EVALUATION OF STORAGE CONDITIONS FOR ASSESSING DNA DAMAGE USING THE COMET ASSAY

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DEDICATION

I would like to dedicate this work to my lord and savior Jesus Christ. I give praise and glory to God who makes all things possible. I would also like to dedicate this work to my wife Rachel and my parents Carol and Cesar. I thank all of you for your love and support.

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ABSTRACT

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The single cell gel electrophoresis assay (comet assay) is a useful tool for monitoring individuals who may be at risk of DNA damage and the ensuing process of carcinogenesis or other disease states. Leukocytes in blood samples provide a means of obtaining cells for use in the comet assay. However instances may arise when samples must be stored for later analysis. The present study investigated the effects of storage conditions on DNA damage in the form of strand breaks and oxidized bases in rat and human leukocytes using the comet assay. Whole blood and buffy coat samples were stored at room temperature or 4ºC for 1, 2, 24, and 48 hours or cryopreserved at -80ºC for 1 day and 1, 2, 3, and 4 weeks. The results show that the time of storage is limited if the whole blood or buffy coat samples are stored at room temperature or 4ºC. However, if cryopreserved using glycerol or DMSO as the cryoprotectant, the samples may be stored for at least 4 weeks without DNA strand breaks or oxidative damage deviating significantly from the fresh samples.

James E. Klaunig, Ph.D.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

4-OH-8-oxo-dG	4,8-digydro-4-hydroxy-8-oxo-2'-deoxyguanosine
8-OHG	8-hydroxyguanine
8-OHdG	8-hydroxydeoxyguanosine
A	Adenine
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
C	Cytosine
CAT	Catalase
CIRBC	Central Indiana Regional Blood Center
Cr	Chromium
Cu	Copper
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EPA	Environmental Protection Agency
FAD	Flavin Adenine Dinucleotide
Fapy	Formamidopyrimidine
Fe	Iron
Fpg	Formamidopyrimidine-DNA Glycosylase
G	Guanine
GSH-Px	Glutathione Peroxidase
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric Acid
He	Helium
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic Acid
IGF-II	Insulin-like Growth Factor II
LMA	Low Melting Point Agarose
Na	Sodium
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
Ni	Nickel
PAH	Polycyclic Aromatic Hydrocarbon
PBS	Phosphate Buffered Saline
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
S9	Supernatant of a 9000 xg Centrifugation
SOD	Superoxide Dismutase
T	Thymine
TGF-α	Transforming Growth Factor α
UV	Ultraviolet
Zn	Zinc

I. Introduction

A. Experiment Background

The present study focuses on the detection of DNA damage, strand breaks and oxidized bases, in leukocytes stored under various conditions by use of the single cell gel electrophoresis assay or more commonly referred to as the comet assay. This is important because DNA damage can lead to cancer and other diseases.

Other methods of detecting DNA damage include: the alkaline-elution assay [60] which measures the rate in which single strands of broken DNA pass through a filter, the alkaline unwinding assay [1] which measures the rate at which double-stranded DNA unwinds in alkali dependent on the number of strand breaks, zonal centrifugation [59] which measures the average molecular weight of DNA fragments, sedimentation of nucleoids [20] which measures the distance nucleoids are sedimented in a sucrose gradient dependant on the amount of DNA supercoiling, and the DNA precipitation assay [83] which measures the percent of undamaged DNA precipitated after centrifugation. Although these methods have been shown to provide a sensitive measure of the overall DNA damage to cells, there are several drawbacks when compared to the comet assay. For example, generally a large number of cells are required, radiolabeling of DNA does not permit analysis of DNA damage in noncycling cells, and information on the response of individual cells is not possible.

The comet assay has been used to detect DNA damage in a variety of animal and human cell types. One of these cell types is leukocytes (white blood

cells) in whole blood samples. Many studies have been published using fresh whole blood samples, buffy coats (leukocyte fraction of whole blood obtained by centrifugation), separated lymphocytes, and separated neutrophils. However, little has been published relating the effects that storage conditions could have on the use of leukocytes for the comet assay. One such study conducted by Anderson et al. [5] reported no change in DNA strand breaks when human blood was stored at room temperature or 4ºC for up to 4 days. However, Narayanan et al. [77] reported an increase in DNA strand breaks when human blood was stored at room temperature or 4ºC for 24 and 48 hours. Another study conducted by Chuang et al. [18] investigated the effect of 4ºC storage on DNA strand breaks by the use of human and rat whole blood, isolated lymphocytes, and isolated leukocytes obtained by the hemolysis of red blood cells. In all cases, there was no change in DNA strand breaks for up to 4 hours. Chuang et al. also investigated the effect of cryopreservation on DNA strand breaks in rat whole blood and isolated lymphocytes which showed no change after 60 days of storage. Similar results were found by Tice et al. [118] and Visvardis et al. [126] who reported no change in DNA strand breaks of cryopreserved human isolated lymphocytes and also Duthie et al. [25] who reported no change in DNA strand breaks or oxidative damage of cryopreserved human isolated lymphocytes.

Due to the discrepancy found in the literature regarding the storage of human whole blood at room temperature or 4ºC, which may be attributed to protocol variation, the evaluation of DNA strand breaks after such storage conditions must be re-evaluated. Furthermore, additional information regarding

the effect on oxidative damage is needed. This study will provide information on the detection of DNA strand breaks as well as oxidative damage after short term storage (1, 2, 24, and 48 hours) at room temperature or 4ºC and long term storage(1 day and 1, 2, 3 and 4 weeks) via cryopreservation with glycerol or DMSO. It will use rat and human leukocytes which will be useful in genotoxicity studies involving rodent models and human biomonitoring studies. The leukocytes will be stored as whole blood or isolated by centrifugation and stored as a buffy coat. This will also provide information about the effect red blood cells could have on the detection of DNA strand breaks and oxidative damage.

B. Multistage Carcinogenesis

Carcinogenesis is a multistage process in which a normal cell is transformed into a malignant cell. This transformation is a multistage process which includes initiation, promotion and progression (figure 1) [74].

