) of the statins under investigation have been researched. Although pravastatin is hydrophilic (Christians, *et al.*, 1998) and is not thought to enter the central nervous system (Christian, *et al.*, 1998; Hamelin and Turgeon, 1998) it has been shown to have an effect on the brain (Lütjohann, *et al.*, 2004). Simvastatin and atorvastatin, on the other hand, are lipophilic (Christians, *et al.*, 1998) and are known to enter the central nervous system (Christian, *et al.*, 1998; Hamelin and Turgeon, 1998). The absorption and central nervous system distribution of statins in the mice model could therefore be further investigated to establish whether the mouse represents an adequate model.
- It is known that pravastatin is excreted unchanged and its main metabolite has a slightly shorter terminal plasma half-life, and that simvastatin and atorvastatin are eliminated mostly as metabolites that accumulate in plasma and tissues (Christians, *et al.*, 1998). The study conducted by Shin, *et al.* (2005) was on the long-term (8 week) treatment of hypercholesterolemic patients with commercially available simvastatin. The effect of the duration of statin treatment on DNA damage and repair therefore also require further investigation.
- It was assumed that the beneficial effect of the statins on DNA damage would be exhibited regardless of the formation of metabolites. Future studies could include a comprehensive investigation into the effects of the metabolites of the statin drugs as well.

Chapter 5 - Conclusion

PD is one of the most common neurodegenerative disorders affecting about 4 million people worldwide (Von Bohlen und Halbach, *et al.*, 2004) and causes a disruption to normal motor function. Age-related degeneration of the dopaminergic neurons located in the SNpc and a subsequent loss of nerve terminals in the striatum are responsible for most of the movement disturbances (Chase et al., 1998; Nagatsu et al., 2000).

Accumulating evidence supports the hypothesis that oxidative stress may contribute to the pathogenesis of PD (Yoshikawa, 1993). The brain depends mostly on mitochondrial energy supply which is associated with the production of ROS (Von Bohlen und Halbach, *et al.*, 2004). Endogenous DNA damage arises from the intermediates and the products of oxygen reduction or other cellular components (Marnett, 2000) that interact with either the bases or the deoxyribosyl backbone of the DNA. Furthermore, the post mortem frontal cortexes of PD patients have been demonstrated to have accumulated excessive levels of H_2O_2 (Kienzl, *et al.*, 1995).

The neurotoxin MPTP is widely used in an animal model reflecting many of the features of human PD (Speciale, 2002). MPTP causes apoptotic cell death (Fall and Bennett, 1999) selectively to the striatum and brings about many responses that regulate this process, including an increase in oxidative stress (Cassarino, *et al.*, 1997).

Previous studies have shown that simvastatin (part of a group of drugs known as the statins) reduces DNA damage in lymphocytes of hypercholesterolemic patients and has a beneficial effect DNA repair (Shin, *et al.*, 2005).

In a search for novel defensive therapies against PD, the potential neuroprotective effects of simvastatin, pravastatin and atorvastatin on DNA damage in mice striatum were investigated.

MPTP treatment increased the level of DNA damage in the striatum. Treatment with statins also increased levels of DNA damage, but left the repair processes intact, increasing the amount of repair that took place as well. The DNA repair of mice treated with MPTP and statins however, was decreased.

The results obtained do not substantiate the hypothesis that the beneficial effects of statins in PD patients may be ascribed to their capacity to reduce DNA damage. This is in contrast with the findings of Shin *et al.* (2005) who established that simvastatin reduces the DNA damage in hypercholesterolaemic patients. This contradiction in results may be due to the differences in human and mice DNA and/or the fact that Shin *et* al. used leucocyte DNA while we used DNA from the striatum. The amount of blood we were able to obtain from the mice proved to be insufficient to analyse.

It may also be argued that the protective mechanism of the statins in PD patients may be attributed to mechanisms other than protection against DNA damage, such as its antioxidative or anti-inflammatory properties.

