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DNA DAMAGE AND REPAIR DURING THE MAMMALIAN HORMONAL CYCLE.

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"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'"

- Isaac Asimov -

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ABSTRACT

The aim of this study was to determine the pattern of DNA damage and repair during the mammalian female hormonal cycle. This was done to determine whether the hormonal cycle must be taken into account when DNA damage and repair studies are done in female subjects, especially so when surrogate tissue such as white blood cells are involved. Because certain factors including gender, age and diet may influence the comet assay responses in lymphocytes when monitoring human genotoxicity, we posed the question whether the menstrual cycle would influence the comet assay responses in lymphocytes to any significant extent. The study approach was to determine DNA damage and repair during the hormonal cycle in both Sprague-Dawley rats and female individuals. Firstly, sexually matured adult female Sprague-Dawley rats were investigated. Vaginal smears were carried out to determine the phase of the estrous cycle of each individual. Differences in baseline DNA damage in white blood cells as measured in whole blood, and in isolated lymphocytes were observed for the different phases of the estrous cycle. The metestrous phase showed the most DNA damage in both the isolated lymphocytes and the white blood cells. The estrous phase has the least amount of DNA damage over the estrous cycle. Results obtained for the human female individuals showed that DNA damage peak at different times for different individuals during the menstrual cycle. The extent of DNA damage also varied between individuals. We could not, however, answer the question whether the extent of DNA damage we have observed was beyond the limits of physiological variation. In summary: In spite of inconclusive results we are of the opinion that the hormonal cycle of the mammalian female proves to be a factor of sufficient importance that needs to be taken in account when studies involving oxidative DNA damage are performed. A more extensive study in this field is required to address the influence of varying hormonal levels on DNA damage during the cycle.

OPSOMMING

Die doel van hierdie studie was om die patroon van DNA-skade en -herstel tydens die soogdier hormonale siklus te bepaal en is gedoen om vas te stel of hierdie siklus in aanmerking geneem moet word wanneer DNA-skade en -herstel ondersoek word by vroulike proefpersone, veral wanneer surrogaatweefsel soos witbloedselle gebruik word. Aangesien sekere faktore soos geslag, ouderdom en dieët die komeet-analise se respons in limfosiete kan beïnvloed wanneer genotoksisiteit gemoniteer word, is die vraag of die menstruele siklus in hierdie verband ook belangrik is. Die benadering van die studie was om DNA-skade en -herstel tydens die hormonale siklus in beide Sprague-Dawley rotte en vroulike proefpersone te bepaal. Eerstens is seksueel volwasse wyfie Sprague-Dawley rotte bestudeer. Vaginale smere is gedoen om elke rot se fase binne die siklus te bepaal. Die komeet-analise is uitgevoer om DNA-skade en -herstel gedurende die siklus te bepaal. Verskille in basislyn DNA-skade in wit bloed selle soos gemeet in heelbloed en in geïsoleerde limfosiete is waargeneem vir die verskillende fases van die estroussiklus van die rot. Die meeste DNA-skade kom voor in die metestrous fase in beide witbloed selle en geïsoleerde limfosiete. Die minste DNA-skade kom voor tydens die estrous fase. Die resultate van die vroulike proefpersone toon dat die hoogste vlak van DNA-skade op verskillende tye tydens die siklus by die verskillende individue voorkom. Die mate van DNA-skade varieer ook tussen die verskillende individue. Met hierdie studie kon die vraag of die mate van DNA-beskadiging wat waargeneem is buite die grense van fisiologiese variasie val, egter nie beantwoord word nie. Opsommend: Alhoewel dit nie finaal aangetoon is in hierdie studie nie, is ons tog van oortuiging dat die hormonale siklus van die soogdier wesentlik 'n belangrike faktor is wat in ag geneem behoort te word wanneer studies gedoen word waar oksidatiewe DNA-skade gemoniteer word. 'n Meer intensiewe studie behoort in hierdie veld gedoen te word ten einde die invloed van variërende hormoonvlakke op DNA-skade tydens die hormonale siklus ook te bepaal.

CHAPTER 1
INTRODUCTION

This study is aimed at indicating the importance of considering the estrous cycle where it involves studies in which oxidative stress is a factor, and this work is part of a bigger study in this laboratory using the comet assay in determining the effect of oxidative stress on the integrity of the mammalian genetic material.

The estrous and menstrual cycle is a cyclic event which in turn can influence other biochemical and physiological factors. Vantyghem *et al*, 2003 hypothesized that tumor cells may vary in their ability to establish metastases when introduced into circulation at different phases of the estrous cycle and that host tissues may also vary in their ability to support metastatic growth at different phases of the cycle.

The estrous cycle of the rat and the menstrual cycle of the human female together with some of the factors which may influence the integrity of the DNA during the hormonal cycle will be discussed followed by a description of the factors that induce possible damage during this cycle (Chapter 2). This is done to provide a better understanding of the hormonal- and physiological changes associated with the cycle. It may shed light on possible DNA damage taking place during the cycle. Chapter 3 consists of the materials and methods that were used to investigate DNA damage and repair during the cycle. The results and discussion are given in Chapter 4, followed by a short summary of the study in Chapter 5. The first draft of a paper based on this work is included as Chapter 6.

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

When a search of the Medline Database were done over the last 20 years for studies in which rats were employed as research subjects a preference of males were used over females by a ratio of approximately 3:1. This proportion can even become 4:1 or higher when search terms become more specific, like "memory" or some drug of abuse. Physiological and behavioral variations across the estrous cycle can influence the results, therefore the concern. This preference is acknowledged to be in part because of concerns over how physiological and behavioral variations across the estrous cycle can influence results (Parker *et al*, 2001).

Why is it then necessary to consider the menstrual- and estrous cycle when any bioassay is being performed using female subjects? Some clinical studies suggest that timing of surgery during specific menstrual phases may influence the chances of survival for premenopausal women with breast cancer, whereas other studies failed to demonstrate this effect. The interactions of circulating tumor cells with secondary tissues in the establishment of metastases may differentially be affected by the fluctuating hormonal milieu of the host. Also the *timing of surgery* concept may be more broadly applicable than just to breast cancer (Vantyghem *et al*, 2003). Previous studies have also shown that the biochemical profiles of female urine samples differ significantly from those of males, for instance, sex-related differences in the elimination of citrate in the urine have been demonstrated. Female rat urine is also more varied in composition than that of males due to the influence of hormonal changes in the estrous cycle (Bollard *et al*, 2001). Also, gender had an influence on the increased risk for the presence of some pathological and physiological situations. Sex hormones, estrogens and testosterone, have been suggested

to play a role in this gender-associated response (Fortoul *et al*, 2004). Most importantly, chromosomal aberrations have been observed to take place during the hormonal cycle (Landi & Barale, 1999, D'Souza *et al*, 1988). These observations support the aims of this study to investigate whether the phase of the hormonal cycle must be taken into account when studies concerning the effect of oxidative stress are performed when subjects are involved, e.g. are DNA damage and repair affected during the hormonal cycle?

2.2 ESTROUS CYCLE OF THE RAT

2.2.1 Introduction

Herewith a short summary of the estrous cycle of the rat taken from Andres & Strange (1999). Female mammals prepare at regular intervals - estrous (rat) and menstrual (human) cycle - for a possible pregnancy – reference will be made to the menstrual cycle where applicable. Maturation and release of the oocyte from the ovary, a build-up of the endometrium in the uterus, and priming of the mammary epithelium for initiation of pregnancy-induced differentiation, takes place during this cycle. If pregnancy does not occur, the changes are reversible, in preparation for a next possible pregnancy. The duration of the cycle between species is highly variable ranging from a few days to several months.

The estrous cycle can be divided into four distinct phases as shown in figure 2.1: *proestrous* (follicular development, endometrial proliferation); *estrous* (ovulation), *metestrous* (endometrial differentiation) and *diestrous* (desquamation of endometrium leading to menstruation in humans).

The estrous cycle is controlled by a complex interplay between ovarian and pituitary hormones (fig. 2.1). The main controllers of the estrous cycle comprise of follicle stimulating hormone (FSH) and luteinizing hormone (LH), pituitary-derived peptide hormone from the ovarian steroid hormones estrogen and progesterone. During proestrous (the follicular phase in woman), increasing levels of FSH stimulate maturation

of the estrogen-producing ovarian follicles. FSH and LH production (mainly LH in woman) increases due to estrogen in the pituitary, while LH induces increased estrogen production. Maximal estrogen and LH levels lead to ovulation at estrous. LH supports the differentiation of empty follicles into the progesterone-producing corpus luteum (CL) after ovulation. Progesterone suppresses FSH and LH production - a negative feedback effect. During this progesterone-dominated, metestrous phase of the cycle (the initial part of the luteal phase in humans), the endometrium is maximally receptive for implantation of the fertilized egg into the uterine lining. In the absence of fertilization, CL ceases to produce progesterone and collapses. The final, diestrous phase of the cycle (menstrual phase in woman) is characterized by minimal hormone levels. Adaptation of the organism for a potential pregnancy is reversed and, due to the decline in progesterone, FSH and estrogen initiate a new round of the cycle (Andres & Strange, 1999).

Table 2.1: Description of the different phases of the estrous cycle

Stage	**Ovary**	**Histology of vaginal mucosa**	**Uterus**
I Proestrous (12h)	Follicles rapidly growing. CL of previous cycle degenerating.	Cornified layer under surface layer epithelium. Epithelium thick.	Uterus becomes distended with fluid increasing the diameter.
II Estrous (12h)	Follicles largest. Germ cells undergoing maturation	Cornified layer well formed and on the surface. Epithelium thick.	Reaches greatest distention and thinness of epithelium.
III Early metestrous (15h)	Ovulation	Cornified layer shedding and finally completely detached.	Epithelium undergoing vacuolar degeneration.
Late metestrous (6h)	Young CL. Eggs in oviduct. Follicles smallest	Cornified layer gone. Epithelium thin. Many leukocytes	Some vacuolar degeneration, but also regeneration.
IV Diestrous (57h)	Follicles various sizes. CL continue to grow	Epithelium thin. No cornified layer yet. Some leukocytes.	Epithelium undergoing regeneration.

(Anon 1, 2004)

The estrous cycle (present in most mammals) and the menstrual cycle (present in most primates) are very similar. The estrous- and menstrual cycle have follicle development

and the CL dominant phase in common. One difference though is that the menstrual cycle has a longer period of follicle development after the CL has regressed prior to ovulation. Another difference is that the uterine endometrium develops prior to implantation to a much greater degree in menstrual cycles than estrous cycles. If the woman (or other primate that has estrous cycles) does not become pregnant, this endometrial thickening is sloughed causing bleeding (Anon 2, 2005). There are various factors that can bring about variation within the cycle. Some of these variations will be discussed.

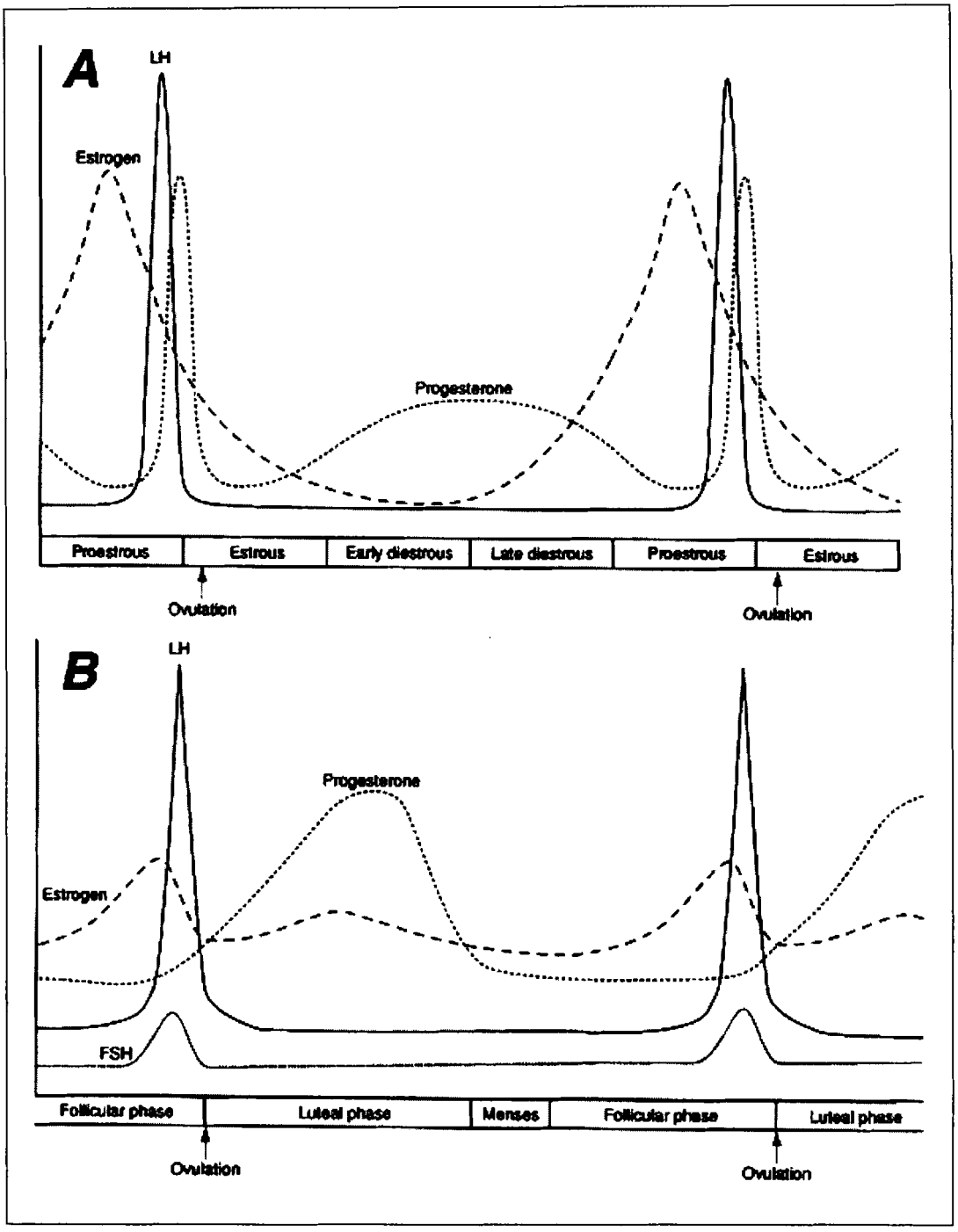


Figure 2.1: Illustration showing the different hormone levels during the different stages of the estrous cycle. A) Estrous cyclicity, B) menstrual cyclicity.

2.2.2 Factors that are influenced by the cycle

During the estrous cycle, certain biochemical and physiological factors may also exhibit some cyclic characteristics. It is imperative to address some of these factors as an illustration that the estrous cycle (and menstrual cycle) may influence other non-related functions. Some of these factors will be briefly discussed.

- Metabolism

During normal metabolism, reactive by-products is formed which causes oxidative damage to DNA. Smaller animals - like the rat - consume greater volumes of oxygen due to the higher metabolic rate. A cascade of events take place as the production of harmful free-radical by-products increase, leading to a higher rate of damage to cellular targets (Adelman *et al*, 1988). In the process of aging, it has been found that whole-body metabolic rate plays a determining role in the rate of DNA oxidative damage. When comparing levels of various measures of DNA oxidative damage and whole-body energy metabolic rate in mice, rats, monkeys and humans, it can be concluded that the rate of DNA oxidative damage is directly correlated with energy metabolic rate (Greenberg *et al*, 2000).

The following results from the literature illustrate the difference in various metabolic activities during the estrous cycle.