Initiation occurs upon genetic damage to cellular DNA [7]. Several chemical agents can produce initiation and will be discussed in more detail in the following section. The insult can cause strand breakage of the DNA double helix or an alteration of bases leading to a mutation, which can give the cell a selective growth advantage and/or an inability to regulate growth. For example, proto-oncogenes and tumor-suppressor genes are two gene families associated with neoplastic development. Proto-oncogenes are normal cellular genes encoding proteins that stimulate the progression of cells through the cell cycle [107]. These proteins include growth factors and growth factor receptors; intracellular signal transducers such as G-proteins, protein kinases, cyclins, and cyclin-

dependent protein kinases; and nuclear transcription factors [57]. Tumor-suppressor genes encode proteins that inhibit the progression of cells through the cell cycle [57]. These proteins include cyclin-dependent protein kinase inhibitors, transcription factors that transactivate genes encoding cyclin-dependent protein kinase inhibitors, and proteins that block transcription factors involved in DNA synthesis and cell division [57]. A constant activation and/or over expression of the products of proto-oncogenes or and inactive product of tumor-suppressor genes as a result of mutation can give the cell this selective growth advantage and/or an inability to regulate growth. In many cases the lesion is repaired before the cell undergoes division and the mutation is not set in the genome. However, if the cell is unable to repair the damage before cell division the mutation is locked in. In this sense, the stage of initiation is irreversible and exhibits no threshold since an infinite number of cells may be initiated [57]. Not all initiated cells result in carcinomas. Many initiated cells undergo apoptosis, are removed by the immune system or simply die due to a defect in a crucial regulatory gene.

Promotion does not involve a direct genotoxic event. It is defined as the clonal expansion of initiated cells, induced by a promoting agent, resulting in a preneoplastic lesion [57]. In contrast to initiation, promotion is reversible because withdrawal of the promoting agent results in a regression of the clonal expansion [74]. Thus, in order for promotion to continue, the initiated cells must be subjected to the promoting agent for extended periods of time.

Progression is marked by a permanent selective growth of preneoplastic cells into neoplastic cells [57]. Neoplasms can be either benign or malignant. Malignant neoplasms are capable of metastatic growth to other regions of the body while benign neoplasms are confined to the primary area of growth. The alterations that bring about progression can arise from continued exposure to the carcinogen, additional spontaneous or induced mutations, or genomic instabilities [74]. The result is a more aggressive phenotype. The agents that effect the transition from the promotion stage to the progression stage are termed progressor agents while agents that effect the transition of normal cells to the progressive stage are termed complete carcinogenic agents [57].

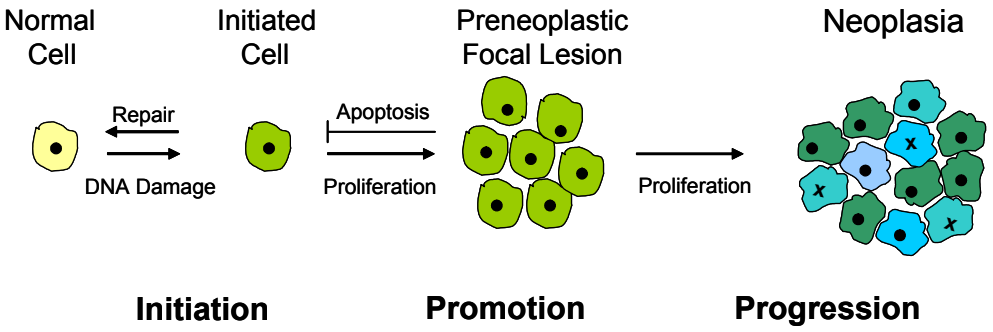

Figure 1. Multistage Carcinogenesis [58]

Carcinogenesis begins with damage to a normal cell's DNA and results in an initiated cell. The initiated cells may undergo clonal expansion in the promotion stage to form a preneoplastic lesion. Continued cell proliferation may lead to neoplasia which can be benign or malignant.

C. Chemical Carcinogenesis

Carcinogenesis caused by harmful chemicals has been well established. Chemicals may induce cancer by genotoxic or non-genotoxic mechanisms. Genotoxic carcinogens may interact directly with DNA and cause mutations by covalently binding to DNA [7]. In contrast to genotoxic carcinogens, non-genotoxic or epigenetic carcinogens are non-mutagenic and commonly act at the promotion stage [62]. These types of carcinogens act by increasing cell growth and/or proliferation as a result of an increase in DNA synthesis, a decrease in apoptosis, a modification of intercellular communication by inhibition of gap junctions, a modification of gene expression by altering DNA methylation, or by the modification of cell surface receptors [57].

Chemical carcinogens may be organic, inorganic, hormones, or induced by radiation. In many cases the carcinogen must be metabolized or biotransformed into the active carcinogen. The term indirect carcinogen refers to the parent compound which must be activated, usually by endogenous enzymes, while the term ultimate carcinogen refers to the metabolite that produces the carcinogenic effects [7].

One class of chemical carcinogens is organic compounds, which can be alkylating, aralkylating, or arylhydroxylamine agents [74]. Alkylating agents transfer alkyl groups to nucleotides resulting in the formation of DNA adducts [74]. Some examples of these are the N-nitroso compounds dimethyl nitrosamine, methylnitrosurea, and also the micotoxin aflatoxin B_1. Aralkylating agents transfer aromatic groups to nucleotides resulting in the formation of DNA

adducts [74]. Some examples of these are the polycyclic aromatic hydrocarbons (PAHs) benzo[a]pyrene, dimethylbenzanthracene and methylcholantrene. The arylhydoxylamines function by transferring aromatic amines to nucleotides resulting in the formation of DNA adducts [74]. Examples of these include the aromatic amines benzidine, 2-naphthylamine, and 2-acetylaminofluorene.

Inorganic compounds represent another class of chemical carcinogens. The metals arsenic, cadmium, chromium and nickel have been shown to be carcinogenic in humans [104]. Arsenic may cause skin and lung cancer by chronic exposure via inhalation [28, 49]. It has also been shown to produce cancer of internal organs via ingestion [9]. However the mechanism of arsenic carcinogenicity has not been determined. Cadmium exposure via inhalation has been shown to be related to lung cancer in humans [48, 116]. Other studies have shown that it can produce a variety of tumors in rats after exposure by inhalation [79, 115], injection [128] and ingestion [129]. Chromium exposure via inhalation has been shown to be associated with cancer of the respiratory tract in humans [67]. The proposed mode of action of chromium carcinogenicity in the lung is the reduction of Cr (VI) to Cr (III). This reduction generates reactive intermediates that can damage DNA [87]. Other studies have loosely associated Cr (VI) with other types of cancer but there is currently not enough evidence for the EPA to classify it as a known carcinogen other than by inhalation. Nickel and compounds of nickel have been shown to produce cancer of the respiratory tract in humans, mainly lung and nasal cancers [6, 52]. The mechanism of this carcinogenicity is unknown. However, it is believed that nickel carcinogenesis is

attributed to enhanced DNA chromatin condensation [21], production of reactive oxygen species [73] or by the replacement of Zn^{2+} with Ni^{2+} in the zinc fingers of DNA-binding proteins [114]. Inorganic carcinogens do not necessarily have to be metals. For example, asbestos has been shown to be associated with lung cancer [22, 130]. Asbestos is a silica fiber used for insulation and fireproofing. It exists in many forms but the most relevant ones to carcinogenicity are crocidolite and tremolite. Once inhaled into the lungs, fibers longer than 10 μm have been associated with lung cancer because they are incompletely phagocytized by alveolar macrophages [57]. The result is a sustained state of macrophage activation which can lead to DNA damage through the production of reactive molecules or an increase of cell replication in the lung [57].