Chapter 6 - References

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APPENDIX A – Data

Table A-1 **Mean levels of DNA damage and change in DNA damage in group A expressed as % DNA in Tail, Tail Length and Tail Moment, with SD between mice in each group**

		Group A1	Group A2	Group A3	Group A4
Tail DNA %	Baseline	2.51 ± 2.25	3.63 ± 2.36	9.24 ± 1.48	2.92 ± 1.04
	t_0	6.02 ± 2.61	22.31 ± 3.51	15.86 ± 2.89	6.85 ± 2.55
	t_{20}	6.33 ± 2.06	22.54 ± 4.58	8.74 ± 3.04	7.40 ± 1.43
	t_{40}	4.05 ± 2.68	10.45 ± 3.48	7.82 ± 2.43	4.37 ± 2.34
	Δ_{Damage}	3.52 ± 3.12	18.68 ± 3.59	7.24 ± 2.83	3.93 ± 2.56
	$\Delta_{Repair\ 1}$	-0.31 ± 2.08	0.23 ± 6.42	-6.95 ± 5.42	0.56 ± 2.78
	$\Delta_{Repair\ 2}$	-1.92 ± 3.60	-12.09 ± 2.40	-0.92 ± 2.09	-3.03 ± 3.20
	$\Delta_{Total\ Repair}$	-1.97 ± 1.65	-11.86 ± 4.95	-8.29 ± 3.72	-2.47 ± 2.95
	RC	0.36 ± 0.26	0.52 ± 0.16	0.51 ± 0.17	0.32 ± 0.37
Tail Length	Baseline	8.20 ± 4.32	12.30 ± 7.44	42.24 ± 6.86	9.80 ± 1.27
	t_0	18.19 ± 4.01	52.78 ± 5.87	63.09 ± 8.37	22.84 ± 7.27
	t_{20}	17.85 ± 4.25	68.32 ± 15.40	38.01 ± 13.36	25.38 ± 3.05
	t_{40}	11.14 ± 5.05	28.78 ± 11.27	36.56 ± 13.17	15.48 ± 6.62
	Δ_{Damage}	9.98 ± 5.47	40.48 ± 4.13	23.14 ± 10.17	13.04 ± 6.85
	$\Delta_{Repair\ 1}$	-1.57 ± 4.20	15.53 ± 14.78	-24.75 ± 18.37	2.54 ± 8.03
	$\Delta_{Repair\ 2}$	-6.08 ± 7.91	-39.54 ± 9.05	-1.45 ± 12.10	-9.90 ± 8.23
	$\Delta_{Total\ Repair}$	-7.04 ± 3.30	-24.01 ± 10.65	-27.87 ± 14.84	-7.36 ± 9.21
	RC	0.40 ± 0.19	0.46 ± 0.19	0.44 ± 0.22	0.28 ± 0.33
Tail Moment	Baseline	0.46 ± 0.50	0.95 ± 0.90	7.70 ± 1.89	0.67 ± 0.28
	t_0	3.51 ± 2.72	13.01 ± 2.00	16.63 ± 7.20	2.79 ± 1.68
	t_{20}	2.17 ± 0.51	17.56 ± 6.80	7.68 ± 3.94	2.99 ± 0.46
	t_{40}	1.03 ± 0.43	4.06 ± 3.04	6.52 ± 3.51	1.36 ± 0.98
	Δ_{Damage}	3.05 ± 2.88	12.06 ± 1.18	9.48 ± 7.80	2.12 ± 1.56
	$\Delta_{Repair\ 1}$	-1.98 ± 3.01	4.54 ± 6.85	-9.00 ± 7.96	0.20 ± 1.70
	$\Delta_{Repair\ 2}$	-1.17 ± 0.88	-13.50 ± 4.82	-1.16 ± 2.17	-1.63 ± 1.22
	$\Delta_{Total\ Repair}$	-2.48 ± 3.08	-8.96 ± 2.97	-10.49 ± 8.36	-1.43 ± 1.70
	RC	0.55 ± 0.30	0.69 ± 0.19	0.58 ± 0.29	0.47 ± 0.33

Table A-2 **Mean levels of DNA damage and change in DNA damage in group B expressed as % DNA in Tail, Tail Length and Tail Moment, with SD between mice in each group**