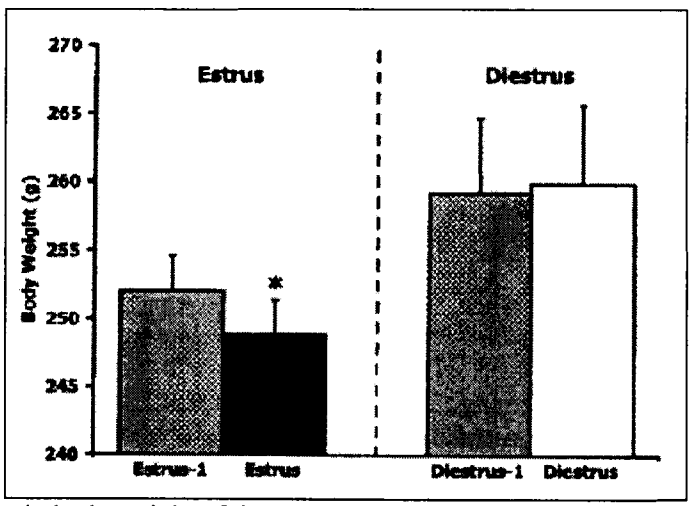


Figure 2.2: Variation in body weight of the rat during the estrous cycle (Parker *et al*, 2001).

Female Sprague-Dawley rats showed significant changes in whole-body metabolism between days associated with the estrous vs. diestrous phases of their ovarian cycle. Parker *et a,l* (2001) chose these two phases because rats' food intake and body weight changes vary maximally between them.

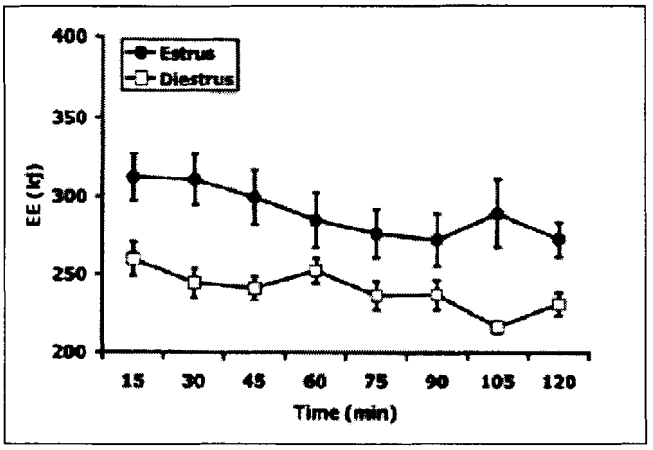


Figure 2.3: Energy Expenditure of the rat during the estrous cycle (Parker *et al*, 2001).

Energy expenditure (EE) provides an index of how many total calories are burned for all physiological and behavioral processes. It is believed that the EE differences observed represent true shifts in basal bioenergetic processes between phases of the ovarian cycle (Parker *et al*, 2001).

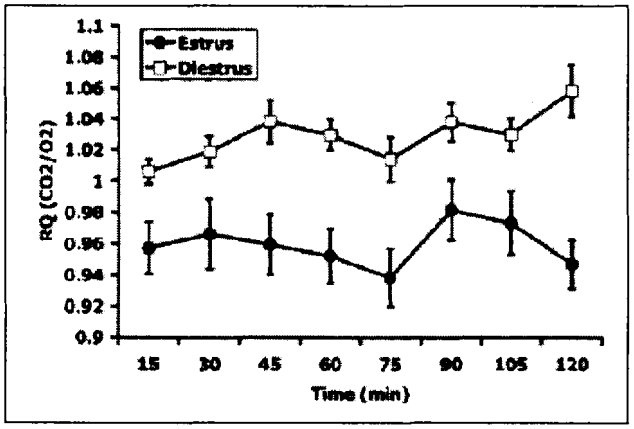


Figure 2.4: Respiratory Quotient, of the rat during the estrous cycle (Parker *et al*, 2001).

Respiratory Quotient (RQ) provides more selective information about the relative contribution of carbohydrate vs. triacylglyceride metabolism to support energetic needs. The decrease in RQ seen on days associated with estrous vs. diestrous (fig. 2.4), indicates a shift in energy metabolism towards greater utilization of triacylglycerol (fat) instead of carbohydrates (Parker *et al*, 2001).

- Sister chromatid exchange (SCE)

In mammals, characteristically the time structures of biologic rhythms at all levels of biology functions are complex. Significant changes occur in the general physiological condition of a given organism, especially of the hematopoetic and immune systems in the form of prominent rhythmicity in cell proliferation and cell function during different periods of the day (circadian), of the week (circaseptal), of the month (circamensual) and of the year (circannual). Not only the number and population but also the reactivity of circulating blood lymphocytes varies periodically as a function of time as shown by their differential response to *in vitro* mitogen (D'Souza *et al*, 1988). D'Souza *et al* (1988) determined SCE frequencies and compared ovulatory and estrogenic stages with the progestogenic stage during the menstrual cycle of woman. Their results indicate that the hormonal variations during menstrual cycle play an important role in bringing about variation in the base line frequency of SCE in woman.

- Cell Proliferation.

During the estrous cycle, cyclic proliferation and death of uterine epithelial cells occur. To evaluate this, proliferating cell nuclear antigen (PCNA) is used which reveals proliferating cells. PCNA was found expressed during tissue regression, such as in the CL during structural luteolysis, the prostate gland after castration and the neural apoptosis after dopamine stimulation. A good model with which to evaluate the role of PCNA in cell proliferation and cell death is the uterus, which undergoes growth and regression under the regulation of ovarian hormones. At estrous, when ovarian steroid concentrations decrease, PCNA was not expressed and the appearance of apoptosis was obvious. These observations suggest that the expression of PCNA and mitosis in uterine

epithelial cells during the estrous cycle were influenced by ovarian steroids (Lai *et al*, 2000).

- Natural killer (NK) cells

Natural killer (NK) cells are cytotoxic lymphocytes that play an important role in host immune responses to viruses, parasites, intracellular bacteria and tumour cells and constitute about 10% of all blood lymphocytes (Klingemann, 2000). In a study done by Yovel *et al,* (2001) it was found that the increase in numbers of NK cells in woman with regular menstrual cycle seemed to occur during the periovulatory period in which LH levels are high. They also reported that both in humans and in rats, adrenergic suppression of NK activity *in vitro* was stronger if blood was drawn during the luteal phase (proestrous and estrous phase in rats) and the ovarian phase characterized by high levels of estradiol.

- Liver enzymes.

Drug metabolizing enzyme activity has also been shown to vary during the estrous cycle. Estrogen exposure during the menstrual cycle of women are quite longer, compared with rats, therefore the effect of the cycle on the hepatic microsomal drug-metabolizing enzyme activity in human may be clearer than that in rats (Watanabe *et al*, 1997). They also established that the physiological dose of estradiol (E_2) as well as the estrous cycle, affected NADPH-cytochrome c reductase (fP_T) and uridine diphosphate glucuronyltransferase (UDP-GT) activities in liver microsomes of female rats.

- Antioxidant capacity

The total antioxidant capacity refers to a full spectrum of antioxidant activity against various reactive oxygen/nitrogen radicals (Anon 3, 2003). One of these enzymes is superoxide desmutase (SOD). SOD is an enzyme which causes the dismutation of superoxide free radical anions to generate hydrogen peroxide (H_2O_2). This enzyme has been localized in the ovary of the rat and exhibits cyclic changes during the cycle (Laloraya *et al*, 1988). It is suggested that SOD may play a role in regulating follicular development, ovulation and luteal functions (Laloraya, M *et al*, 1989). On proestrous,

the superoxide anion (O2-) rise six time more than the other stages, while its regulating enzyme, SOD, decreases. Superoxide anion is also connected with the increase of fluidity and polarity of the membranes during the implantation. SOD and cytosolic glutathione peroxidase were elevated in exercising animals. As previously mentioned, SOD exhibit cyclic changes during the cycle, but are also elevated in rat skeletal muscle during exercise (Leeuwenburgh & Heinecke, 2001). These defenses may be critical for preventing chronic oxidative damage to muscle during exercise and even at rest. In a study on the activity of three antioxidant enzymes (SOD, CAT (catalase) and GPx) in red blood cells of male and female rats done by D'Almeida *et al,* (1994) the results indicate that there were no detectable effects of the estrous cycle on the activity of SOD, CAT and GPx. These different observations regarding SOD activity during the estrous cycle may be attributed to the cells used, because alterations found in SOD activity were observed in the uterus, brain, ovary and the thyroid (D'Almeida *et al,* 1994).

To summarize: The female hormonal cycle is a cyclic event, which causes variation in a variety of physiological and biochemical entities. These variations may directly or indirectly influence the integrity of the genetic material of the subjects involved. In the following section, the factors that may influence DNA integrity during the hormonal cycle will be discussed.

2.3 FACTORS THAT CAN INDUCE DNA DAMAGE DURING THE HORMONAL CYCLE

2.3.1. Introduction

DNA undergoes several types of spontaneous modifications, and it can also react with many physical and chemical agents, of which some are endogenous products of the cellular metabolism (e.g. reactive oxygen species or ROS) while others, including ionizing radiation and ultraviolet light, are threats from the external environment. The result is alterations in the DNA structure and is generally incompatible with its essential role in preservation and transmission of genetic information (Wiesmüller *et al*, 2002). ROS generated endogenously as by-products of aerobic or xenobiotic metabolism can also cause oxidative damage to cellular macromolecules. DNA lesions resulting from exposure to ROS include modified bases, abasic sites, single and double strand breaks, and DNA-protein cross links. Continuous damage to DNA by free radical mechanisms may contribute to aging, cancer and other age-related degenerative diseases (Cadenas *et al*, 1997).

Any DNA damage must by repaired to maintain the integrity of the genetic material. The biological importance of DNA repair is indicated by the great variety of such pathways possessed by even relatively simple organisms such as *E. coli*. In fact, the major DNA repair processes in *E. coli* and mammalian cells are chemically quite similar (Voet & Voet, 1995).

DNA repair consists mainly out of five mechanisms, which will now be discussed briefly.

Nucleotide excision repair (NER)

NER involves the elimination of a damaged section of a DNA chain, followed by the action of first DNA polymerase and then DNA ligase to regenerate a covalently closed duplex at the site of the original damage. This system also repairs DNA damage that results when two strands covalently crosslink to each other. In this case, the two strands

are repaired sequentially (one after the other) in order to preserve an intact template strand (Mathews *et al*, 2000).

Base excision repair (BER)

One or more nucleotides from a site of base damage are removed with the BER process. The process initiates with enzymatic cleavage of the glycosidic bond between the damaged base and deoxyribose. Oxidative damage to DNA is repaired primarily by BER (Mathews *et al*, 2000).

Mismatch repair (MMR)

The mismatch repair system is responsible for removal of base mismatches caused by spontaneous and induced base deamination, oxidation, and methylation and replication errors. The main targets of MMR are base mismatches such as G/T, G/G, A/C and C/C (Christmann *et al*, 2003).

Non-homologous End- Joining (NHEJ)

In simple eukaryotes like yeast, homologous recombination is the main pathway, whereas in mammals the NHEJ pathway predominates. NHEJ occurs mainly in G0/G1 cell cycle. The NHEJ system ligates the two ends of a double strand break (DSB) without the requirement of sequence homology between the DNA ends (Christmann *et al*, 2003).

Homologous Recombination (HR)

HR occurs during the late S and G2 phases. During HR, the damaged chromosome enters into physical contact with an undamaged DNA molecule with which it shares sequence homology and which is used as template for repair (Christmann *et al*, 2003).

The observation of variations during the hormonal cycle noted in section 2.2.2 may lead to the belief that a woman's life expectancy would be lower than that of males. Life expectancy at birth indicates the total number of years a person could expect to live, based on the mortality rates of the population at each age in a given year (Anon 4, 2004). This is not the case. A Woman's life expectancy is usually higher than that of males

(Anon 5, 2004). Why is it then that a female show higher life expectancy over a male? DNA damage is a certain process during the estrous- and menstrual cycle. Repair seem to be the important factor as a woman's cycle seem to have no influence on the life expectancy of a female.

2.3.2. Apoptotic cell death

Apoptosis functions as a homeostatic mechanism that ensures that cell proliferation is balanced by a corresponding rate of cell death, which is a fundamental component in uterine remodeling prior to and during implantation in normal rats (Mendoza-Rodríguez *et al*, 2003). To obtain homeostasis of ovarian function in human and other mammalian species, ovarian cell death plays an important role. It ensures the selection of the dominant follicle and the demise of excess follicles (Amsterdam *et al*, 2003).

In each estrous cycle only one follicle, the dominant follicle, reaches full maturation while the other recruited follicles become atretic in a process characteristic of programmed cell death. Moreover, the old CL formed in a previous cycle undergoes luteolysis by a mechanism also characteristic of programmed cell death. Granulosa cells comprise the largest cell population of the ovarian follicle and are the main source of estradiol and progesterone in the ovary. Their cyclic nature of differentiation and death determines the cyclic secretion of female sex hormones and therefore serve as an excellent model for steroid regulation during apoptosis. However, local factors that determine the dominant follicle that will survive and the rest of the recruited follicles that undergo atresia, have yet to be determined (Amsterdam *et al*, 1998).

The rodent uterus and vagina show marked histological changes during the estrous cycle. Sato *et a,l* (1997) found that apoptotic cell death was encountered in the uterus and vagina during estrous cycle in rats. They also found that there is an inverse correlation between cell death and cell proliferation in rat uterine and vaginal epithelial cells during the estrous cycle.

An involvement of increased ROS generation or antioxidant completion in apoptosis has been shown in various cell lines. Apoptosis can often be blocked or slowed by antioxidants, and apoptosing cells have a general more 'oxidized' cytoplasm; they may extrude antioxidants such as glutathione (GSH), for example. Since apoptosis involves dismantling the cellular architecture, the involvement of a cascade of proteases is not surprising: proteins degraded during apoptosis include histone H1, poly(ADP-ribose) polymerase and β-actin (Halliwell & Gutteridge, 1999).

2.3.3 Estrogen metabolism

Estrogen is one of the most important hormones involved in the estrous cycle (fig 2.1). The metabolism of estrogen and its derivatives may lead to DNA damage. Furthermore estrogen proved to be a hormone that shows evidence of both pro- and anti-oxidant traits. Some of these characteristics will be further explored.

Here follows a short summary prepared from Raftogianis *et a,l* (2000).
Estrogens exert biological responses in steroid hormone-responsive cells largely via interaction with estrogen receptors (ER), members of a super family of nuclear hormone receptors, which act as ligand-activated transcription factors. The two most potent endogenous estrogens, estrone and 17β-estradiol, are both ligands for the ER's, although those receptors have higher affinity for 17β-estradiol than for estrone. 17β-estradiol is believed to be the predominant endogenous activator of ER-mediated cellular processes. Specifically, oxidative reactions, often catalyzed by isoforms of the cytochromes P450, can result in the formation of catechol estrogens (CE's) from parent estrogens and, subsequently, semiquinones and quinones derived from CE's that are capable of forming either stable or depurinating DNA adducts. The DNA damage accompanying exposure to estrogens is highly complex, involving direct adduction by bioactivation products as well as an oxidative component due to redox-cycling metabolites (Burcham, 1999).

Biological role of Estrogen conjugation.

The endogenous formation of estrogen conjugates has long been recognized as a major route of estrogen metabolism. The most abundant circulating estrogen conjugates are the sulfates, followed by the glucuronides. Direct or indirect DNA damage can play a role in estrogen carcinogenesis, with multiple bioactivation products likely to contribute to genetic damage (Burcham, 1999).

Biological role of CE conjugation.

The biotransformation of estrone and estradiol to CE's involves hydroxylation at the 2^{nd} or 4^{th} position of the steroidal A ring of these parent estrogens. Those reactions are catalysed by multiple cytochrome P450 isoforms. Both the 2- and the 3-hydroxy CE's can be further oxidized to CE quinones (CE-Qs) or semiquinones. The 2-hydroxy CE-Qs have been shown to form stable DNA adducts, whereas the 4-hydroxy CE-Qs have been shown to form depurinating adducts. There are good evidence suggesting that those depurinating adducts can lead to apurinic DNA sites and permanent mutations that, when inflicted upon critical DNA sequences, can lead to tumorigenesis. CE's can also enter into redox cycling and, thereby, become a source of ROS. Hence, unless CE's are inactivated, they may contribute to carcinogenesis by causing DNA damage mediated by ROS and by direct interaction of CE-Qs with DNA to form depurinating adducts. Generally, the reactive CE's are detoxified by biotransformation to predominantly methyl conjugates, to a lesser extent glucuronides, and possibly sulfate conjugate.