Hormones represent yet another class of chemical carcinogens. Hormones modulate a variety of cell processes including signal transduction and cell replication. If these cell processes are disrupted by an under- or overproduction of a specific hormone, carcinogenesis may occur. Gonadotropins are hormones released by the pituitary that act on the testes or ovaries. The testes and ovaries then release androgens and estrogens, respectively, which activate a feedback mechanism to regulate the production and release of the gonadotropins from the pituitary. When normal ovaries were transplanted into the spleen of castrated mice, neoplasms developed in the implanted ovaries [12], apparently because of a break in the hormonal feedback loop in which estrogen produced by the ovary implants was completely metabolized by the liver and could not reach the pituitary in order to suppress the production of

gonadotropins. Similar results were seen in the ovariectomies of mice [55] and administration of antiandrogens to rats [78] which resulted in adrenocortical neoplasms and interstitial (Leydig's) cell neoplasms, respectively. Thyrotropin is another hormone released by the pituitary but it acts on the thyroid gland. The thyroid releases another hormone called thyroxin that acts as a feedback to pituitary production of thyrotropin. Excessive production of thyrotropin has been associated with thyroid neoplasms in rats. This has been demonstrated by the administration of goitrogens [35], which inhibit the synthesis and/or production of the thyroid hormone, and transplantation of pituitary neoplasms [123]. Prolactin has also been shown to be a hormonal carcinogen. The induction of high levels of estrogen inhibits dopamine formation in the hypothalamus. Dopamine inhibits prolactin synthesis and release by the pituitary. When this inhibition is eliminated, an excessive amount of prolactin is formed and along with estrogen it has been shown to result in mammary neoplasms in rats [78]. Transforming growth factor α (TGF-α) and insulin-like growth factor II (IGF-II) are other hormones that may induce carcinogenesis. This has been shown by the use of transgenic mice that over express TGF-α [68] or IGF-II [93] which resulted in liver neoplasms.

Although radiation is considered to be a physical carcinogen, it may also induce chemical carcinogenesis. The two most common types of radiation include ultraviolet and ionizing radiation.

Ultraviolet radiation exists at wavelengths from 200 nm to 400 nm (visible light ranges from 400 nm to 700 nm). It is subdivided into UV-A (320 to 400 nm),

UV-B (280 to 320 nm) and UV-C (200 to 280 nm). UV-B radiation can cause DNA strand breakage and the formation of thymine dimers (cyclobutane rings and 6-4' photoproducts) [74]. This can result is skin cancers such as squamous and basal cell carcinomas and malignant melanomas [62].

A more serious form of radiation is ionizing radiation caused by free neutrons or alpha particles (He^{2+}). Some sources for chronic low levels of exposure include chest x-rays, dental exam x-rays, endogenous isotopes and cosmic or terrestrial irradiation. Ionizing radiation can cause direct or indirect damage to DNA. Indirect damage involves the ionization of water to form reactive oxygen species (ROS) which can then react with DNA. In either case, ionizing radiation can cause DNA strand breaks or base and sugar damage which can lead to mutations [74]. The result may be leukemia, lung cancer, bone cancer, skin cancer, and thyroid cancer [62].

D. Oxidative DNA Damage

Oxidative DNA damage has gained more attention over the past few decades as a cause of carcinogenesis. Reactive oxygen species have been attributed to over 20 different types of DNA damage [105] in addition to over 100 diseases [43].

The main types of reactive oxygen species are hydrogen peroxide, the hydroxyl radical, singlet oxygen, the superoxide radical, and the peroxyl radical. These reactive oxygen species are normally kept in balance by enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidases such as glutathione peroxidase (GSH-Px) (figure 2). In addition to enzymes, antioxidants

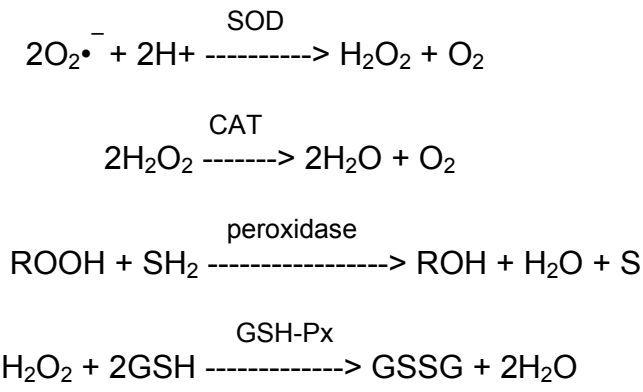

Figure 2. Enzymatic Removal of Reactive Oxygen Species

or radical scavengers can help in the protection against damage induced by reactive oxygen species. They may be present endogenously (uric acid, melatonin, carnosine) or present in the diet (vitamins A, C, and E) [27].

Reactive oxygen species are constantly being formed in the body by, for example, the mitochondrial electron transport chain. This chain catalyzes a series of oxidation/reduction reactions that are driven by a proton gradient across the mitochondrial membrane. In the process, four electrons are transported to O_2 to form $2H_2O$ and coupled with the phosphorylation of ADP to ATP. In a minor pathway, flavin adenine dinucleotide (FAD) and ubiquinone (coenzyme Q) are able to accept one electron forming a semiquinone radical which in turn can generate the superoxide radical [27]. Coenzyme Q is the major source of the superoxide radical, producing 1.85 ± 0.2 nmoles/min per mg of protein [120].