		Group B1	Group B2	Group B3	Group B4
Tail DNA %	Baseline	4.24 ± 1.94	11.20 ± 3.19	8.88 ± 3.71	6.57 ± 0.69
	t_0	15.52 ± 4.84	20.18 ± 4.62	12.48 ± 5.85	14.19 ± 3.65
	t_{20}	15.21 ± 5.26	19.69 ± 5.71	12.73 ± 5.13	18.16 ± 5.77
	t_{40}	7.34 ± 2.14	10.62 ± 2.76	6.77 ± 1.92	9.33 ± 4.57
	Δ Damage	11.28 ± 6.00	8.99 ± 4.00	3.60 ± 8.71	7.62 ± 4.11
	Δ Repair 1	-0.64 ± 3.15	-0.50 ± 8.02	0.01 ± 7.84	1.45 ± 6.55
	Δ Repair 2	-7.86 ± 4.93	-8.00 ± 5.07	-8.70 ± 1.52	-8.84 ± 5.04
	Δ Total Repair	-8.51 ± 5.44	-8.30 ± 4.33	-5.04 ± 5.81	-5.22 ± 9.43
	RC	0.48 ± 0.25	0.42 ± 0.18	0.17 ± 0.64	0.27 ± 0.61
Tail Length	Baseline	15.72 ± 5.20	41.72 ± 9.21	34.14 ± 11.16	18.27 ± 0.85
	t_0	52.65 ± 15.34	71.21 ± 18.35	45.86 ± 18.99	40.16 ± 11.56
	t_{20}	36.24 ± 19.47	57.60 ± 23.36	55.96 ± 21.73	44.16 ± 11.22
	t_{40}	22.60 ± 6.77	35.70 ± 11.07	37.17 ± 6.77	22.45 ± 8.23
	Δ Damage	36.93 ± 17.83	29.48 ± 16.48	11.72 ± 21.45	21.89 ± 12.41
	Δ Repair 1	-15.08 ± 10.98	-13.61 ± 32.28	11.88 ± 19.21	-3.45 ± 16.60
	Δ Repair 2	-13.64 ± 18.54	-17.11 ± 15.16	-29.00 ± 6.09	-21.72 ± 11.25
	Δ Total Repair	-28.73 ± 15.13	-31.04 ± 24.31	-10.48 ± 16.61	-18.30 ± 21.69
	RC	0.53 ± 0.18	0.40 ± 0.36	0.03 ± 0.58	0.38 ± 0.42
Tail Moment	Baseline	1.43 ± 0.65	6.59 ± 3.03	6.54 ± 4.13	2.69 ± 0.37
	t_0	10.34 ± 4.41	19.98 ± 7.78	9.00 ± 5.26	7.84 ± 3.05
	t_{20}	8.08 ± 4.36	13.93 ± 7.88	12.70 ± 7.18	10.64 ± 4.43
	t_{40}	2.76 ± 1.18	4.96 ± 2.26	4.40 ± 2.08	3.52 ± 2.22
	Δ Damage	8.92 ± 4.77	13.39 ± 6.79	2.46 ± 7.14	5.14 ± 3.22
	Δ Repair 1	-2.18 ± 1.10	-6.05 ± 12.37	3.54 ± 6.09	0.41 ± 5.24
	Δ Repair 2	-5.32 ± 3.83	-7.46 ± 6.40	-11.67 ± 2.80	-7.12 ± 4.20
	Δ Total Repair	-7.50 ± 4.43	-12.82 ± 7.97	-4.96 ± 5.55	-4.30 ± 5.79
	RC	0.68 ± 0.21	0.63 ± 0.33	0.14 ± 0.84	0.43 ± 0.57