High levels of oxidatively damaged DNA have been found in breast tissue from women in the United States. It is reasonable to propose that in human breast tissue, like the kidneys of hamsters treated with estrogen, oxidative metabolism of CE's might contribute to this oxidative damage.

In short, during normal cell metabolism, the genome is exposed to numerous reactive substances. A range of endogenous processes has been surveyed with regard to their ability to generate DNA-reactive substances for example, oxidants, lipid peroxidation products, alkylating agents, estrogens, chlorinating agents and many others. In each case,

as stated in the review by Burcham (1999), it has been observed that there is at least strong *in vitro* evidence for such damaging potential. For some substances, however, evidence that they cause DNA damage *in vivo* is currently lacking (Burcham, 1999). It is for this reason that the study was done, to provide a better understanding of possible DNA damage during the estrous- and menstrual cycle.

2.3.4 Estrogens – Good or bad?

Controversial reports exist in the literature on the good or bad effect of estrogen. Here follows some examples of the observations found.

Estrogen has a significant influence on body growth by broadening the pelvis, stimulating breast development, and increasing fat deposition, particularly in the thighs and hips. This increase in fat deposition in the thighs and hips is the result of increased lipoprotein lipase activity in these areas. This enzyme is considered the gatekeeper for storing fat in adipose tissue. Estrogen also increases the growth rate of bone, allowing the final bone length to be reached within 2 to 4 years following the onset of puberty. As a result, female grow very rapidly for the first few years following puberty and then cease to grow (Wilmore & Costill, 2004).

Redox cycling of estrogens has been shown to generate free radicals which may react to form the organic hydroperoxides needed as cofactors for oxidation to quinones (Liehr, 1990). The formation of endogenous DNA adducts both in humans and animals are induced by estrogens, and it is suggested that these DNA adducts could ultimately lead to cancer development (Hundal *et al*, 1997).

The administration of estrogen to laboratory animals present evidence for inducing kidney tumors in hamsters, uterine tumors in mice, Leydig cell tumors in mice, or pituitary tumors in rats (Liehr, 1990). In a study done by Dhillon & Dhillon (1995), their results together with other reports on the ability of the estrogens (estradiol) to cause formation of DNA adducts and inhibition of DNA synthesis and cell proliferation rates, strongly indicate that, although these drugs are unable to induce mutations in the

prokaryotic system, their prolonged use can cause serious genetic health hazards. The oxidation-induced DNA damage in estrogen-treated hamsters has been taken as evidence for the generation of oxygen radicals by metabolic redox cycling of catecholestrogens of diethylstilbestrol and for the participation of this process in the induction of estrogen-induced cancer in animals or humans (Markides *et al*, 1998).

In contrast to the above mentioned, estrogens are considered reversible cellular signals. The antioxidant properties of estrogens have been demonstrated in many studies *in vitro* and *in vivo*. These antioxidant effects of estrogens were used as an explanation of lower heart disease rates in premenopausal women or postmenopausal women treated with estrogen compared to men (Markides *et al*, 1998). Administration of estrogen to an ovariectomized mouse resulted in organ growth, cell proliferation and target gene expression in the uterus. When estrogen was withdrawn, uterine size and weight, as well as expression of estrogen-regulated genes, returned to close to the unstimulated state (McLachlan *et al*, 2001).

The role of sex hormones in lipid peroxidation has been investigated in rat liver homogenates. Male rats have a higher content of products of lipid peroxidation than females. There is substantial evidence that estrogens show signs of antioxidant properties. The antioxidant effect of estrogen has been regarded as the main mechanism for this hormone to protect skeletal and cardiac muscles, uterus and liver from damage. In brain, progesterone instead of estrogen exerts a significant antioxidant effect. Contrary to female steroids, testosterone has been shown to decrease the activities of SOD, CAT and glutathione peroxidase (GSH-Px), leading to lipid peroxidation (Azevedo *et al*, 2001).

When any study is done where estrogen is involved, it is very important to consider both mechanisms. As can be seen in the literature, estrogen proves to be both a tumor inducing hormone, but also shows antioxidant capacity. Markides *et al,* (1998) observed that estrogens may exhibit either pro- or antioxidant activities depending on the nature of the estrogen metabolites and their concentrations. Micromolar, i.e., pharmacological

concentration of parent hormones or their metabolites, clearly have antioxidant properties. Thus, the pro-oxidant activity of estrogens *in vivo* may be dependent on their conversion to catecholestrogen metabolites in a specific organ or species.

In summary, a balance of pro- and antioxidant effects of estrogens has been established which depends on the nature (structure) of the estrogen and on its concentrations. Under physiological conditions, the metabolic profile of estrogen metabolites in a specific tissue fluid will determine its pro- or antioxidant activities (Markides *et al*, 1998).

2.4 The aim and approach of the study

The aim of this study was to determine the extent of DNA damage and repair during the estrous cycle of the rat and the menstrual cycle in humans.

The following approach was formulated for this study.

1. A group of research animals were obtained from the Animal Care Facility. The different phases of the rats were determined, blood was collected and the Comet Assay was carried out to establish DNA damage and repair.

2. Blood was collected from female individuals once a week over a period of four weeks and the Comet Assay was carried out to establish DNA damage and repair.

CHAPTER 3
MATERIALS AND METHODS

3.1 Animals

Adult female Sprague-Dawley rats were obtained from the Experimental Animal Centre, Potchefstroom Campus, North-West University. They were maintained under a 12h dark-light cycle in a temperature-controlled colony, and had food and water *ad libitum*. All animal care and handling was carried out in accordance with the Ethical Committee (number 04D12). Sexually, matured healthy female rats were used.

3.2 Vaginal smears

3.2.1 Principle of the method

In most mammals, the vaginal epithelium goes through a series of cytological changes (table 1) that corresponds with hormonal and ovarian events. The rat is a polyestrous, spontaneous ovulator undergoing repetitive estrous cycles of about 5-days duration unless interrupted by mating and pregnancy. Vaginal status can be determined by using a lavage technique to sample the cells that constantly slough from the vaginal epithelium (Anon 1, 2004).

3.2.2 Method

The method was carried out by a trained laboratory animal technician, as described by Oettle and Weldhagen: A modified Shorr's stain: A practical rapid stain for canine vaginal cytology. 1982. *Journal of the South African Veterinary association.*

3.3 The Comet Assay

3.3.1 Principle of the method

Singh *et al* (1988) modified the microgel electrophoresis technique to permit an evaluation of DNA damage in single cells under alkaline conditions. This approach optimizes DNA denaturation and the migration of single-stranded DNA, thus permitting an evaluation of single-stranded DNA breaks and alkali-labile sites (Rojas *et al*, 1999., Collins, *et al*, 1997., McKelvey-Martin *et al*, 1993., Singh *et al*, 1988).

The comet assay (single cell gel electrophoresis assay or SCGE) is a rapid, simple, visual and sensitive technique for the assessment of DNA damage in individual mammalian cells. Briefly, cell membranes are lysed, protein bonds are broken and the DNA is unwound. During electrophoresis, broken strands of DNA are drawn out and a cell with damaged DNA gives the appearance of a 'comet', i.e. the head of the comet contains undamaged DNA content of the cell, which does not migrate with electrophoresis, and the comet tail contains damaged DNA pieces which migrate freely (Baltaci & Zeyneloglu, 2004).

3.3.1 Materials

All reagents were from the highest grade available and were purchased from Sigma-Aldrich, South Africa, unless otherwise stated. See Appendix A.

3.3.2 Method

Blood were collected in both heparin coated (comet assay) and blood coagulating tubes (ORAC assay) and were kept cool, not exposed directly to the ice. Microscope slides were coated evenly with 300 µl high melting point agarose (HMPA) gel and left to solidify. For the whole blood: 60 µl blood was taken from the heparin coated tube and placed in an Eppendorf tube. To this was added 250 µl phosphate buffered saline (PBS) and quickly vortexed. It was then centrifuged at 5 50 g for 5 minutes. The supernatant was removed and the process was repeated three times. From the cell suspension 50 µl

together with 150 µl low melting point agarose (LMPA) was placed in an Eppendorf tube and 130 µl of this mixture was spread evenly on the microscope slide.

For the isolation of leukocytes, 2ml blood was placed on top of 2ml Histopaque® in a Falcon tube and were centrifuged at 5 50 g for 30 minutes at room temperature. The top layer was carefully removed followed by the buffy coat which was transferred to an Eppendorf® tube. To this was added 300 µl PBS and it was then centrifuged at 5 50 g for 5 minutes. The supernatant was removed and the process was repeated. The cells were then resuspended in 500 µl PBS.

Control

From the cell suspension, 50 µl was placed in an Eppendorf® tube together with 150 µl LMPA and vortexed briefly. Of this 130 µl was spread evenly on the agarose coated microscope slides and put on an aluminium plate on ice.

H_2O_2 induced damage and repair

The remaining cells were incubated for 20 minutes at 37°C with 60 µM H_2O_2. The cells were then washed with PBS and centrifuged for 1 minute at 500 g. The supernatant was removed and cells were suspended in 450 µl HAMS. 50 µl from this cell suspension and 150 µl LMPA were mixed in an Eppendorf® tube; 130 µl were used to coat the slides and kept on ice. The remaining cells were incubated for 10 minutes at 37°C and 50 µl from the cell suspension and 150 µl LMPA were placed in an Eppendorf® tube, 130 µl were coated on a slide and kept on ice. The remaining cells were incubated for another 10 minutes at 37°C. Another 50 µl from the cell suspension were placed together with 150 µl LMPA in an Eppendorf® tube, 130 µl were coated on the slides and kept on ice. The remaining cells were incubated for 10 minutes at 37°C. Another 50 µl from the cell suspension were placed together with 150 µl LMPA in an Eppendorf® tube, 130 µl were coated on the microscope slide and kept on ice.

The microscope slides were placed overnight in the lyses buffer (see Appendix). The slides were then placed in the electrophoresis buffer for 30 minutes. The slides were

electrophoresed for 45 minutes at 30 Volt and 500 mA in the cold room (4°C). The microscope slides were now placed in the TrisHCl buffer (pH 7.5) for 15 minutes. Ethidium bromide was used to stain the DNA in the agarose for one hour. The slides were then washed with ddH_2O for at least half an hour before the pictures were taken.

Cells were counted using the Trypan Blue staining method. 50µl cell suspension was mixed with 50µl Trypan Blue and incubated for 5 minutes at room temperature. Of this mixture, 10µl was placed on a hemacytometer and viable cells counted under a light microscope using a 100x magnification. Based on the number of cells counted, the cell suspension volume was adjusted so that each 50µl cells suspension contained approximately 5000 cells. After the slides were washed with ddH2O, pictures were taken using the Olympus X70 fluorescence microscope (200 x magnification). A minimum of 50 cells/sample were taken for statistical significance. The data was scored using the Comet Assay Software Project (CASP program). This program calculates the percentage of DNA in the tail of the comet. This is then representative of the percentage of DNA damage. The comets were grouped in different classes according to the percentage DNA present in the tail. Examples are given in table 3.1. The data was then processed in Excel.

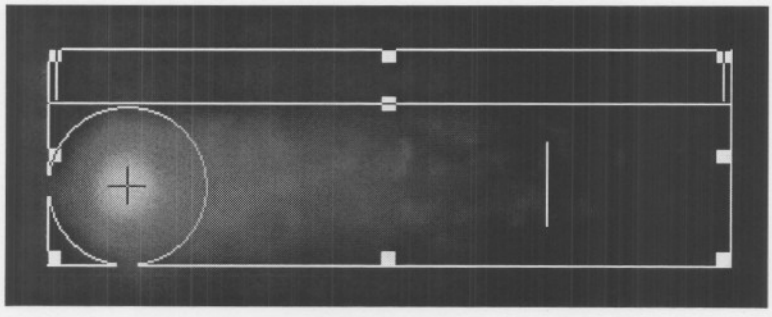
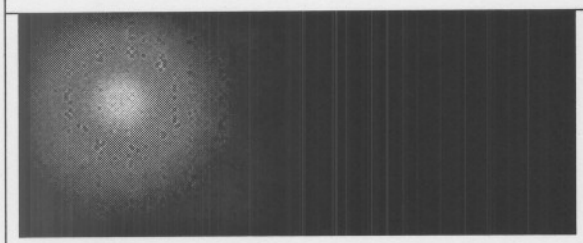
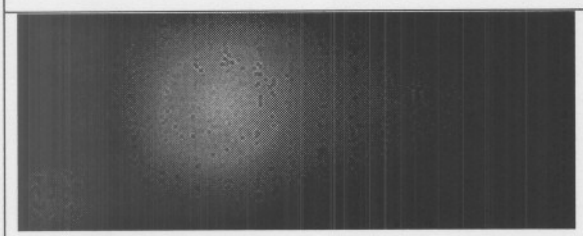
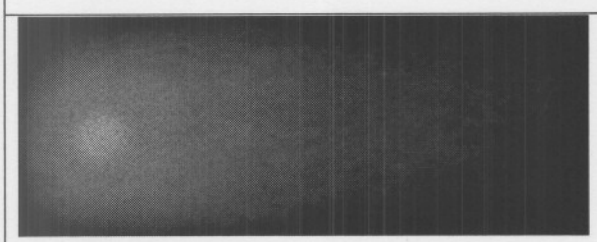
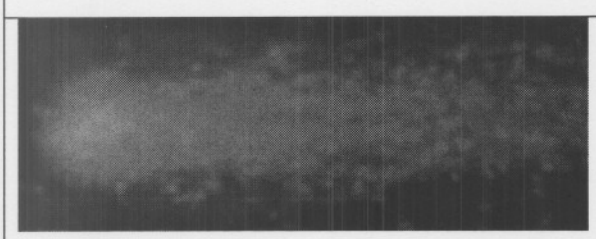


Figure 3.1: The CASP program used to determine the DNA% present in the tail. This data is exported to Excel.

Table 3.1: Comet images showing the different classes of DNA damage.

Comet image	Tail DNA% (CASP)	Class of DNA damage
	0-6 %	Class 0 (no damage)
	6.1-17%	Class 1
	17.1-35%	Class 2
	35.1-60%	Class 3
	60.1-100%	Class 4 (most damage)

(Giovannelli *et al*, 2002).

3.4 Oxygen Radical Absorbance Capacity (ORAC)

3.4.1 Principle of the method

The ORAC procedure measures the ability of the sample being tested to protect against attack by free radicals. This method utilizes a free radical source that produces the peroxyl radical, the most common free radical in the body. In the ORAC assay, B-phycoerythrin (B-PE) is used as an indicator protein or target of free radical attack, 2,2'-azobis-2-amidinopropane-dihydrochloride (AAPH) is the peroxyl radical generator, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, a water-soluble vitamin E analogue) is the control standard. Results are expressed as ORAC units, where 1 ORAC unit equals the net protection produced by 1 µM Trolox (Anon 6, 2005).

3.4.2 Materials

All reagents were from the highest grade available and were purchased from Sigma-Aldrich, South Africa, unless otherwise stated. See Appendix A.

3.4.3 Method

Sample preparation

Blood was collected in a blood coagulating tube. The blood was centrifuged for 10 min at 4 00 g and the top serum layer collected. To measure the ORAC in the non-protein fraction of serum, the serum and plasma were diluted with 0,5M PCA (1:1 v/v). Treating serum with PCA preserves ascorbate. (Blood plasma was stored at -70°C until analysis was done).