The microsomes of cells are another location of ROS formation. Amino acid oxidases in the endoplasmic reticulum of the liver and kidney can be oxidized by O_2 to form H_2O_2 [27]. Another family of microsomal enzymes, the

cytochrome P450's, can initiate ROS production. These enzymes metabolize xenobiotics by a variety of mechanisms (hydroxylation, epoxidation, ester cleavage, dehydrogenation, etc.) in order to make them more water soluble for excretion. As a side product they can produce hydrogen peroxide or the super oxide radical [57].

Phagocytosis also generates ROS and is associated with carcinogenesis as a result of chronic inflammation. Phagocytes destroy invading microbes or foreign matter. They constitute granulocytes (neutrophils, eosinophils, and basophils) and agranulocytes (monocytes and macrophages) both of which produce the superoxide radical via NADPH catalysis of molecular oxygen [36, 53]. The superoxide radical dismutates spontaneously to form hydrogen peroxide and singlet oxygen or is catalyzed by SOD to form hydrogen peroxide and molecular oxygen [27]. In either case, the presence of hydrogen peroxide can result in the formation of the hydroxyl radical via the Fenton reaction [57] or the Haber-Weiss reaction [27] (figure 3).

a) $H_2O_2 + Fe^{2+}$ -------> $Fe^{3+} + OH^- + \bullet OH$

b) $2O_2\bullet^- + H_2O_2$ -------> $O_2 + OH^- + \bullet OH$

Figure 3. Fenton and Haber-Weiss Reaction

Generation of the hydroxyl radical via a) Fenton reaction, b) Haber-Weiss reaction.

Photosensitization reactions are another source of ROS production. These types of reactions are prevalent in the skin, which is under constant attack by UV radiation. Photosensitization reactions involve the absorption of light by certain phototoxic chemicals (porphyrins, polycyclic aromatic hydrocarbons, methylene blue, etc.) and the energy is then transferred to a nearby molecular oxygen exciting it to singlet oxygen [27]. The formation of singlet oxygen can lead to cellular and DNA damage.

Important biomolecules such as fatty acids, proteins, and DNA can be damaged as a result reactive oxygen species. Reactive oxygen species can attack fatty acids and cause lipid peroxidation. The dismutation of poly unsaturated fatty acids to lipid peroxyl radicals (L-OO•) may lead to singlet oxygen formation, cyclic peroxides by adding to a double bond of the same molecule, or react with olefins to produce epoxides [27]. Lipid hydroperoxides (L-OOH) can also react with metal ions (Fe^{2+}, Cu^{2+}) to form alkoxyl radicals (L-O•), which can in turn produce reactive aldehydes or induce further lipid peroxidation [27]. Lipid peroxidation compromises the integrity of the cellular or microsomal membrane. This causes leakiness and a loss of chemical and ionic homeostasis necessary for a variety of cellular processes. Furthermore, the products of lipid peroxidation can diffuse away from their site of formation and cause damage at other sites by reacting with other biomolecules.

Reactive oxygen species can also oxidize proteins, which can lead to the amino acid residue conversion of histidine to 2-oxohistidine, tryptophan to kynurenine or N-formylkynurenine, tyrosine to dihydroxy derivatives, methionine

to methionine sulfoxide or methionine sulfide derivatives, leucine and valine to hydroxyl derivatives and cysteine to disulfide derivatives [111]. It is believed that the oxidation of amino acid residues occurs at metal binding sites on the protein in which hydrogen peroxide or alkyl peroxides (R-OOH) react by the Fenton reaction to form the hydroxyl radical and alkoxyl radical, respectively [111, 112]. The damaged proteins are subject to degradation by proteases or, in the case of the sulfur containing amino acids, they can be repaired by reduction. For example, upon oxidation by ROS, methionine residues are converted to methionine sulfoxide derivatives which can be reduced back to methionine by methionine sulfoxide reductase in the presence of thioredoxin [111]. In this way methione residues can act as an antioxidant defense against ROS. However, in cases of oxidative stress the protein damage may be too extensive to be removed by degradation or repaired by reduction. Many studies have associated oxidative stress and protein oxidation to conditions such as ischemia-reperfusion [8, 50, 84, 88], hyperoxia [108, 113, 131, 137], cigarette smoke [92], artificial ventilation [39], forced exercise [108, 132], paraquat toxicity [131], oxidative burst of neutrophils [54, 85], Alzheimer's disease [45, 46, 106], Parkinson's disease [4, 32], diabetes [10, 51, 121], and induction of renal tumors [122].

Most importantly to my study is the damage ROS can inflict on DNA which can lead to initiation and the process of carcinogenesis. The ROS responsible for the direct damage to DNA are the hydroxyl radical and singlet oxygen (however, other ROS like superoxide or H_2O_2 are involved in its production) [27].

The damage to DNA may be in the form of abasic sites, base modifications, sugar lesions, base-protein cross-links or single and double strand breaks.

The hydroxyl radical is a very reactive ROS and reacts with DNA indiscriminately. It may react with pyrimidines to cause base modifications. For example, it may react with thymine to form the allyl radical, 5-hydroxy-6-yl radical, or the 6-hydroxy-5-yl radical (figure 4) [23].