APPENDIX B – Statistical Analysis

Table B-1 Two-tailed Student's T-test for independent samples by group for group A1 and group B1

		df	p (2-sided)	F-ratio Variances	p Variances
% DNA in Tail	Baseline	6.021781	0.267895	1.35070	0.753999
	Δ Damage	6.213897	0.044995	3.69939	0.311031
	Δ Repair 1	4.977902	0.871979	2.29617	0.635497
	Δ Repair 2	4.994977	0.124908	1.87538	0.732672
	Δ Total Repair	3.551083	0.091270	10.79501	0.081652
	RC	5.988411	0.539123	1.09203	0.944007
Tail Length	Baseline	6.965043	0.049816	1.44611	0.793409
	Δ Damage	4.906692	0.024747	10.61643	0.081392
	Δ Repair 1	4.053539	0.086614	6.83273	0.260670
	Δ Repair 2	4.256233	0.502439	5.49696	0.315545
	Δ Total Repair	3.284105	0.061079	21.07147	0.032295
	RC	5.969128	0.344723	1.15498	0.908512
Tail Moment	Baseline	6.999634	0.038942	1.69124	0.694480
	Δ Damage	6.638664	0.058853	2.74211	0.433528
	Δ Repair 1	2.401620	0.919450	7.54381	0.135095
	Δ Repair 2	3.408383	0.115939	19.10816	0.100281
	Δ Total Repair	5.354952	0.117993	2.06312	0.567135
	RC	5.334660	0.508142	2.09194	0.559876

Table B-2 One-way analysis of variance (ANOVA; F-Test) for group A1, group A2, group A3 and group A4

Degr. of Freedom		SS	MS	F	p	SS	MS	F	p
		% DNA in Tail - Baseline				% DNA in Tail - Δ_{Damage}			
Intercept	1	316.272	316.272	96.078	0.0000	1176.11	1176.118	121.364	0.0000
Group	3	96.110	32.037	9.732	0.0012	679.594	226.531	23.376	0.0000
Error	13	42.794	3.292			125.981	9.691		
Total	16	138.904				805.575			
		% DNA in Tail - $\Delta_{Repair\ 1}$				% DNA in Tail - $\Delta_{Repair\ 2}$			
		42.554	42.554	1.889	0.1926	343.389	343.389	43.004	0.0000
		159.198	53.066	2.356	0.1194	352.545	117.515	14.717	0.0002
		292.869	22.528			103.806	7.985		
		452.067				456.351			
		% DNA in Tail - $\Delta_{Total\ Repair}$				% DNA in Tail - RC			
		627.708	627.708	44.891	0.0000	3.105	3.105	43.121	0.0000
		291.936	97.312	6.959	0.0049	0.132	0.044	0.610	0.6206
		181.780	13.983			0.936	0.072		
		473.716				1.068			
		Tail Length - Baseline				Tail Length - Δ_{Damage}			
		5011.98	5011.98	177.31	0.0000	7864.18	7864.180	160.772	0.0000
		2710.41	903.47	31.96	0.0000	2435.82	811.942	16.599	0.0001
		367.463	28.266			635.896	48.915		
		3077.87				3071.72			
		Tail Length - $\Delta_{Repair\ 1}$				Tail Length - $\Delta_{Repair\ 2}$			
		69.098	69.098	0.412	0.5320	3496.47	3496.474	36.260	0.0000
		3686.93	1228.97	7.330	0.0040	3913.94	1304.647	13.530	0.0003
		2179.49	167.653			1253.57	96.429		
		5866.42				5167.51			
		Tail Length - $\Delta_{Total\ Repair}$				Tail Length - RC			
		4548.62	4548.62	39.893	0.0000	2.583	2.583	40.101	0.0000
		1445.18	481.728	4.225	0.0273	0.093	0.031	0.480	0.7021
		1482.25	114.020			0.837	0.064		
		2927.43				0.930			
		Tail Moment - Baseline				Tail Moment - Δ_{Damage}			
		87.720	87.720	91.554	0.0000	757.897	757.897	45.078	0.0000
		126.090	42.030	43.867	0.0000	305.767	101.922	6.062	0.0082
		12.456	0.958			218.567	16.813		
		138.545				524.334			
		Tail Moment - $\Delta_{Repair\ 1}$				Tail Moment - $\Delta_{Repair\ 2}$			
		39.578	39.578	1.263	0.2815	321.851	321.851	35.674	0.0000
		419.775	139.925	4.464	0.0230	511.386	170.462	18.894	0.0001
		407.513	31.347			117.285	9.022		
		827.288				628.672			
		Tail Moment - $\Delta_{Total\ Repair}$				Tail Moment - RC			
		587.156	587.156	27.299	0.0002	5.714	5.714	71.948	0.0000
		253.202	84.401	3.924	0.0339	0.135	0.045	0.565	0.6474
		279.613	21.509			1.032	0.079		
		532.815				1.167			