Reaction

The frozen plasma were thawed and centrifuged for 10 min. Supernatant was recovered for ORAC assay. Standards were prepared as described in Appendix A. The samples and the buffer were added together to wells in duplicate to a total of 20 µl in the plate. To this was added 160 µl fluorescein solution in each well. The plate was pre-incubated at 37°C for 15 min. 'The ORAC-FI' program on BioTek plate reader was used to record fluorescence (excitation 485 nm, emission 530 nm, static mode) every 5 min for 2 hours.

The reaction was started by adding 20 µl AAPH (240 mM). The reader was initialised. The readings should decline until the final reading is ± 5% of the initial reading.

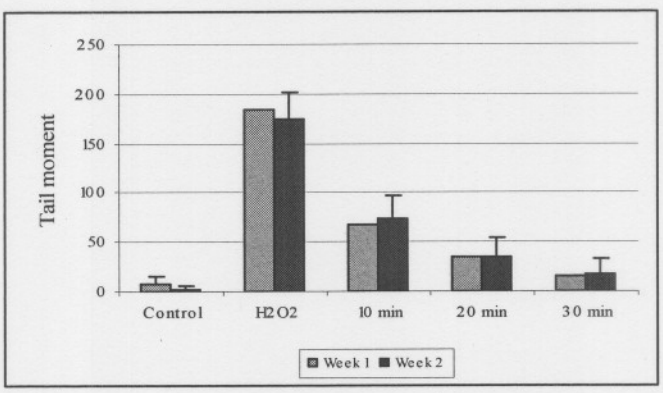
CHAPTER 4

RESULTS AND DISCUSSION

4.1 The method

The comet assay is a quick and sensitive method for measuring DNA strand breaks, as described in Chapter 3. However, to perform a study like this in which possible changes in DNA integrity is studied over time, questions like reliability and reproducibility of a particular method need to be addressed. For this reason a study has been performed using material from two male individuals, in which DNA damage and repair were measured over a period of two weeks.

(a)



(b)

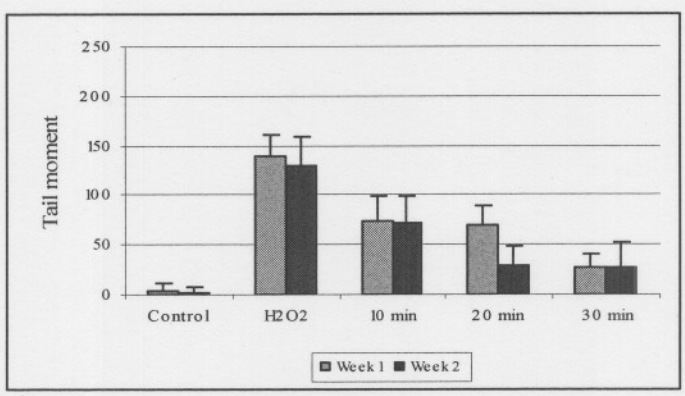


Figure 4.1: Reproducibility of the comet assay. DNA damage and repair were measured a week apart in lymphocytes freshly isolated from two male individuals A and B, presented in (a) and (b) respectively.

Comparing the results given in fig. 4.1, it is noticeable that the control or baseline value of both A and B show very little DNA damage for both the measurements a week apart and after an exposure of the lymphocytes to 60 µl H_2O_2, the amount of DNA damage increased drastically for both individuals. No sinificint was observed between these measurements. Furthermore, after a time period of 30 minutes allowed for DNA repair to take place, no real difference in the results was apparent. This proves the method's reproducibility and that the comet assay can be used with confidence for this study and that any observed differences in these parameters in female subjects can be ascribed to be the result of physiological effectors and not experimental variation.

4.2 DNA damage during the rat estrous cycle

The estrous cycle of the Sprague-Dawley rat lasts for four days and consists of four phases (Table 2.1). To determine the different phases of each rat, a vaginal smear was carried out (Chapter 3). In every experiment performed the occurrence of the different phases was irregular. This complicated the interpretation of the results to a certain extent. In total fifty five rats were investigated. In some cases, some of the results were pooled for statistical reasons. In this experiment, baseline DNA damage in individual white blood cells in whole blood was determined.

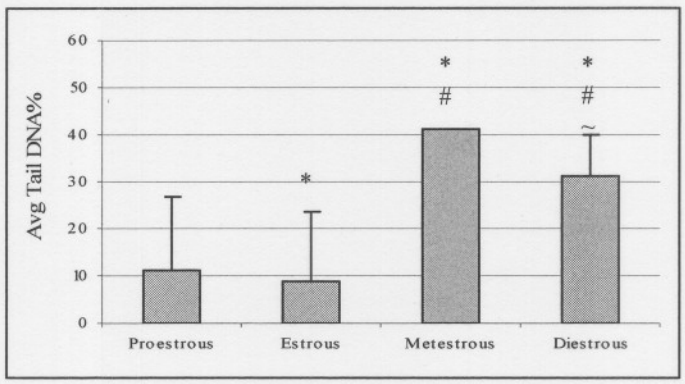


Figure 4.2: Baseline DNA damage in white blood cells measured in whole blood of the control group. Proestrous consists of nine rats, estrous with seven, metestrous one, and diestrous with two. Standard deviation is also shown where it could be calculated. * ($p < 0,05$) statistical significant difference between proestrous and diestrous, metestrous and estrous, # ($p < 0,05$) statistical significant difference between estrous and metestrous and diestrous, ~ ($p < 0,05$) statistical significant difference between metestrous and diestrous.

In the metestrous phase more DNA damage was observed than in the other phases with the least amount of DNA damage in the estrous phase (fig. 4.2). The proestrous differs statistically significant from estrous, metestrous and diestrous. The estrous phase differs statistically significant from metestrous and diestrous and the diestrous phase differ statistically significant from metestrous. The data used in fig. 4.2 consists of two separate occasions when the rats were sacrificed. The control group consists of 19 rats, with proestrous consisting of nine rats, estrous with seven, metestrous one, and diestrous with two. To further analyze the extent of DNA damage in the different phases; the comets were grouped into different classes as described in Chapter 3. These results are presented in fig. 4.3.

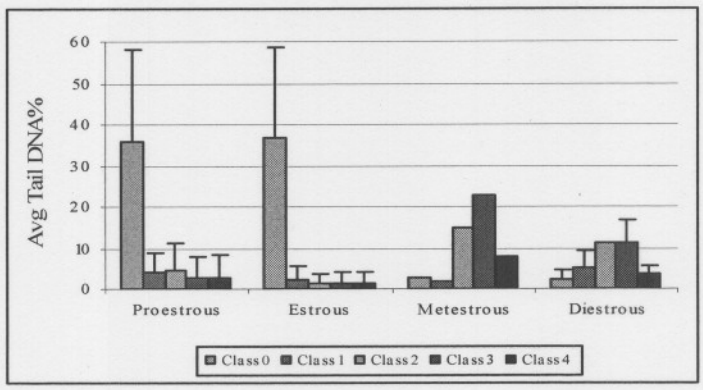


Figure 4.3: Comet distribution of baseline DNA damage in white blood cells measured in whole blood obtained from female rats during the estrous cycle. Proestrous consists of nine rats, estrous with seven, metestrous one, and diestrous two. Standard deviation is also shown where it could be calculated.

In the proestrous and estrous phase, class 0 is prominent with little or no real difference in the number of comets in the remaining classes. DNA damage in the metestrous phase is presented mainly by class 2 and 3 comets as well as class 4 comets, as it is shown in fig. 4.2. Since relatively small differences in DNA damage were observed in the comets from whole blood obtained in the various phases of the estrous cycle, the question was asked whether the same picture is true for isolated lymphocytes. We therefore isolated lymphocytes from heparinized blood and measured DNA damage in these cells. The results are given in fig. 4.4. DNA damage is more prominent in the metestrous phase (~40% tail DNA) relative to the other phases. The amount of DNA damage observed in

the proestrous phase differs statistically significant ($p < 0,05$) from that in the estrous and metestrous phase and the amount of DNA damage in the metestrous phase differs significantly from the estrous phase.

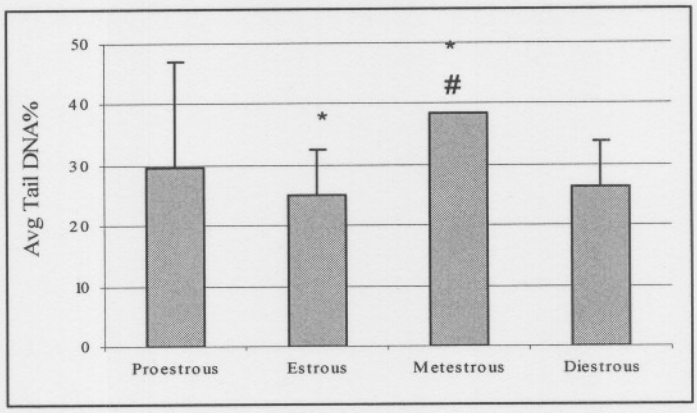


Figure 4.4: Baseline DNA damage in isolated rat lymphocytes of the control group. Proestrous consists of four rats, estrous with three, metestrous one, and diestrous with three. Standard deviation is also shown where it could be calculated. * ($p < 0,05$) statistical significant difference between proestrous and diestrous, metestrous and estrous, # ($p < 0,05$) statistical significant difference between estrous and metestrous and diestrous, ~ ($p < 0,05$) statistical significant difference between metestrous and diestrous.

Fig. 4.5 is given to further illustrate the various degree of DNA damage in the different phases of the estrous cycle. It is evident from this result that the metestrous phase consists mainly out of class 4 comets. No difference in classes is observed in the proestrous phase. The estrous phase, however, consists mainly of class 0-2 comets with little class 4 comets. Class 3 and 4 comets are present in the metestrous phase. Diestrous phase consists mainly of class 0-3 comets with little class 4 comets. These results indicate that DNA damage varies across the different phases of the estrous cycle. The white blood cells measured in whole blood (fig. 4.2, 4.3) and the isolated lymphocytes both point toward the metestrous phase consisting with the most DNA damage followed by the diestrous phase, and with the least DNA damage present in the estrous phase.

From these results it is evident that to a large extent, the same pattern in DNA damage in the various phases is observed in the white blood cells in whole blood (fig. 4.2 & 4.3) as in the isolated lymphocytes (fig. 4.4 & 4.5).

The reactions of steroid hormone biosynthesis are accompanied by the formation of oxygen radicals. Steroidogenic tissues contain high levels of antioxidants such as ascorbate, α- tocopherol, β-carotene and the enzymes superoxide desmutase (SOD), catalase, glutathione peroxidase, glutathione-S-transferase and glutathione reductase (Rapoport *et al*, 1998). One function of these antioxidants may be protection against oxygen radicals produced during steroidogenesis.

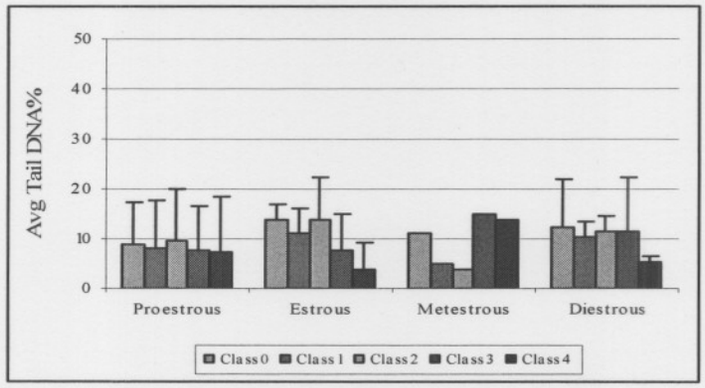


Figure 4.5: Comet distribution of baseline DNA damage in isolated lymphocytes from female rats during the estrous cycle.

However, oxygen radicals may also be functional in leading to luteolysis and apoptosis in the corpus luteum (CL) during each reproductive cycle (Rapoport *et al*, 1998). Small deviations from the physiological activity of antioxidant enzymes may have a dramatic effect on the resistance of cells to oxidant-induced damage to the genome and cell killing (Matés *et al*, 1999). The ORAC assay (Chapter 3) was performed using a different group of normal rats to determine the antioxidant capacity of each of the different phases of the estrous cycle. In this experiment, 25 rats were killed and each phase was determined. Of the 25 rats, only one rat was in the diestrous phase. The results are given in fig. 4.6.

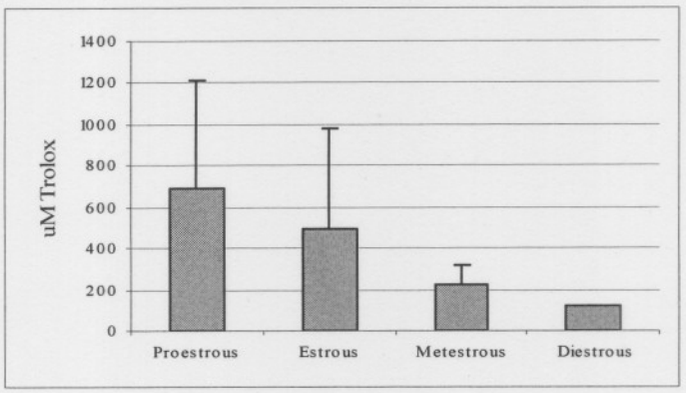


Figure 4.6: Antioxidant capacity during the estrous cycle of the rat. Proestrous phase consists of 11 rats, estrous of eight, metestrous of five and diestrous of one rat. The higher µl Trolox, the better the antioxidant capacity. Standard deviation is also shown.

The antioxidant capacity present in the proestrous phase is higher in relation to the other phases. The diestrous phase showed the least antioxidant capacity when compared to the other phases. There is a continuously decline in antioxidant capacity from the proestrous phase, through to the diestrous phase. How does this figure compare to the hormonal variations (fig. 2.1) that takes place during the estrous cycle? During the proestrous phase (beginning of the cycle), estrogen levels are high. It starts to fall drastically and reaches a minimum at metestrous, where it slowly begins to increase towards the next proestrous phase, when the next cycle begins. Estrogen metabolism, as previously described in Chapter 2, and its derivatives may lead to possible DNA damage. This may explain the increased DNA damage observed in the proestrous phase (fig 4.4), where estrogen levels are elevated. With this increased DNA damage the antioxidant capacity for the proestrous phase is also elevated. It could be a possible defense mechansism against the prominent estrogen level (fig. 2.1) present at the proestrous phase.

Summary

When it is necessary to determine the phase of the rat's estrous cycle for a study like this, there are certain difficulties hampering its execution. The collecting and staining of the vaginal epithelium cells is a technique requiring experience. Although the method was performed by a trained technician, it still remained a difficult (tricky) technique. Because the duration of the estrous cycle in the rat is only 4-5 days (Table 2.1), each phase is

relatively short. This made it difficult – especially in this study – to obtain rats in some of the phases, the metestrous phase in particular. A possible explanation for this 'phenomenon' maybe that the rats live together, which can lead to synchronization of the cycles of the rats.

Measuring the baseline DNA damage in both white blood cells in whole blood and in isolated lymphocytes gave similar results over the proestrous, estrous and metestrous phases. The serum antioxidant capacity showed a decline in capacity from proestrous to diestrous. Are these differences in DNA damage that we have observed big enough to be taken into account when using female rats in studies where oxidative damage and some form of intervention are studied, or are these differences within the limits of normal physiological cyclic variation?

Although we observed small but significant differences in DNA damage between the various phases of the rat estrous cycle, we could not make a definitive conclusion from the studies in rats with reference to the real significance of these variations. We therefore decided to shift the focus of the study to human subjects.

4.3 Female individuals

4.3.1 Average baseline DNA damage

Whole blood can easily be obtained by peripheral venous puncture and can be directly used for comet assay (Chuang, Hu, 2004). DNA damage was determined in the white blood cells of whole blood of eight female individuals, once a week. This was done for a period of four weeks. The results are given in fig. 4.7.