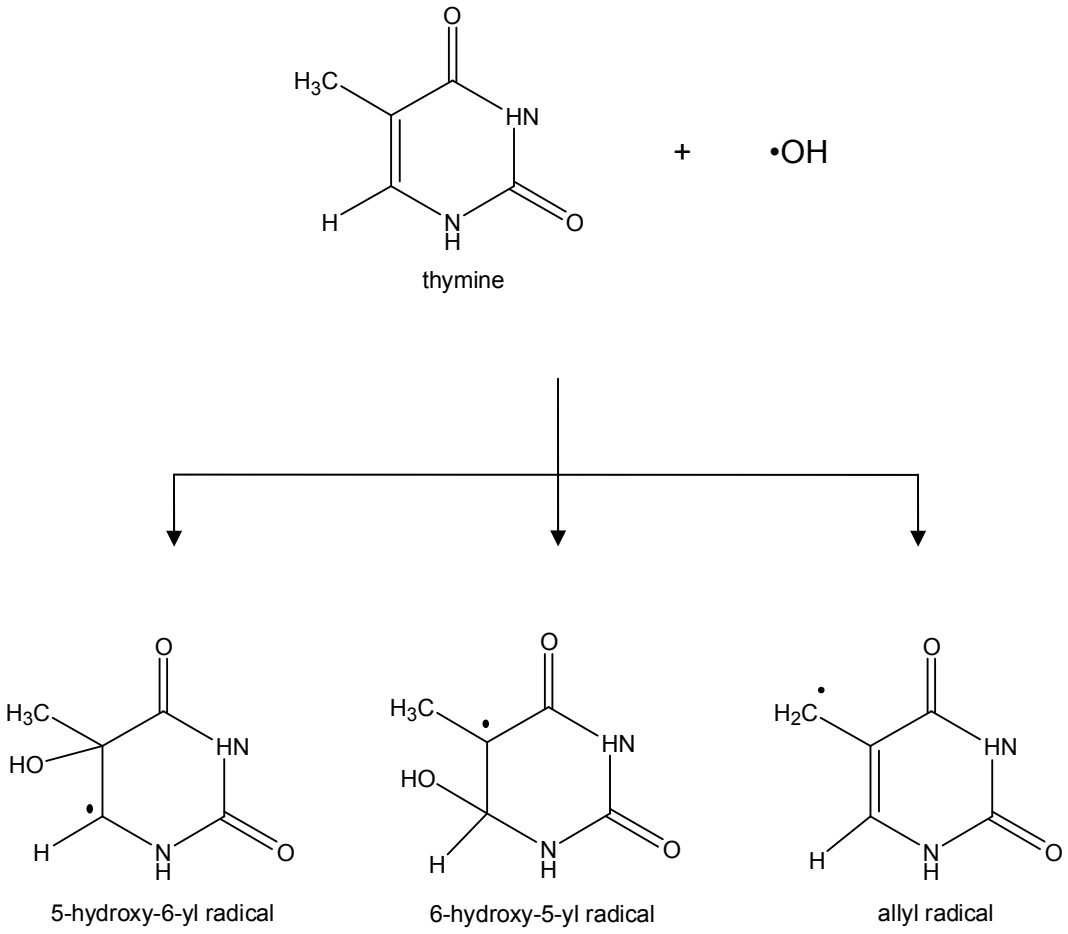

Figure 4. Reaction of Thymine with the Hydroxyl Radical

The hydroxyl radical may also react with purines. For example, it can cause the conversion of guanine to the C4-OH-adduct radical, C5-OH-adduct radical and the C8-OH-adduct radical [23]. The C8-OH-adduct radical can then lead to the formation of 8-hydroxyguanine (8-OHG), 7-hydro-8-hydroxyguanine or 2,6-diamno-4-hydroxy-5-formamidopyrimidine (Fapy G) (figure 5) [23]. The hydroxyl radical can also react with the pentose sugars of the nucleic acid backbone by abstracting hydrogen from any of the C atoms [127]. For example, hydrogen abstraction from the C-2' position in the presence of molecular oxygen can lead to base elimination (abasic site) [23]. Abstraction from the C-5' position in the presence of molecular oxygen can lead to strand breakage while abstraction in the absence of molecular oxygen can lead to addition to a neighboring guanine forming a cyclic product [24]. Additionally, the hydroxyl radical may also form base-protein cross-links. DNA is surrounded by proteins called histones which are highly basic due their rich composition of the amino acids arginine and lysine. The cross-links can occur by radical-radical combination, base radical addition to an aromatic amino acid or protein radical addition to DNA bases [27].

As compared to the hydroxyl radical, singlet oxygen is more selective in its reaction with DNA. It preferentially reacts with deoxyguanosine to form 8-OHdG, Fapy G or 4,8-digydro-4-hydroxy-8-oxo-2'-deoxyguanosine (4-OH-8-oxo-dG) [14, 91]. Singlet oxygen has also been shown to produce strand breaks however this event is minimal compared to the hydroxylation of guanosine [29, 64, 99].