Table B-3 One-way analysis of variance (ANOVA; F-Test) for group B1, group B2, group B3 and group B4

Degr. of Freedom		SS	MS	F	p	SS	MS	F	p
		% DNA in Tail - Baseline				% DNA in Tail - Δ_{Damage}			
Intercept	1	705.00	705.005	170.143	0.0000	1242.562	1242.562	50.406	0.0000
Group	3	89.344	29.781	7.187	0.0074	42.544	14.181	0.575	0.6442
Error	10	41.436	4.144			246.512	24.651		
Total	13	130.78				289.056			
		% DNA in Tail - $\Delta_{Repair\ 1}$				% DNA in Tail - $\Delta_{Repair\ 2}$			
		11.533	11.533	0.264	0.6185	836.336	836.336	40.731	0.0001
		31.123	10.374	0.237	0.8682	6.417	2.139	0.104	0.9557
		436.82	43.682			205.334	20.533		
		467.94				211.751			
		% DNA in Tail - $\Delta_{Total\ Repair}$				% DNA in Tail - RC			
		651.44	651.444	15.471	0.0028	1.424	1.424	6.408	0.0298
		31.651	10.550	0.251	0.8592	0.269	0.090	0.403	0.7538
		421.06	42.106			2.223	0.222		
		452.71				2.492			
		Tail Length - Baseline				Tail Length - Δ_{Damage}			
		8806.7	8806.7	412.70	0.0000	11473.3	11473.31	41.733	0.0001
		1425.9	475.33	22.275	0.0001	447.718	149.239	0.543	0.6639
		213.39	21.339			2749.20	274.921		
		1639.3				3196.92			
		Tail Length - $\Delta_{Repair\ 1}$				Tail Length - $\Delta_{Repair\ 2}$			
		142.92	142.92	0.261	0.6207	4770.240	4770.240	24.506	0.0006
		2540.1	846.72	1.544	0.2632	471.260	157.087	0.807	0.5183
		5482.2	548.22			1946.576	194.658		
		8022.4				2417.836			
		Tail Length - $\Delta_{Total\ Repair}$				Tail Length - RC			
		6564.6	6564.6	15.574	0.0027	1.404	1.404	7.965	0.0181
		1006.6	335.54	0.796	0.5236	0.611	0.204	1.155	0.3743
		4215.0	421.50			1.762	0.176		
		5221.6				2.373			
		Tail Moment - Baseline				Tail Moment - Δ_{Damage}			
		175.24	175.243	147.223	0.0000	998.606	998.606	32.952	0.0002
		43.333	14.444	12.135	0.0011	98.606	32.869	1.085	0.3996
		11.903	1.190			303.046	30.305		
		55.236				401.652			
		Tail Moment - $\Delta_{Repair\ 1}$				Tail Moment - $\Delta_{Repair\ 2}$			
		0.903	0.903	0.013	0.9114	729.168	729.168	37.055	0.0001
		237.62	79.209	1.143	0.3786	92.841	30.947	1.573	0.2567
		693.17	69.317			196.778	19.678		
		930.79				289.619			
		Tail Moment - $\Delta_{Total\ Repair}$				Tail Moment - RC			
		781.39	781.397	19.325	0.0013	2.781	2.781	8.789	0.0142
		152.34	50.781	1.256	0.3412	0.766	0.255	0.807	0.5184
		404.35	40.436			3.164	0.316		
		556.69				3.930			

Table B-4 Dunnett's post-hoc test comparing group A2, group A3 and group A4 to group A1, probabilities for post-hoc tests (2-sided)