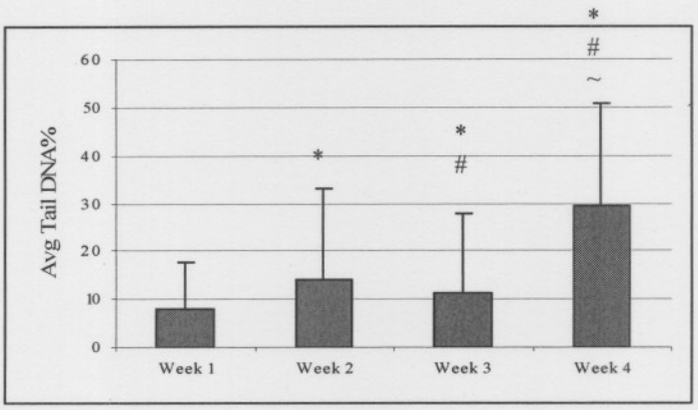


Figure 4.7: Average baseline DNA damage in whole blood from eight female individuals during the menstrual cycle. Standard deviation is also shown. * ($p < 0,05$) statistical significant difference between week one and week two, three and four, # ($p < 0,05$) statistical significant difference between week two and week three and four, ~ ($p < 0,05$) statistical significant difference between week three and four.

Week one differs statistically significant from week two, three and four. Week two differs statistically significant from week three and four and week three differs statistically significant from week four. During week four of the menstrual cycle, the follicle releases the egg; it closes and forms the corpus luteum (CL). The CL secretes progesterone. If pregnancy does not occur, the CL can lasts up to 14 days before breakdown begins. During this period the estrogen and progesterone levels are low (fig. 2.1). Because of a lack of hormones, the blood vessels go into spasm and cut off blood supply to the top layers of the endometrium. Little or no oxygen or nutrients are available, the endometrial cells die, this causes the tissue to break down, bleeding occurs and a new cycle commences (Anon 7, 2002; Guyton & Hall, 1996). This may explain the extended baseline DNA damage that was observed during week four, because of the physical changes and breakdown of tissue that takes place.

In a study done by Chuang and Hu (2004), they showed that the whole blood can be used for *in vivo* genotoxic studies, but when it comes to *in vitro* studies, the whole-blood technique proved to have one disadvantage: interferences from red blood cell (RBC) components. They also reported that when whole blood was 'replaced' with white blood cells (WBC) and isolated lymphocytes, and incubated with H_2O_2, that whole blood was

completely ineffective. To further analyze the extent of DNA damage in the different phases, the comets were grouped into different classes as described in Chapter 3. These results are presented in fig. 4.8.

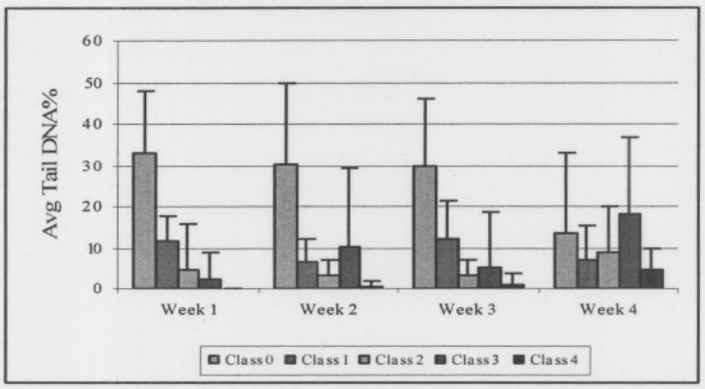


Figure 4.8: Comet distribution of baseline DNA damage of whole blood from four female individuals during the menstrual cycle.

Week one and two show a similar pattern to that of week three, with class 0 comets dominating. In week two, class 3 comets are prominent, with somewhat less class 0 comets in week 4. With this decline in class 0 comets, class 2-4 comets are more elevated. The different classes of comets reflects the DNA damage observed in fig. 4.7, example in week 2, class 3 comets dominate and this is visible in the elevated DNA damage in fig. 4.7.

When using whole blood in the comet assay, we have observed that the most DNA damage is present in week four of the menstrual cycle. Since relatively small differences in DNA damage were observed in the comets from whole blood obtained in the various weeks of the menstrual cycle, the question was asked whether the same picture is true for isolated lymphocytes. Lymphocytes were isolated from blood obtained from four healthy female individuals and DNA damage was determined. The results are given in fig. 4.9.

Baseline DNA damage in the isolated lymphocytes (fig. 4.9) does not compare to the white blood cells measured in the whole blood (fig. 4.7). Week one differs statistically significant from week two and four, week three differs statistically significant from week

two, and week four differs statistically significant from week three. Week two shows a twofold increase in DNA damage to week one (~ 40%). What is the reason for this shift in baseline DNA damage from week four in the white blood cells to week two in the isolated lymphocytes? It is also important to remember that this study was carried out in the isolated lymphocytes and that the DNA damage observed is not in the target organ – the uterus, but it still reflects to some degree the processes that take place in the uterus. During week two (day 6-14 in the menstrual cycle) the follicles grow in the ovary and forms the egg. The lining of the uterus is also repaired during this period. This is stimulated by estrogen (Guyton & Hall, 1996).

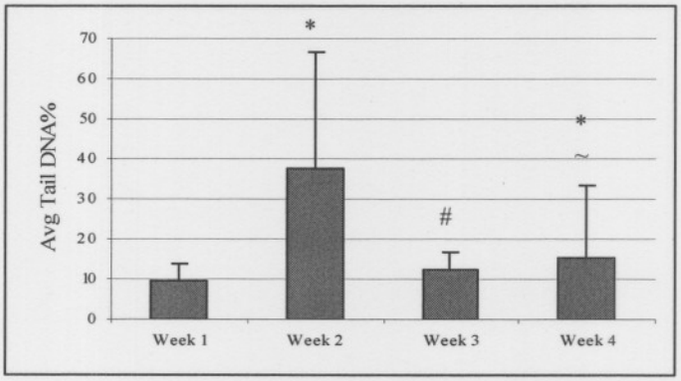


Figure 4.9: Average baseline DNA damage in isolated lymphocytes of four female individuals during the menstrual cycle. Standard deviation is also shown. * ($p < 0,05$) statistical significant difference between week one and week two, three and four, # ($p < 0,05$) statistical significant difference between week two and week three and four, ~ ($p < 0,05$) statistical significant difference between week three and four.

To further analyze the extent of DNA damage in the different phases; the comets were grouped into different classes as described in Chapter 3. These results are presented in fig. 4.9.

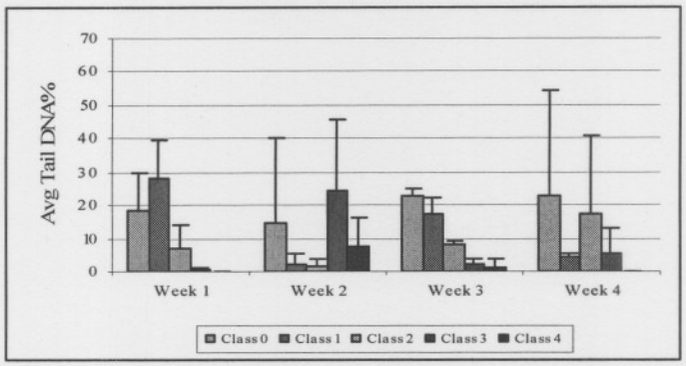


Figure 4.10: Comet distribution of baseline DNA damage in lymphocytes from four female individuals during the menstrual cycle.

In week one, class 1 comets dominate followed by class 0 comets. In week two there is a shift in classes mainly to class 3 and 4 comets. This is also reflected in fig 4.9 showing the average DNA damage. Week three contains class 0 and 1 comets with week four consisting out of class 0 and 2 with no class 4 comets. The individual baseline DNA damage of the three females is included (fig. 4.11-13).

4.3.2. Individual baseline DNA damage

It was decided to look at the baseline DNA damage in isolated lymphocytes of the three individuals separately, to observe possible inter-individual variation. The results are given in fig. 4.11-4.13.

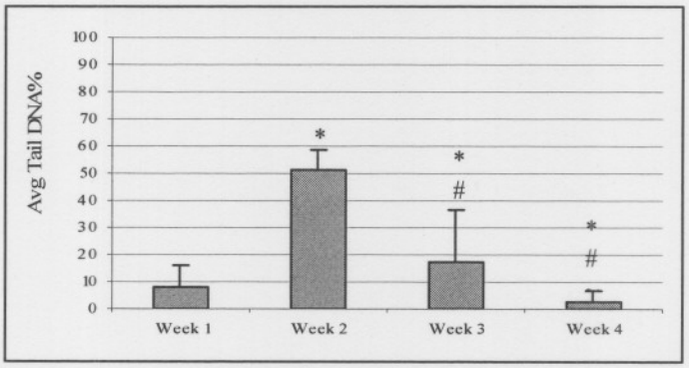


Figure 4.11: Baseline DNA damage in isolated lymphocytes of Person A over a period of four weeks. * ($p < 0,05$) statistical significant difference between week one and week two, three and four, # ($p < 0,05$) statistical significant difference between week two and week three and four, ~ ($p < 0,05$) statistical significant difference between week three and four.

Week one displays little DNA damage (~10% tail DNA) with more than a twofold increase towards week two. Week one differs statistically significant from week two, three and four. Week two differs statistically significant from week three and four and week three differs statistically significant from week four. Week two dominate with the most DNA damage (~50 % tail DNA).

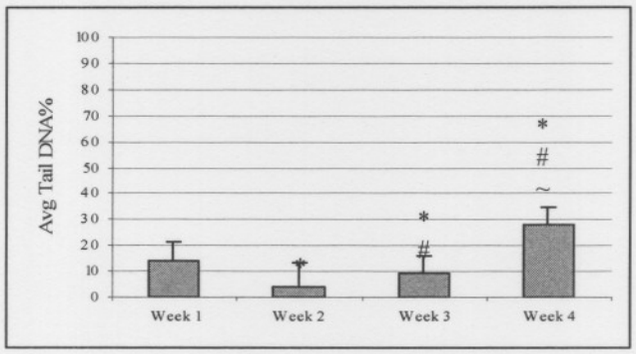


Figure 4.12: Baseline DNA damage in isolated lymphocytes of Person B over a period of four weeks. * ($p < 0,05$) statistical significant difference between week one and week two, three and four, # ($p < 0,05$) statistical significant difference between week two and week three and four, ~ ($p < 0,05$) statistical significant difference between week three and four.

Person B shows a markedly different pattern to that of Person A. From week two onwards the damage slightly increases with week four displaying the most DNA damage. Week one differs statistically from week two, three and four. Week two differs statistically significant from week three and four and week three differs statistically significant from week four. Week four dominate with the most DNA damage (~30 % tail DNA).

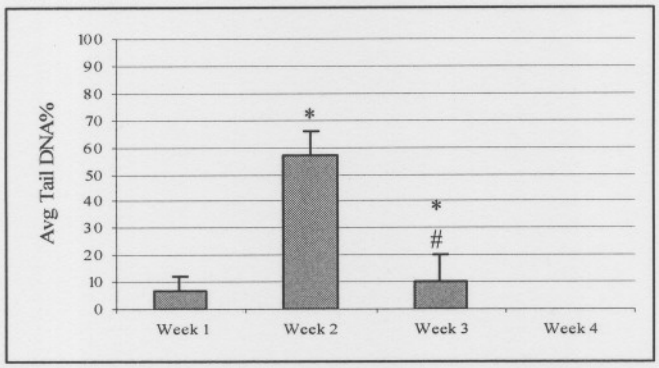


Figure 4.13: Baseline DNA damage in isolated lymphocytes of Person C over a period of four weeks. * ($p < 0,05$) statistical significant difference between week one and week two, three and four, # ($p < 0,05$) statistical significant difference between week two and week three and four, ~ ($p < 0,05$) statistical significant difference between week three and four.

Person C baseline DNA damage pattern is similar to that of Person A, with week two consisting of the most DNA damage. Week one differs statistically significant from week two and three, and week two differ statistically significant from week three. No week four data was available, as the females' menstrual cycle was irregular and for a period of two weeks the individual was in week one of the menstrual cycle.

In a study done by Bajpayee (2005), they measured the baseline DNA damage in lymphocytes from an Indian female population. Their results demonstrate that the menstrual cycle does not influence the basal level of DNA damage in peripheral blood lymphocytes in healthy female subjects. This is not the case in the three female individuals as can be seen in fig 4.11-4.13. Baseline DNA damage is not the most appropriate parameter to use when DNA damage is measured. It is vital to study the repair capacity as well in each of the different weeks of the menstrual cycle to understand the underlying mechanisms involved.

Summary

According to our experience it is not sufficient to measure only the baseline DNA damage to get an idea of the effect of any effector on DNA damage and repair. We have observed in the case of a cancer patient that baseline DNA damage did not differ from controls measured at the same time but that differences in DNA repair came to the fore

only after invoking DNA damage with peroxide (Huysamen, 2005). No difference in DNA integrity would have been observed if we looked only at the baseline DNA damage.

During the four week period the average baseline DNA damage was the highest during week two of the menstrual cycle (fig. 4.9). However, different results were obtained for female individual B (fig. 4.12). This is an important observation as this illustrate that the menstrual cycle shows a certain pattern of DNA damage over the four week period, but individual variation is apparent as can be seen in fig. 4.12. From the results presented in fig. 4.11- 4.13 it is evident that inter individual variation in the degree of DNA damage exist during the menstrual cycle of which one must take cognizance of when doing studies of this kind where female subjects are involved.

Because of this inter individual variation in the degree of DNA damage was during the menstrual cycle and since a definite pattern in DNA damage was observed during the cycle, it is important to consider each individual's own pattern when any bio-assay is performed. Also, the menstrual cycle is a cyclic process and it, as it was described in the literature review, influences a variety of other physiological processes.

To get a more complete picture of the effect of the human hormonal cycle on the integrity of DNA, we deemed it necessary to also study the repair capacity during the different weeks of the menstrual cycle of the same three female individuals.

Lymphocytes were isolated from blood obtained from the three female individuals and after an exposure of the lymphocytes to 60 µl H_2O_2, a time period of 30 minutes was allowed for DNA repair to take place to determine the DNA repair capacity of the cells. The data of female individual A over the four week period is given as an illustration (fig. 4.14-4.21), followed by fig. 4.22 where the average tail DNA% of the three individuals over the four week period is given.

4.4 DNA damage and repair

Week 1

The DNA damage of the control is relative little (~ 9% tail DNA). The H_2O_2 was added and the DNA damage increased almost twofold. A time period of 30 minutes was allowed for DNA repair to take place, and after 20 min, visible repair was apparent.

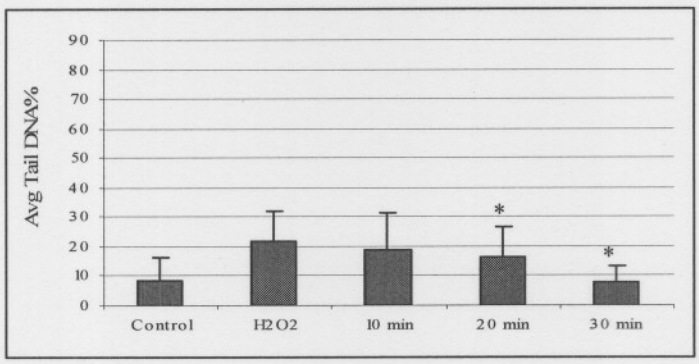


Figure 4.14: Average Tail DNA% in lymphocytes of Person A - week one. * ($p < 0,05$) of the repair differs statistically significant from the H_2O_2 induced damage.

The repair at 30 min. was approximately the same as that of the control. The repair after 20 and 30 minutes differs statistically significant from H_2O_2 induced damage. Fig. 4.15 is also included to illustrate the extent of DNA damage in week one. The comets were grouped into different classes as described in Chapter 3.