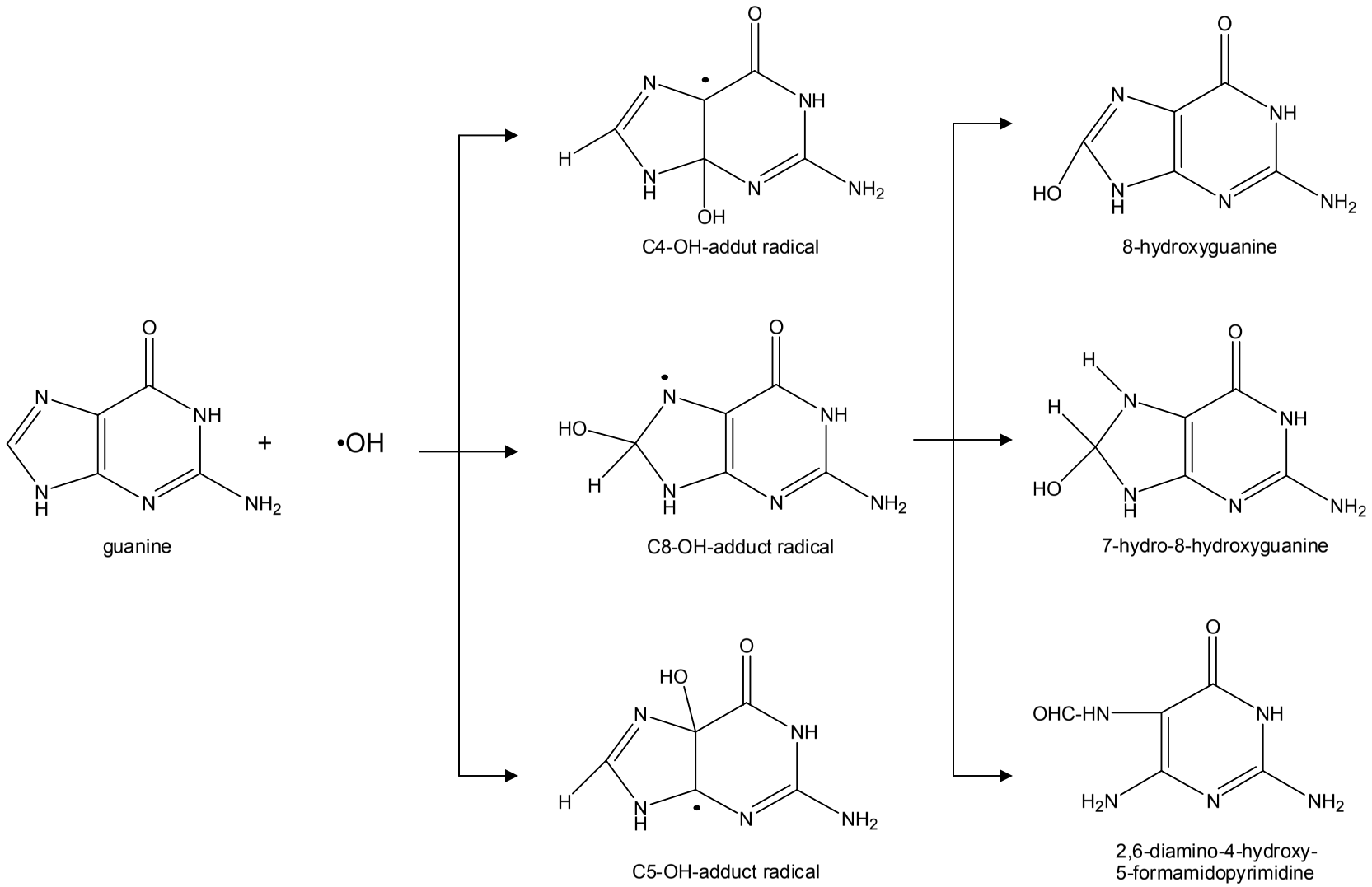

Figure 5. Reaction of guanine with the hydroxyl radical

DNA damage as a result of ROS may lead to mutations if the damage is not repaired. The most studied DNA lesion is the 8-OHdG, which has been used as a marker for oxidative damage in vivo and in vitro. The 8-OHdG can be present in three forms: 6-keto, 8-enol form, 6,8-diketo form and the 6-enol, 8-keto form (figure 6) [33]. The 6,8-diketo tautomer is the most common and can exist in the syn conformation as opposed to the normal anti conformation [17]. While in the normal anti configuration, 8-OHdG can pair with deoxycytidine as expected. However, in the syn conformation, 8-OHdG pairs with deoxyadenosine (figure 7) [100]. In this way 8-OHdG is able to cause mutations