% DNA in Tail						
	Baseline	**Δ Damage**	**Δ Repair 1**	**Δ Repair 2**	**ΔTotalRepair**	**RC**
MS	3.2918	9.6908	22.528	7.9851	13.983	0.07201
df	13.000	13.000	13.000	13.000	13.000	13.000
	{1} 2.4753	{1} 4.1594	{1} -0.3067	{1} -1.923	{1} -2.229	{1} 0.39022
A1						
A2	0.695794	0.000072	0.996636	0.000734	0.009348	0.824139
A3	0.001765	0.426644	0.194327	0.983101	0.120669	0.859475
A4	0.969576	0.999044	0.986815	0.895149	0.999370	0.966164
Tail Length						
	Baseline	**Δ Damage**	**Δ Repair 1**	**Δ Repair 2**	**ΔTotalRepair**	**RC**
MS	28.266	48.915	167.65	96.429	114.02	0.06440
df	13.000	13.000	13.000	13.000	13.000	13.000
	{1} 8.1424	{1} 11.280	{1} -1.568	{1} -6.077	{1} -7.645	{1} 11.28
A1						
A2	0.562494	0.000196	0.201917	0.001158	0.125489	0.989404
A3	0.000016	0.101854	0.081941	0.952228	0.064430	0.997299
A4	0.940993	0.967114	0.938277	0.896183	0.999971	0.797407
Tail Moment						
	Baseline	**Δ Damage**	**Δ Repair 1**	**Δ Repair 2**	**ΔTotalRepair**	**RC**
MS	0.95813	16.813	31.347	9.0220	21.509	0.07942
df	13.000	13.000	13.000	13.000	13.000	13.000
	{1} 0.51588	{1} 3.6350	{1} -1.978	{1} -1.168	{1} -3.146	{1} 0.62656
A1						
A2	0.856393	0.035096	0.280518	0.000228	0.233548	0.971882
A3	0.000010	0.183896	0.261158	0.997486	0.130613	0.993427
A4	0.991713	0.908994	0.896650	0.991841	0.908774	0.753734

Table B-5 **Dunnett's post-hoc test comparing group B2, group B3 and group B4 to group B1, probabilities for post-hoc tests (2-sided)**

	% DNA in Tail					
	Baseline	**Δ_{Damage}**	**$\Delta_{Repair\ 1}$**	**$\Delta_{Repair\ 2}$**	**$\Delta_{TotalRepair}$**	**RC**
MS	4.1436	24.651	43.682	20.533	42.106	0.22225
df	10.000	10.000	10.000	10.000	10.000	10.000
	{1} 3.5593	{1} 12.291	{1} -0.6427	{1} -7.865	{1} -8.507	{1} 0.48285
B1						
B2	0.003635	0.697342	0.999819	0.999968	0.999965	0.995158
B3	0.021400	0.753160	0.802596	0.990392	0.884613	0.642997
B4	0.191933	0.506165	0.955680	0.973389	0.852796	0.886003
	Tail Length					
	Baseline	**Δ_{Damage}**	**$\Delta_{Repair\ 1}$**	**$\Delta_{Repair\ 2}$**	**$\Delta_{TotalRepair}$**	**RC**
MS	21.339	274.92	548.23	194.66	421.51	0.17622
df	10.000	10.000	10.000	10.000	10.000	10.000
	{1} 13.778	{1} 13.778	{1} -15.08	{1} -13.64	{1} -28.73	{1} 0.53456
B1						
B2	0.000071	0.809114	0.999838	0.971923	0.997171	0.947816
B3	0.001466	0.775147	0.191778	0.389617	0.507160	0.236306
B4	0.481133	0.502851	0.859278	0.998977	0.851833	0.937296
	Tail Moment					
	Baseline	**Δ_{Damage}**	**$\Delta_{Repair\ 1}$**	**$\Delta_{Repair\ 2}$**	**$\Delta_{TotalRepair}$**	**RC**
MS	1.1903	30.305	69.317	19.678	40.436	0.31641
df	10.000	10.000	10.000	10.000	10.000	10.000
	{1} 1.2214	{1} 9.0415	{1} -2.183	{1} -5.318	{1} -7.501	{1} 0.67640
B1						
B2	0.000917	0.729326	0.911798	0.841761	0.535653	0.998992
B3	0.002676	0.968493	0.464803	0.210746	0.960609	0.401705
B4	0.246721	0.696307	0.957630	0.996072	0.855443	0.892280