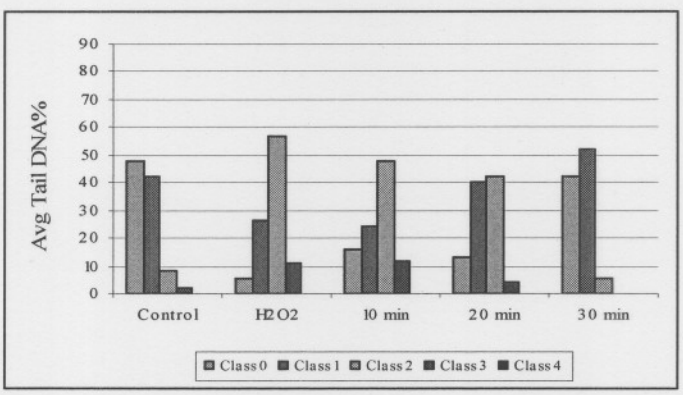


Figure 4.15: Comet distribution in lymphocytes of Person A (week one), showing H_2O_2 induced damaged and repair. Class 0-4 the degree of DNA with class 0 no damage to class 4 most damaged DNA.

DNA damage is represented by class 0 and 1 comets, during the control. After the H_2O_2 was added the comets shifted from 0 and 1 to class 2 and class 3. No class 4 comets were present. When time was allowed for repair, class 0 comets were elevated with a slight decrease in class 2 comets. Class 1 comets increased from 10 minutes to 30 minutes repair. Repair is apparent in week one of the menstrual cycle.

Week 2

Baseline DNA damage is relatively large in this case and exposure of the cells to peroxide did not cause an increase in DNA damage (fig. 4.16). During the repair time of 10, 20 and 30 minutes, an initial phase of quick repair is seen after which the rate of repair leveled off. The relative high degree of DNA damage in this case is also evident in the results given in fig. 4.11.

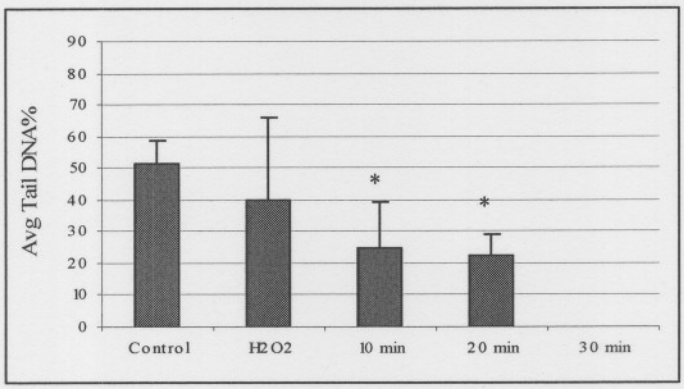


Figure 4.16: Average Tail DNA% in lymphocytes of Person A - week two. * ($p < 0,05$) of the repair differs statistically significant from the H_2O_2 induced damage.

The repair after 10 and 20 minutes differs statistically significant from the H_2O_2 induced damage. It cannot be argued that this result may be due to a possible experimental outlier because person C showed the same elevated baseline DNA damage in fig. 4.13. The comets were grouped into the different classes and the results are given in fig 4.17.

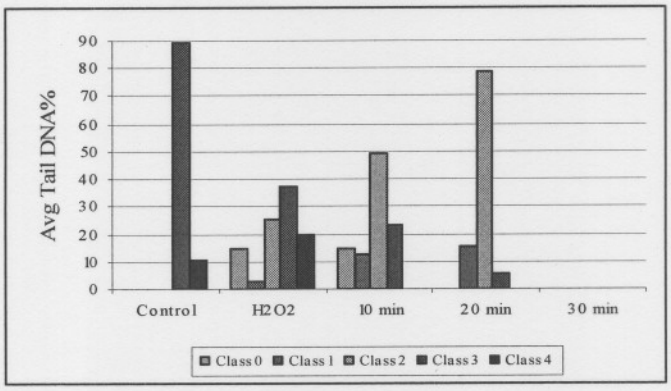


Figure 4.17: Comet distribution in lymphocytes of Person A (week two), showing H_2O_2 induced damaged and repair. Class 0-4 the degree of DNA with class 0 no damage to class 4 most damaged DNA.

This figure confirms the results given in fig. 4.16 and shows that repair of the DNA after exposure to peroxide was relatively good after 10 minutes but that after 20 minutes the number of comets in class 2 and 3 increased again. This may explain the relatively high level of DNA damage in the control cells in that the cells' inherent capability to repair DNA is compromised during this stage of the cycle.

Week 3

During this phase of the cycle a low level of DNA damage was observed in the control cells and good initial DNA repair after exposure to peroxide. This fast initial phase of repair was followed by a plateau in the repair capacity of the cells. The repair period of both 10, 20 and 30 minutes differs statistically significant from the H_2O_2 induced damage. DNA damage in the control cells is mainly represented by class 0 comets (fig. 4.19).

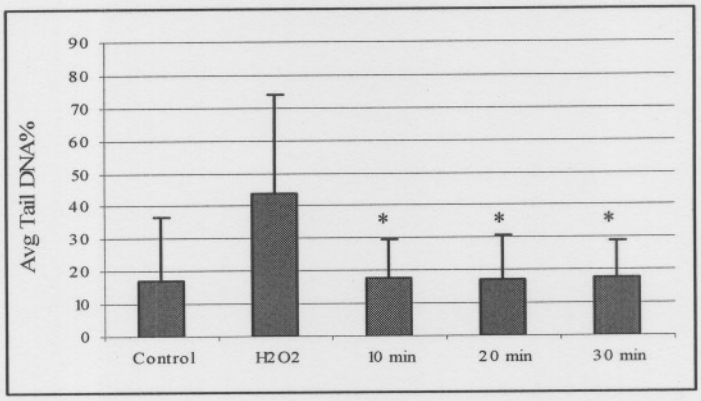


Figure 4.18: Average Tail DNA% in lymphocytes of Person A - week three. * ($p < 0,05$) of the repair differs statistically significant from the H_2O_2 induced damage.

When damage is induced, the number of comets in class 4 is elevated with the presence of heavily damaged DNA (fig. 4.19). The CASP program was unable to detect the tail of these comets since most of the DNA was in the tail. These comets were scored manually. Class 0 and class 1 comets increased during the initial 20 minutes of the repair period, but class 1 declined towards 30 minutes repair time. In general, the repair capacity of these cells seems to be insufficient to repair the peroxide damaged DNA properly.

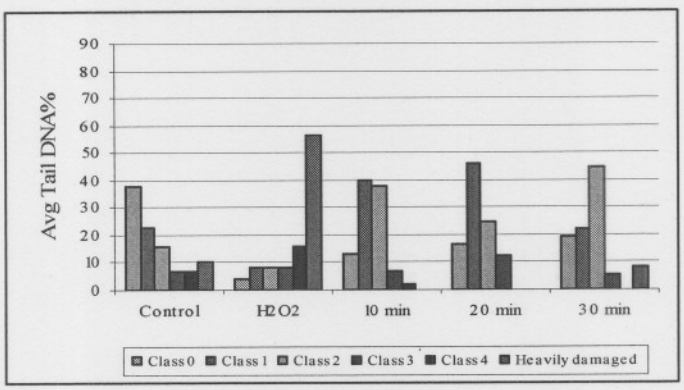


Figure 4.19: Comet distribution in lymphocytes of Person A (week three), showing H_2O_2 induced damage and repair. Class 0-4 the degree of DNA with class 0 no damage to class 4 most damaged DNA.

DNA damage during the control (fig. 4.19) is mainly represented by class 0 comets. When damage is induced, class 4 is elevated with incidence of heavily damaged DNA.

The CASP program was unable to detect the tail of the DNA, therefore this damage was classified as heavily damaged DNA. Class 0 increases during the repair period, with class 1 decreasing towards 30 minutes.

Week 4

Although the comet assay was shown to be a reproducible method (fig 4.1), there might still be unexplainable errors that can occur during the experimental procedure. This may be the case in fig. 4.20.

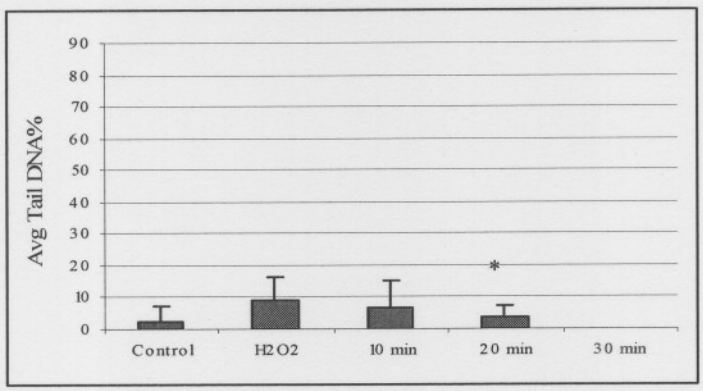


Figure 4.20: Average Tail DNA% in lymphocytes of Person A - week four. * ($p < 0,05$) of the repair differs statistically significant from the H_2O_2 induced damage.

Even though the comet length (DNA damage) was not as high as in the previous cases, the tendency is still visible, with 20 minutes repair that differs statistically significant from the H_2O_2 induced damage.

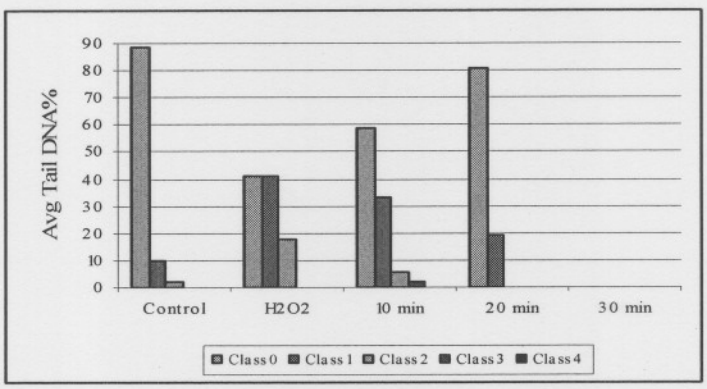


Figure 4.21: Comet distribution in lymphocytes of Person A (week four), showing H_2O_2 induced damaged and repair. Class 0 to 4 representing the level of DNA with class 0 no damage to class 4 most damaged DNA.

Class 0 comets are dominant in the control, with a sharp decrease after H_2O_2 exposure, and then increase reaching a maximum after 20 minutes repair. This is also visible in fig. 4.20. Fig. 4.22 shows the average DNA damage and repair of the three female individuals during the four week period. During week one, visible repair is apparent after the H_2O_2 induced damage. Little standard deviation is observed between the three female individuals.

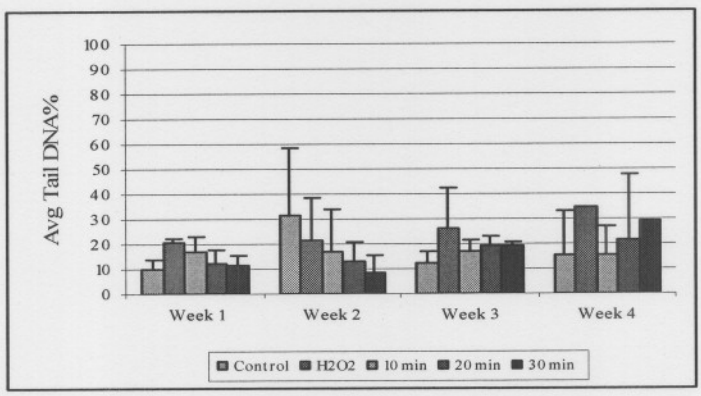


Figure 4.22: Average Tail DNA% of DNA damage and repair of three female individuals during the menstrual cycle. Standard deviation is also shown.

DNA damage of the control is relative high in week two – note the standard deviation – followed by good repair. The standard deviation is a significant marker of individual variation between the three females. The DNA damage observed during week three is low for the control and increases after the H_2O_2. A slight decrease in damage is visible, but no repair is apparent. This can also be seen in fig. 4.23. Week four displays the same pattern of DNA damage and repair as week three, though after 30 minutes of repair, the damage was more elevated than that of week three. Fig. 4.23 is included to demonstrate the repair capacity of the three female individuals during the four weeks.

4.5 DNA repair capacity and antioxidant capacity

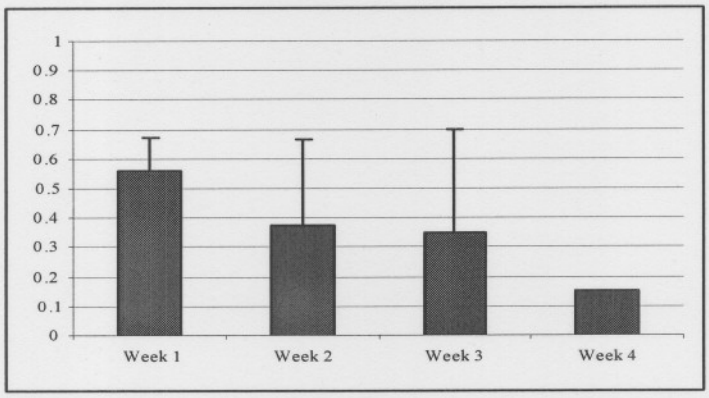


Figure 4.23: Average calculated DNA repair capacity (Rc) measured in the lymphocytes from three female individuals during the menstrual cycle. Standard deviation is shown.

The Rc was calculated using the formula (Huysamen, 2005):

$$Rc = 1 - \left[\frac{\text{Average tail DNA\% (30 min repair)}}{\text{Average tail DNA\% } (H_2O_2 \text{ exposed})} \right]$$

Week one displays the best Rc during the menstrual cycle. Week two and three is the same with week four exhibiting relatively very poor Rc. The low Rc of week four correlate with the extent of DNA damage observed in week four when whole blood was used in the assay (fig. 4.7). Although the Rc during week one displays a 'high' value, in comparison to week four, Rc in week one is only ~ 0,55, which is average on the scale of 0 to 1. On the face of it a decreasing trend is seen in the Rc as the cycle progresses, however, overall the Rc is not that good and the SD is of such an extent that no definite conclusion could be drawn from this result.

It was decided to determine the antioxidant capacity during the four week period of four female individuals. The results are given in fig. 4.24.

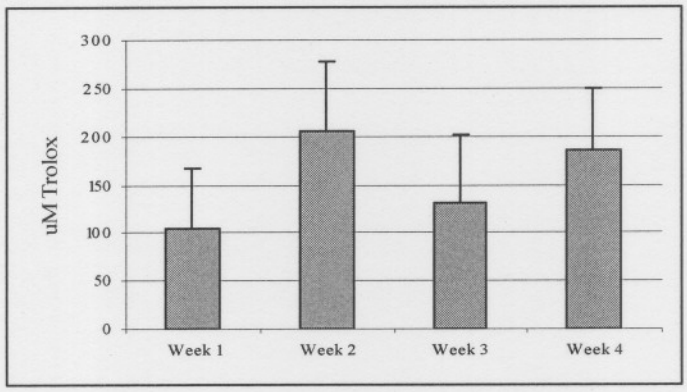


Figure 4.24: Average antioxidant capacity in the serum of four female individuals during the menstrual cycle. Standard deviation is shown.

Week one and three is relative similar with week two and four exhibiting more antioxidant capacity. During the four weeks, the antioxidant capacity differs in pattern to that of the repair capacity (fig. 4.23). The reason might be because the repair capacity is the capability of the lymphocytes to repair themselves. The antioxidant capacity is a series of enzymes protecting the cell from any free radicals formed during the hormonal cycle.

Summary

The observed pattern in baseline DNA damage in the white blood cells (measured in whole blood) did not correspond to the observed pattern baseline DNA damage in isolated lymphocytes. In white blood cells week four displayed the most baseline DNA damage (fig. 4.7), in contrast with week two in the isolated lymphocytes (fig. 4.9). Also, when we looked at the baseline DNA damage in isolated lymphocytes in the three individuals separately, we found significant inter individual differences. This supports our proposal that the menstrual cycle is a significant factor to consider in studies where female subjects are involved. However, the question may be asked whether the differences or variations in DNA damage that we have observed are big or significant enough to be taken into account when using female individuals in studies where oxidative damage and some form of intervention are studied, or are these differences within the limits of normal physiological cyclical variation? The availability of the baseline DNA

damage levels and the repair capacity of a large number of control individuals of both sexes will resolve this problem. We also believe that the level of baseline DNA damage alone is not a true representative parameter of events taking place during the hormonal cycle (Huysamen, 2005).