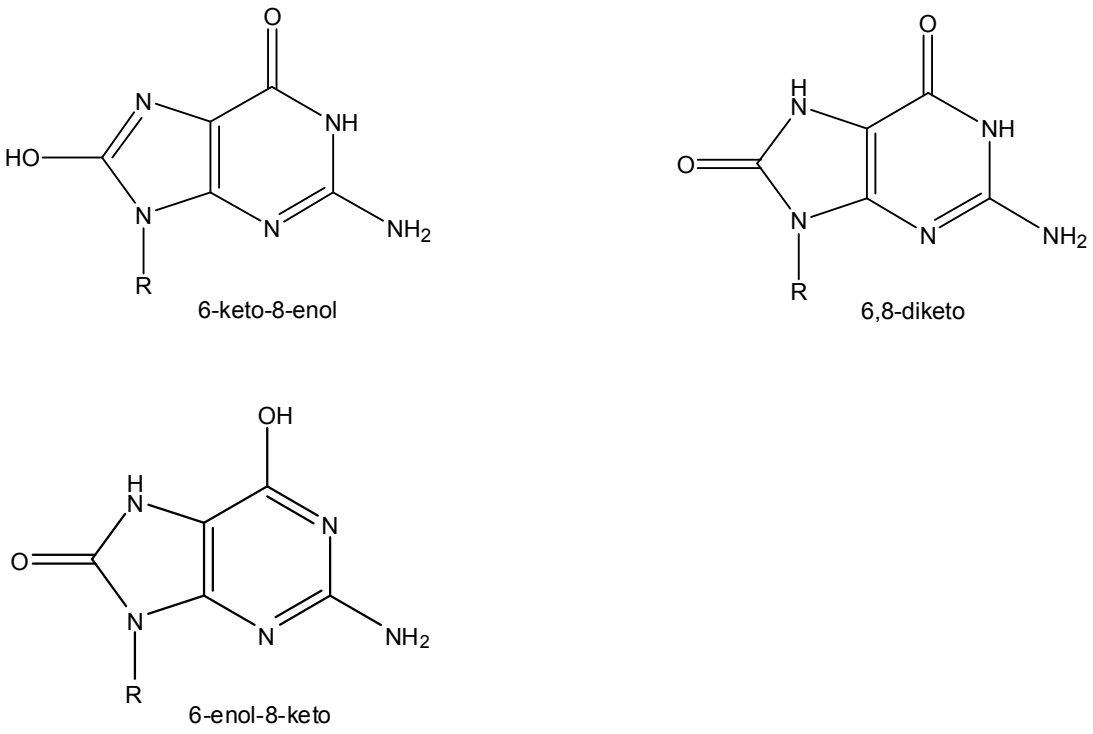

Figure 6. Tautomeric Forms of 8-OHdG

R = 2'-deoxy-D-ribose

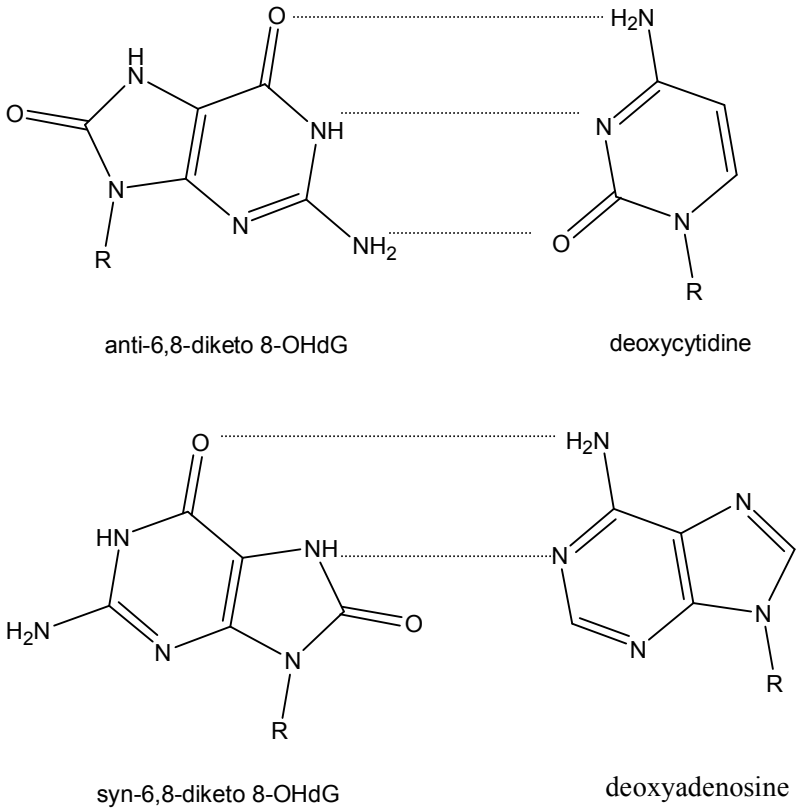

Figure 7. Base pairing of 8-OHdG with dA or dC

R = 2'-deoxy-D-ribose

by converting G-C base pairs to T-A base pairs if it is the template or by converting A-T base pairs to C-G base pairs if it is used as a substrate [16]. There is controversy as to whether 8-OHdG can cause mutations in adjacent base pairs. Kuchino et al. reported that deoxycytosine adjacent to the 3'-side of 8-OHdG directs insertion of all four nucleosides [63]. However, Shibutani et al. reported insertion of only deoxyguanosine no matter what nucleoside was present to the 5'-side of the 8-OHdG lesion [100]. Never the less, 8-OHdG has been the reported cause of G to T and A to C transversions [2, 16, 56, 124].

Increased 8-OHdG levels have been associated with gynecologic cancer [136], bladder cancer [3], breast cancer [69, 72], lung cancer [30], and colorectal cancer [61].

E. Comet Assay

1. Development

The single cell gel electrophoresis assay (comet assay) is becoming an increasingly more common method for the detection of DNA damage. Its development began with Rydberg and Johanson [95] in which they embedded irradiated Chinese hamster cells in agarose, lysed them in alkali and observed the extent of DNA strand separation after staining with acridine orange. This method was later modified by Ostling and Johanson [86] by lysing in a neutral detergent solution and applying a weak electric field prior to fluorescent evaluation with acridine orange. They observed that the amount of DNA migration toward the anode increased in irradiated cells in a dose responsive manner. The cell had the appearance of a comet in which the nucleus represents the head of the comet and the migrated DNA represents the tail. In doing so Ostling and Johanson were able to quantitate this dose/response relationship by measuring the fluorescent intensity at the head of the comet and at various positions of the tail.

Currently there are two versions of the comet assay generally accepted, one introduced by Singh et al. [103] and another by Olive et al. [82]. The versions are similar in principle but the major difference is at the pH of electrophoresis. Singh's method involves electrophoresis at a pH > 13 and is

commonly referred to as the alkaline comet assay. Conversely, Olive's method involves electrophoresis at a pH of 8.3 and therefore commonly referred to as the neutral comet assay. Both methods enable detection of single strand breaks as well as double strand breaks but the alkaline method also reveals alkali labile sites [94]. For this reason, the alkaline comet assay is more commonly used.

2. Methodology

The basic steps in the alkaline comet assay are: obtaining a single cell suspension, preparing the microscope slides, cell lysis, enzyme treatment (optional), alkali unwinding, electrophoresis, neutralization and DNA staining for visualization of the "comet".

A single cell suspension can easily be obtained from cell cultures, leukocytes from whole blood, or from tissues. In the preparation of cell suspension from cell cultures, DNA damaged has been shown to be induced by both cell scraping and trypsinization as compared to control [101]. In order to overcome the damage induced by trypsin a weak solution of Trypsin-EDTA may be used or the cells may be allowed to sit for a period of time after digestion in order to recover [40]. The comet assay can also be performed on whole blood [18, 38, 117], separated leukocytes [18], separated lymphocytes [18, 25, 26, 38, 70, 77, 103, 117, 126], or even more specifically, separated neutrophils [71]. Cell suspension generated from tissues can be prepared by enzymatic digestion [47, 98], mincing [65, 119] or homogenization [47, 76, 98]. The cells may then be suspended in either PBS or their respective media.

Once a single cell suspension has been obtained, the cells are embedded in 1% low melting agarose and mounted on a microscope slide. In the past the comet slides where prepared by layering cells suspended in agarose on microscope slides pre coated with agarose and then applying another layer of agarose as to sandwich the cells in between. Presently, specialized comet slides with hydrophobic barriers to contain the agarose within wells are available. The cell/agarose suspension is placed directly in the wells which greatly reduces preparation time. The agarose is then cooled and allowed to solidify.

After the agarose has solidified the slides are then subjected to a pre-chilled lysis solution at pH 10 containing detergent and a high salt concentration. The purpose of the lysis solution is to compromise the cellular and nuclear membranes in order to expose the nucleus to the high salt which solubilizes the histone proteins that stabilize the negatively super-coiled DNA. What is left is the nucleoid skeleton bound within the agarose cavity previously occupied by the entire cell.

When cell lysis is complete the slides are placed in an alkaline solution of pH>13. The purpose of this step is to allow the lysis solution to diffuse out of the agarose. More importantly, the high alkali disrupts the hydrogen bonding between opposing base pairs of the DNA double helix. This results in the unwinding of the DNA double helix from points where strand breakage has occurred and the generation of DNA fragments.

After alkali unwinding, the cells are subjected to electrophoresis in the alkaline solution. This allows for expression of double strand breaks, single

strand breaks and alkali labile sites. The negatively charged fragments of DNA are pulled toward the anode giving the nucleus its characteristic comet tail. The ability of the DNA fragments to migrate through the agarose depends on the agarose density, number of breaks, fragment size, and the electrophoretic conditions [31, 82].

The next step is to neutralize the slides by submersion in tris-buffer and water. The slides are stained with fluorescent DNA binding dyes. These dyes include acridine orange [86], ethidium bromide [103], and propidium iodide [82]. It should be noted that ethidium bromide is more commonly used but the use of acridine orange can distinguish double stranded DNA (which fluoresces green) from RNA or single stranded DNA (which fluoresces red).

The type of comet scoring varies in the literature. Visual scores can be obtained by categorizing the resulting comets in 5 classes from 0 (no tail) to 4 (almost all DNA in tail) and reported in arbitrary units. They can also be scored by reporting the proportion of cells with damage (comet tails), the extent of DNA migration measured in μm, or as a ratio of length to width with cells exhibiting no migration or tails as a ratio of 1. Alternatively, image analysis software has become available which can produce various types of endpoint measurements. One of these endpoints which has become increasingly popular is referred to as the tail moment and was developed by Olive et al. [82]. The tail moment is defined as the product of the percentage of DNA in the tail (intensity) and the distance between the mean head and tail positions. This parameter takes into

account the size of the fragments (tail length) and the number of fragments (tail intensity).