Although inter-individual variation in baseline DNA damage was observed at the weekly measurements during the hormonal cycle, significant DNA repair, albeit to different extents, was also apparent. It is also true that small numbers tend to amplify variation in a study like this which compels one to interpret the results with caution. However, we are of the opinion that the variation in DNA damage and repair that we have observed in both the rat and human subjects, is of such an extent (although within physiological limits) that it needs to be reckoned with in studies where the effects oxidative stress are studied in females. Especially so, since we have not seen even this level of variation in the male subjects (fig. 4.1).

Main observation:

- The level of observed DNA damage peaked in different parts of the hormonal cycle for different individuals.
- No correspondence was observed between the levels of DNA damage on the one hand and on the other hand the repair capacity and serum antioxidant status, at the various points measured during the cycle.

CHAPTER 5
SUMMARY

The aim of this study was to determine the pattern of DNA damage and repair during the mammalian female hormonal cycle. This was done to determine whether the hormonal cycle must be taken into account when DNA damage and repair studies are done in female subjects. Some observations in the literature stressed the importance of the menstrual cycle and that effect of associated ovarian hormonal levels must be considered in for instance breast cancer related studies (Dumitrescu & Cotarla, 2005; Ratajczak *et al*, 1988).

The comet assay, which proved to be a simple and sensitive technique, was used to determine DNA damage and repair in individual white blood cells in whole blood and in isolated lymphocytes. Because certain factors including gender, age and diet may influence the comet assay responses in lymphocytes when monitoring human genotoxicity (Møller *et al*, 2000), we posed the question whether the menstrual cycle would influence the comet assay responses in lymphocytes to any significant extent.

Initial experiments were done with female rats because these animals are frequently used in genotoxicity experiments. Research animals were obtained from the animal care facility, North-West University and the phase of the hormonal cycle was determined for each individual. The rats were sacrificed and DNA damage in the white blood cells of whole blood and in isolated lymphocytes was determined using the comet assay. The collecting and staining of the vaginal epithelium cells is a technique requiring experience, and although the method was performed by a trained technician, it still remained a difficult (tricky) technique. Because the duration of the estrous cycle in the rat is only 4-5 days (table 2.1), each phase is relatively short which made it difficult to obtain sufficient numbers of rats in some of the phases, the metestrous phase in particular. Differences in baseline DNA damage in white blood cells as measured in whole blood,

and in isolated lymphocytes were observed for the different phases of the estrous cycle. The metestrous phase showed the most DNA damage in both the isolated lymphocytes and the white blood cells and the least amount of DNA damage was observed during the estrous phase. Although the extent of DNA damage did vary across the estrous cycle, the question can be raised whether the differences in DNA damage that we have observed were significant enough to force one to take it into account when using female rats in studies where oxidative damage are studied, or are these differences within the limits of normal physiological variation? From the results that we have obtained in the rats, we were, however, unable to make definitive conclusions with reference to the real significance of these variations. The focus of the study was then shifted to human subjects. Using the comet assay DNA damage and repair were studied in samples taken once a week on Mondays for four consecutive weeks. Inter-individual variation in the degree of DNA damage was indeed observed during the menstrual cycle.

While this study was nearing its end a paper was published in which the baseline DNA damage in isolated lymphocytes from Indian females was measured (Bajpayee, *et al,* 2005). An illustration of the results obtained is given in fig 5.1. Based on these results, these authors concluded that the human menstrual cycle does not significantly influence the basal level of DNA damage in peripheral blood lymphocytes in healthy female subjects.

When comparing for instance female individual 8 with female individual 13 (tail DNA %, the same as what we have measured) individual variation is apparent with different peaks of DNA damage during different days of the cycle. This is illustrated with the following examples from the results in fig.5.1. Female individual 8 shows a DNA damage peak during cluster 5 (days 24-30 of the menstrual cycle), with the lowest DNA damage peak during cluster 3 (days 12-18). Female individual 13 shows a DNA damage peak during cluster 3, with the lowest DNA damage during cluster 5.

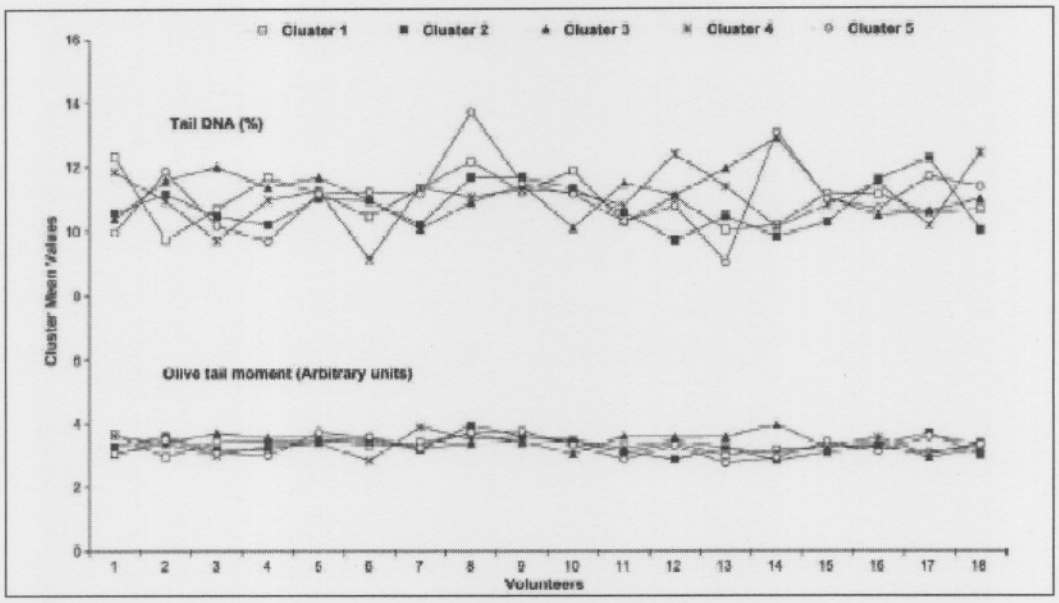


Figure 5.1: The influence of the menstrual cycle on comet assay parameters in lymphocytes from healthy Indian female volunteers. Each point is a mean of cluster (comparable physiological period) for each individual. Clusters 1-5 represent data of 6 days each (Bajpayee, *et al,* 2005).

Our results, although obtained from a much smaller sample, correspond to a large extend with their observations: our results also showed that DNA damage peak at different times for different individuals during the menstrual cycle. For example, person A (fig 4.11) showed the highest DNA damage present in week two with the least DNA damage in week four and Person B (fig 4.13) showed the highest DNA damage present in week four with the least DNA damage in week two. However, we are not convinced that baseline DNA damage is really a true reflection of the possible DNA damage occurring during the estrous and menstrual cycle. The patterns in DNA damage observed in the different individuals do not demonstrate the same cyclic pattern as the estrogen fluctuations during the hormonal cycle (fig 2.1). This suggests that the levels of DNA damage that we have observed are indeed within physiological limits, as Bajpayee (2005) have mentioned. However, we are of the opinion that, because of the degree of DNA damage and also due to the fact that the time during the cycle when DNA damage peaks varies between individuals, when measuring DNA damage in female individuals one must take the specific phase of the hormonal cycle into consideration. To stress this point: when the initial analysis in the case of individual A (fig. 4.11) is made in the fourth week of the

cycle and after some kind of intervention the following analysis is made in the second week, the effect of the intervention may be not significant enough to be detected. The reverse is obviously also true which can give a false positive result. This same argument can be applied to the results of Bajpayee (2005).

Although our results show that there was inter-individual variation in DNA damage it did not, however, correspond to the fluctuation in estrogen levels during the hormonal cycle (fig 2.1). Although inter-individual variation is present, it is still an open question whether these variations lie between normal ranges of DNA damage. Currently it is not possible to address this problem, as there is not yet an extensive comet assay baseline DNA damage (in both white blood cells and lymphocytes) database available. If it is true that the observed DNA damage occurs within the normal physiological range, we need to ask what the significance is of the extent of baseline DNA damage variation (white blood cells in whole blood and lymphocytes) we have observed in the menstrual cycle. Even though it is possible that the DNA damage may be within normal physiological limits, the DNA may be more exposed and fragile at these times. In a study done by Ratajczak *et al* (1988) in mice they concluded that the estrous stage at the time of surgical removal of breast tumors, markedly affected whether or not metastases ultimately appeared. Because the estrous cycle in mice is comparable to the human menstrual cycle it may be that the hormonal status of a woman at the time of tumor resection is an important determinant of whether or not that breast cancer ultimately metastasizes. This is important as this supports our findings that the hormonal cycle needs to be considered when biochemical studies involving females are conducted.

To continue this argument on the significance of the observed DNA damage during the hormonal cycle: one of the early biomarkers of exposure and effect in chemical toxicology is the occurrence of sister chromatid exchange (SCE) in isolated lymphocytes of exposed individuals. SCE may be the initiator of pathological situations (Dumitrescu & Cotarla, 2005). D'Souza *et al* (1988) determined SCE frequencies and compared ovulatory and estrogenic stages with the progestogenic stage during the menstrual cycle of women. Their results indicate that the hormonal variations during the menstrual cycle

play an important role in bringing about variation in the base line frequency of SCE in women. However, in a study done by Landi & Barale (1999) they observed variation of SCE and MN (micronuclei) in blood lymphocytes during the menstrual cycle, but they stated that is within the physiological normal range. The SCE frequencies showed two peaks, the highest peak at days 6-8 and the second peak just before menstruation. The minimum point was observed around the presumed ovulation time. The minimum point is in correspondence with the low estrogen levels just before menses, as can be seen in fig 2.1. They concluded that methodological, biological and lifestyle factors accounted for about 30% of the total variance observed. The mechanisms involved in the observed SCE and CA fluctuations remain unknown. But SCE proved to be a biomarker of early biological effect and increased frequencies of SCE has been observed due to biological rhythms in women (D'Souza *et al*, 1988; Watson & Mutti, 2004). These observations may implicate a period of genetic vulnerability during the cycle.

The average repair capacity for the three female individuals was calculated but no real conclusions could be drawn from this result. The average repair capacity during week four was very low (fig 4.23) contrary to what is expected since in this phase of the cycle the most physical damage occurs (menstruation). One must remember that we have measured DNA damage and repair in a surrogate tissue which may not reflect the real events taking place in the target organ. This, however, does not affect the aim of this study. Baseline DNA damage in the white blood cells as measured in the whole blood was the most in week four (fig 4.7). Also, during week one and two, estrogen and progesterone levels are low (fig. 2.1). During this period good repair capacity was observed (fig 4.23). A similar observation was made with reference to the repair capacity as with DNA damage during the cycle, i.e. variation in the extent of repair during the cycle.

Recommendations for future studies: To obtain a more complete picture of the DNA damage and repair during the hormonal cycle more female individuals needs to participate in the study. Also, the estrogen concentration of each individual will give a more accurate indication of the phase within the cycle. To obtain a more consistent result

of the effect of the menstrual cycle, the female needs to be monitored over a longer period comprising of at least 3 consecutive cycles.

CHAPTER 6

ARTICLE

The first draft of a paper based on this work for possible submittion to a suitable journal.

DNA DAMAGE AND REPAIR DURING THE MAMMALIAN HORMONAL CYCLE.

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Abstract

The aim of this study was to determine the pattern of DNA damage and repair during the mammalian female hormonal cycle. This was done to determine whether the hormonal cycle must be taken into account when DNA damage and repair studies are done in female subjects, especially so when surrogate tissue such as white blood cells are involved. Because certain factors including gender, age and diet may influence the comet assay responses in lymphocytes when monitoring human genotoxicity, we posed the question whether the menstrual cycle would influence the comet assay responses in lymphocytes to any significant extent. The study approach was to determine DNA damage and repair during the hormonal cycle in both Sprague-Dawley rats and female individuals. Firstly, sexually matured adult female Sprague-Dawley rats were investigated. Vaginal smears were carried out to determine the phase of the estrous cycle of each individual. Differences in baseline DNA damage in white blood cells as measured in whole blood, and in isolated lymphocytes were observed for the different phases of the estrous cycle. The metestrous phase showed the most DNA damage in both the isolated lymphocytes and the white blood cells. The estrous phase has the least amount of DNA damage over the estrous cycle. Results obtained for the human female individuals showed that DNA damage peak at different times for different individuals during the menstrual cycle. The extent of DNA damage also varied between individuals. We could not, however, answer the question whether the extent of DNA damage we have observed was beyond the limits of physiological variation. In summary: In spite of inconclusive results we are of the opinion that the hormonal cycle of the mammalian female proves to be a factor of sufficient importance that needs to be taken in account when studies involving oxidative DNA damage are performed. A more extensive study in this field is required to address the influence of varying hormonal levels on DNA damage during the cycle.

Introduction

A search of the Medline Database over the last 20 years for studies in which rats were employed as research subjects shows that males were used over females by a ratio of approximately 3:1. This preference is acknowledged to be in part because of concerns over how physiological and behavioral variations across the estrous cycle can influence results [1]. The estrous cycle can be divided into four distinct phases: *proestrous* (follicular development, endometrial proliferation); *estrous* (ovulation), *metestrous* (endometrial differentiation) and *diestrous* (desquamation of endometrium leading to menstruation in humans) [2]. Although the estrous (rat) and menstrual cycle (human) differs in length, proestrous corresponds to the estrogen-dominated follicular phase, and metestrous corresponds to the progesterone-dominated luteal phase. During the hormonal cycle, certain biochemical and physiological factors may also exhibit some cyclic characteristics such as metabolism [1], cell proliferation [3], natural killer cells [4], liver enzymes [5], antioxidant capacity [6] and sister chromatid exchange [7]. These variations may directly or indirectly influence the integrity of the genetic material of the subjects involved. Any DNA damage must by repaired to maintain the integrity of the genetic material [8]. The aim of this study was to determine the extent of DNA damage and repair during the estrous- and menstrual cycle.

Materials and Methods

Animals

Adult female Sprague-Dawley rats were obtained from the Lab Animal Centre, North-West University. They were maintained under a 12h dark-light cycle in a temperature-controlled colony, and had food and water *ad libitum*. All animal care and handling were carried out in accordance with the university's Ethical Committee (number 04D12). Sexually matured female rats were sacrificed and blood was collected in heparin containing tubes. The different phases of the estrous cycled were ascertained by vaginal smears [9].

Female individuals

Four normal healthy women aged 23 years were recruited for this investigation. They were non-smokers, had no chronic illnesses, were not taking medications, and had not suffered from any incidental infections in the recent past. Considering the first day of menses as day 1, we divided the menstrual cycle into four weeks as confirmed by each of the individuals.

The comet assay (single cell gel electrophoresis or SCGE)

The comet assay was carried out according to the method developed by Singh [10]. Briefly, for the whole blood: 60 µl blood was washed three times with PBS and 50 µl of the cell suspension was mixed with 150 µl 1% LMPA at 37°C, out of which 130 µl was loaded onto microscope slides pre-coated with 1% HMPA. Slides were put on ice to solidify. For the isolation of lymphocytes, 2ml blood was placed on top of 2ml Histopaque® in a Falcon tube and was centrifuged for 30 minutes at room temperature. The buffy coat was transferred to an Eppendorf® tube. The cells were then washed three times with PBS and then resuspended in 500 µl PBS of which 50 µl were spread on microscope slides as described above. The remaining cells were exposed for 20 minutes at 37°C to 60 µM H_2O_2. The cells were then washed with PBS to remove the H_2O_2 centrifugation for 1 minute at 500 g at room temperature. The supernatant was removed and cells were suspended in 450 µl HAMS and of this cell suspension was spread onto microscope slides. The remaining cells were incubated for 10, 20 and 30 minutes at 37°C and at each time point 50µl was removed and spread onto microscope slides. Finally the slides were immersed for at least 12 hours in freshly prepared and chilled (4°C) lysing solution consisting of 5M NaCl, 0.4 M EDTA, Triton X-100, 10% DMSO and ddH_2O. The slides were briefly rinsed with in distilled water and then transferred to freshly prepared chilled electrophoresis buffer (0.6M NaOH, 0.05M EDTA and ddH_2O). The slides were placed side-by-side and avoiding air spaces between them with the agarose ends nearest to the anode in the electrophoresis tank. Slides were left for 30 min to allow unwinding of DNA and electrophoresis was performed at 30V and 500mA at 4°C for 45 min. Slides were briefly rinsed with distilled water and were then left in TrisHCl buffer (pH 7.5) for at least 15 min. The

DNA in the agarose was stained with ethidium bromide for 1 hour. Excess dye was removed by dipping the slides in ddH_2O. The lymphocytes and white blood cells (whole blood) were checked for viability before the start of the experiment using Trypan blue dye. Pictures were taken using the Olympus X70 fluorescence microscope (200 x magnification). A minimum of 50cells/sample was taken for statistical significance. The comets were scored using the Comet Assay Software Project (CASP program). Standard deviation was calculated using the *stddev* function in Excel and the statistical significant difference was calculated using the *ttest* fuction.

The ORAC assay (Oxygen Radical Absorbance Capacity).

We also determined the antioxidant capacity during the estrous cycle using the ORAC assay. The ORAC procedure measures the ability of the sample being tested to protect against attack by free radicals. This method utilizes a free radical source that produces the peroxyl radical, the most common free radical in the body [11]. Blood was collected in a blood coagulating tube. The blood was centrifuged for 10 min at 4 000 g and the top plasma layer collected. To measure the ORAC in the non-protein fraction of serum, the serum and plasma were diluted with 0,5M PCA (1:1 v/v). Treating serum with PCA preserves ascorbate. (Blood plasma was stored at -70°C until analysis was done). The frozen plasma were thawed and centrifuged for 10 min. Supernatant was recovered for ORAC assay. Standards were prepared. The samples and the buffer were added together to wells in duplicate to a total of 20 µl in the plate. To this was added 160 µl fluorescein solution in each well. The plate was pre-incubated at 37°C for 15 min. 'The ORAC-Fl' program on BioTek plate reader was used to record fluorescence (excitation 485 nm, emission 530 nm, static mode) every 5 min for 2 hours. The reaction was started by adding 20 µl AAPH (240 mM) and the reaction was followed until the final reading ± 5% of the initial reading.

Results and discussion

A statistically significant difference in DNA damage was observed between the various phases of the estrous cycle of the rat. The average baseline DNA damage in white blood cells (WBC, Table 1) as measured in the whole blood of the rats was the highest during the metestrous phase with 41.19 tail DNA%, with the least amount of DNA damage (8.99 tail DNA%) was observed in the estrous phase. The same pattern in DNA

Table 1. DNA damage during the rat estrous cycle

	Proestrous	Estrous	Metestrous	Diestrous
WBC	11.04 ± 15.74 (9)	8.99 ± 14.43 (7)	41.19 (1)	31.08 ± 8.81 (2)
Lymphocytes	29.45 ± 15.34 (4)	25.10 ± 7.58 (3)	38.44 (1)	26.29 ± 7.53 (3)
AC (μM Trolox)	686.69 ± 527.63 (11)	491.65 ± 488.24 (8)	221.83 ± 95.99 (5)	118.51 (1)

The number of rats investigated in each phase of the estrous cycle is indicated in brackets.
Standard deviation is also shown.

damage was seen in the case of isolated lymphocytes (Lymphocytes, Table 1), however, the antioxidant capacity of the blood did not follow the same pattern during the estrous cycle. A continuous decline in antioxidant capacity from the proestrous phase (686.69 μM Trolox) through to the diestrous phase (118.51 μM Trolox) was observed. Because of the short estrous cycles in rats, only 4-5 days, each phase is relatively short which made it difficult to obtain adequate numbers of rats in some of the phases, the metestrous phase in particular. Although the extent of DNA damage did vary across the estrous cycle, the question can be raised whether the differences in DNA damage that we have observed are significant enough to compel one to take it into account when using female rats in studies where oxidative damage are studied, or are these differences within the limits of normal physiological variation?

We then studied DNA damage and repair in the menstrual cycle of human subjects. The results of the comet analysis of DNA damage in white blood cells in whole blood and in isolated lymphocytes are given in Table 2. The average baseline DNA damage in

Table 2: Average baseline DNA damage in human female white blood cells.

	Week 1	Week 2	Week 3	Week 4
WBC	7.34 ● 10.30	14.27 ± 18.94	11.33 ± 16.53	**29.20 ± 22.38**
Lymphocytes	10.06 ± 7.52	**35.91 ● 25.79**	12.20 ± 13.58	15.28 ± 14.56
AC (μM Trolox)	104.95 ± 63.10	206.06 ± 71.21	129.95 ± 71.90	184.27 ± 65.25

DNA damage is given as tail DNA%. AC: antioxidant capacity. Standard deviation is also shown. The peak in DNA damage is in bold.

white blood cells measured in whole blood of human females during the menstrual cycle peaked in week four (29.20 tail DNA%) followed by week two, with the least amount of DNA damage observed in week one. The level of DNA damage observed in week one differed statistically significant ($p < 0.05$) from that in week two and three and in week two it differed statistically significant from week three. When we look at the DNA damage in the isolated lymphocytes we see that the peak in the baseline DNA damage shifted from week four to week two. Week one and three displays somewhat the same level of DNA damage. Week one differs statistically significant ($p < 0.05$) from week two and four, week three differs statistically significant from week two and four. It is evident that the same pattern is not observed in the white blood cells and isolated lymphocytes. During week four of the menstrual cycle, the follicle releases the egg, it closes and forms the corpus luteum (CL), which secretes progesterone. If pregnancy does not occur, the CL can lasts up to 14 days before breakdown begins. During this period the estrogen and progesterone levels are low. Because of a lack of hormones, the blood vessel goes into spasm and cut off blood supply to the top layers of the endometrium. Little or no oxygen or nutrients are available, the endometrial cells die, this causes the tissue to break down, bleeding occurs and a new cycle commences [11, 12]. This may explain the relatively higher baseline DNA damage that was observed during week four, because of the physical changes and breakdown of tissue that takes place in the target organ. The latter may be reflected in the white blood cells in whole blood but not in the isolated lymphocytes.

However, a different picture emerged when we compared the baseline DNA damage in isolated lymphocytes obtained from three individuals in the various phases of the

Table 3: Baseline DNA damage in isolated lymphocytes of three female individuals during the four week period.

	Person A	Person B	Person C
Week 1	8.297 ± 7.913	14.109 ± 7.171	*6.860 ± 5.014*
Week 2	**51.624 ● 6.966**	*4.165 ± 9.450*	**57.016 ● 9.052**
Week 3	17.263 ± 19.308	9.224 ± 6.658	10.025 ± 9.993
Week 4	*2.686 ● 4.287*	**28.127 ● 6.658**	NA

The peak in DNA damage is shown in bold and the minimum in italics. Standard deviation is also shown. NA, no results obtained.

menstrual cycle (Table 3). Our results correspond to a large extent with the observations made by Bajpayee [13] in that we also found that the level of DNA damage peaked at different times during the menstrual cycle for different individuals. This is clearly illustrated by the bold and italic numbers in Table 3.

Table 4: DNA damage and repair in isolated lymphocytes.

	Control	H2O2	10 min repair	20 min repair	30 min repair
Week 1	9.76 ± 3.84	20.69 ± 1.55	17.03 ± 6.15	11.99 ± 5.36	11.53 ± 3.84
Week 2	31.50 ± 26.69	21.54 ± 17.28	17.22 ± 16.26	13.37 ± 7.33	8.65 ● 7.03
Week 3	12.17 ± 4.43	26.47 ± 15.63	16.83 ± 4.90	19.05 ± 3.68	19.05 ± 1.90
Week 4	15.41 ± 17.99	34.38	15.12 ± 11.80	21.81 ± 25.89	29.11

The results are given as tail DNA% and the standard deviation is also given.

We also wanted to see whether the hormonal cycle has any effect on DNA repair in white blood cells. DNA repair after exposure to hydrogen peroxide was studied in isolated lymphocytes obtained in each week of the cycle and the combined results of three individuals are given in Table 4. During week one of the hormonal cycle small but significant repair is apparent after the H_2O_2 induced damage (tail DNA decrease from 20.69 to 11.53) The same can be said for week two except that in the latter case the repair process was continued during the whole of the time allowed for repair to take place. Contrary to the first two weeks of the cycle, the results we obtained for week three and four is rather erratic in the sense that after an initial repair, DNA damage tend to increase.

Our results show that there is significant inter-individual variation in DNA damage and that the estrous and menstrual cycle is a vital physiological process to consider in any

study. It remains, however, unknown if these variations are between normal physiological limits of DNA damage. Since there is not yet a comet assay baseline DNA damage (in both white blood cells and lymphocytes) database available, it is not possible to fully evaluate these data. If it is true that the DNA damage we have observed falls in the normal physiological range, we need to ask what significance is of the increased levels of baseline DNA damage (white blood cells in whole blood and lymphocytes) observed at various times during the menstrual cycle. Even though it is possible that the DNA damage may be within normal limits, the DNA may be more fragile during certain phases of the hormonal cycle, e.g. week two in the case of individuals A and C. This may explain the occurrence of sister chromatid exchange during the human menstrual cycle [7, 15], a phenomenon that is seen in chemical toxicology as a biomarker of effect [16]. Nevertheless, we are of the opinion that the effect of the female hormonal cycle is of such significance that is necessary to take cognizance of when using female subjects in experiments where DNA damage and repair is measured.

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Appendix A.

Comet assay

Lysis buffer

[5M] NaCl	500 ml
[0,4M] EDTA, pH 7-8	250 ml
Triton X-100	10 ml
DMSO [10%]	100 ml
ddH_2O	140 ml
	1 000 ml

Electrophoresis buffer

[0,6 M] NaOH	500 ml
[0,05M] EDTA	20 ml
ddH_2O	480 ml
	1 000 ml

TrisHCl

63,04 g TrisHCl in 500 ml ddH_2O.

After one hour at 4°C, adjust pH to 7.5 and fill up to 1 *l* with ddH_2O.

Phosphate buffered saline

NaCl	8 g
KCl	0,2 g
Na_2HPO_4	1,15 g
KH_2PO_4	0,2 g

Dissolve in 1 000 ml ddH_2O

Adjust pH to 7-8.

Ethidium bromide (Staining solution)

Work with caution → Carcinogenic.

Dissolve 5 mg Ethidium Bromide in 1 000 ml ddH_2O

Keep for at least one hour at 4°C before use, cover container with tinfoil.

Hydrogen peroxide (H_2O_2)

2 µl H_2O_2 in 998 µl PBS

Use 17 µl to induce damage.

HMPA [1 %]

Prepare just enough for one day's work.

1 g → 100 ml 0,1 M EDTA

0,5 g → 50 ml

0,4 g → 40 ml

Heat until dissolved.

LMPA [0,5%]

Prepare just enough for one day's work.

0,5 g → 100 ml 0,1 M EDTA

0,01 g → 2 ml

0,25 g → 50 ml

ORAC assay

Phosphate buffer

Mix 1M K_2HPO_4 and Na_2HPO_4 (61.6:38.9) and dilute to 75 mM.

pH 7.4

Fluorescein

Main stock solution of 265 mM (10% w/v in water, pH 7.4) of fluorescein is made. Before each assay dilution is made in phosphate buffer as follows:

1 µl (265mM) + 999 µl buffer - D1 (265 µM)

50 reactions: D1 + 9998 µl buffer – D2 (56 nM)

2,2' Azobis(2-aminodinopropane) dihydrochloride (AAPH)

240 mM (86.8 mg/ml in buffer)

Prepare fresh before use, keep on ice.

Trolox

500 µM (122,5 µg/ml in buffer) aliquoted in vials and kept at -70°C for 4 months

Prepare from a newly thawed vial each day the standards.

Preparation ofstandard series

	T1	**T2**	**T3**	**T4**	**T5**	**T6**
Buffer (µl)	20	19	18	16	14	12
500 µM Trolox (µl)	0	1	2	4	6	8
End concentration (µM)	0	2.5	5	10	15	20

Table A.1: List of reagents and catalog reference.

Reagents	**Company**	**Catalog #**
K_2HPO_4	Merck	1.05108.0500
DMSO	Sigma-Aldrich	D 5879
EDTA	Sigma-Aldrich	E 5134
Ethidium Bromide	Sigma-Aldrich	E 8751
H_2O_2	Sigma-Aldrich	H1009
HAMS F10 medium	Addock-Ingram	12-618F
Histopaque®	Sigma-Aldrich	10771
HMPA	Techcomp ltd	9201
KCl	Sigma-Aldrich	P 9333
LMPA	Roche	1441345
Na_2HPO_4	Merck	1.06566.0500
NaCl	Sigma-Aldrich	S 9625
NaOH	Sigma-Aldrich	S 5881
Tris HCl	Sigma-Aldrich	T 5941
Triton X-100	Sigma-Aldrich	T 8787
Trypan Blue	Sigma-Aldrich	93595
Fluorescein	Sigma-Aldrich	F6377
AAPH	Merck	99
Trolox	Sigma-Aldrich	23881-3

SYMBOLS

µl	Micro liters
µM	Micro molar
β	Beta
°C	Degrees Celsius
%	Percentage

LIST OF ABBREVIATIONS

A:

A	Adenine
AAPH	2,2' Azobis(2-aminodinopropane) dihydrochloride
ANON	Anonymous

B :

BER	Base excision repair

C :

C	Cytosine
CAT	Catalase
CE	Catechol estrogens
CE-Qs	Catechol estrogens quinones
CL	Corpus luteum

D:

ddH_2O	Double distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DSB	Double strand break

E:

E_2	Estradiol
E. coli	Escherichia coli
EDTA	Ethylenediaminetetra acetic acid
EE	Energy expenditure
ER	Estrogen receptors
et al	Et alii/alia (Latin: and others)

F:

fP_T	NADPH-cytochrome c reductase
FSH	Follicle stimulating hormone

G:

G	Guanine
g	Grams
g	Gravity (9.8 $m.s^{-2}$)
GSH	Glutathione
GSH-Px	Glutathione peroxidase

H:

h	Hours
H_2O_2	Hydrogen peroxide
HMPA	High melting point agarose gel
HR	Homologous recombination

K:

KCl	Potassium chloride
K_2HPO_4	Potassium dihydrogen ortophosphate

L:

LH	Luteinizing hormone
LMPA	Low melting point agarose gel

M:

M	Molar
ml	Milliliters
mM	Millimolar
MMR	Mismatch repair

N:

NaCl	Sodium chloride

NADH	Nicotinamide adenine dinucleotide
Na_2HPO_4	Sodium dihydrogen orthophosphate
NaOH	Sodium hydroxide
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NK	Natural killer cells
nm	Nanometer

P:

PBS	Phosphate buffered saline
PCA	Perchloric acid
PCNA	Proliferating cell nuclear antigen
pH	Potential of hydrogen

R:

RBC	Red blood cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RQ	Respiratory quotient

S:

SCE	Sister chromatid exchange
SCGE	Single-cell gel electrophoresis
-SH	Sulphhydryl
SOD	Superoxide dismutase
SSB's	Single-strand breaks

T:

T	Thymine
TE	Trolox equivalent

Tris HCl	2-Amino-2-(hydroxymethyl)-1,3-propandiol-hydrochloride

U:

U	Uracil
UDP-GT	Uridine diphosphate glucuronyltransferase
UV	Ultraviolet

V:

vs	Versus
v/v	volume/volume

W:

WBC	White blood cells

LIST OF TABLES

LIST OF FIGURES

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