Direct single or double strand breakage of the phosphate backbone is not the only means of observing DNA damage by the comet assay. The comet assay can also reveal damaged bases or apurenic/apyrimidinic sites by use of specialized endonucleases which are treated between the cell lysis and alkali unwinding steps. The more commonly used enzymes include formamidopyrimidine DNA glycosylase (fpg) for the detection of damaged purines [13, 89, 110, 135], endonuclease III for the detection of damaged pyrimidines [110, 133, 134, 135], protein kinase for the detection of DNA-protein cross-links [66, 75, 138], T4 endonuclease V for the detection of UV induced cyclobutane pyrimidine dimers [89, 110, 133, 134], and Alk A for the detection of 3-methyl adenine [13, 135].

3. Applications

The comet assay is advantageous from the perspective of its sensitivity to DNA damage, sample size required, and it is inexpensive to perform. It can be applied to a variety of studies including genotoxicity, DNA repair, environmental and human biomonitoring as well as clinical studies.

Genotoxicity studies using the comet assay have been performed on a variety of metals, pesticides, nitrosamines, and antineoplastic drugs [94]. The assay is most commonly used to investigate the extent to which certain substances cause single or double strand breaks. However, it can also provide useful information as to the mechanism of damage with use of specific

endonucleases that can recognize various types of damaged bases. It may also prove useful in determining if a substance is a pro or a direct carcinogen by the addition of a S9 fraction, which contains enzymes capable of metabolizing an indirect carcinogen into its active form, to one of the test groups. Conversely, the comet assay can also be used to determine the chemoprotective characteristics of a test substance, for example, the ability of antioxidants to reduce the oxidative effects generated by H_2O_2.

In addition to DNA damage, repair studies have also been performed in which a population of cells is exposed to a known DNA damaging agent and the comet assay performed at various time intervals until the cells have returned to control levels of damage [102]. The kinetics of the repair process may also be examined by the use of repair inhibitors, DNA synthesis inhibitors or chain terminators (nucleic acid analogs that prevent stand elongation) [37].

The comet assay has also proved useful in environmental biomonitoring. This has been accomplished by sampling the white blood cells of fish collected from polluted lakes [90], coelomocytes of earthworms from different soil samples [97, 125], and tissues from rodents living in hazardous waste sites [94, 19]. Likewise, the comet assay has been used in human biomonitoring. For example, the comet assay can be performed on human blood samples from individuals suspected of occupational exposure to DNA damaging agents [109] or nasal epithelial cells from individuals living in air polluted cities [15].

The application of the comet assay has also extended to the clinic. Many studies have been published relating smokers to nonsmokers [11, 34], dietary

habits [41], and physical activity [44]. It has also been used to identify hypoxic cells in solid tumors [80, 81]. These cells are poorly perfused and are 3 times more resistant to cell killing and DNA strand breakage by ionizing radiation. The comet assay has also been useful in the diagnosis of xeroderma pigmentosum [37, 42]. Cells from these patients are deficient in excision repair and do not show strand breaks attributed to repair of DNA damage induced by UV irradiation.

F. Present Study

The human body is constantly under attack by endogenous and exogenous agents which may overpower the body's natural defense mechanisms. Therefore, it is important to have a method of monitoring individuals who may be at risk of DNA damage and the ensuing process of carcinogenesis. The comet assay provides this method and leukocytes are an easy and non-invasive way to obtain cells for use in the assay. However, instances may arise when the samples must be stored for later analysis. It is not known if leukocytes in whole blood or buffy coat samples can be frozen and stored for extended periods without causing artifactual oxidative DNA damage. For this reason, this study investigated the effects of processing time on oxidative DNA damage in rat whole blood, a rat buffy coat, and a human buffy coat via the comet assay. Secondly, it investigated the effects of cryopreservation on oxidative DNA damage. Finally, it compared the usage of the cryoprotectants glycerol and DMSO.

II. Materials and Methods

A. Chemicals and Media

Chemical Name	Catalog Number	Supplier
Bovine Serum Albumin	A-6003	Sigma-Aldrich
Deferoxamine Mesylate	D-9533	Sigma-Aldrich
Dimethyl Sulfoxide	D-2650	Sigma-Aldrich
Ethidium Bromide	E-8751	Sigma-Aldrich
Fpg		Klaunig Lab
Glycerol	G-7893	Sigma-Aldrich
HEPES	H-3375	Sigma-Aldrich
Hydrochloric Acid	A144-212	Fisher
Na_2EDTA	E-5134	Sigma-Aldrich
Potassium Chloride	P-9333	Sigma-Aldrich
Potassium Hydroxide	221473	Sigma-Aldrich
RPMI 1640	R-6504	Sigma-Aldrich
Sodium Bicarbonate	S-4019	Sigma-Aldrich
Sodium Chloride	S-9888	Sigma-Aldrich
Sodium hydroxide	S-8045	Sigma-Aldrich
Sodium Lauryl Sarcosinate	BP234	Fisher
Triton X-100	X-100	Sigma-Aldrich
Trizma Base	T-1503	Sigma-Aldrich
Trizma Hydrochloride	T-3253	Sigma-Aldrich

B. Equipment and Supplies

EC 150 Power Supply

Electrophoresis Chamber

Heparinized Capillary Tubes

Microcentrifuge Tubes
- Screw Cap Tube 2.0 ml
- Screw Cap with O-Rings
- Snap Top 2.0 ml
- Snap Top 1.5ml

Nikon Diaphot Fluorescent Microscope

Nitrile Exam Gloves

Polypropylene Containers (1 L)

Respirator (3M600)

Water Bath

C. Computer Software

Komet 4.0.4

Microsoft Excel

Microsoft Word

Microsoft XP

SigmaStat 3.1

D. Rat Whole Blood Sampling

Male Sprague-Dawley rats (6-8 weeks of age) were purchased from Harlan. Animals were housed and maintained in an AAALAC approved facility at Indiana University. Animals were placed in a CO_2 chamber and administered CO_2 until the animal was no longer conscious. The animal was removed from

the chamber and 2 ml blood was drawn from the orbital sinus using heparinized capillary tubes. The blood was collected in a clear 2.0 ml microcentrifuge tube containing 80 µl of a sterile 0.5 M EDTA solution and inverted several times to ensure adequate mixing.

E. Rat Buffy Coat Preparation

The buffy coat was prepared by collecting 1 ml whole blood as described above in a clear 2.0 ml microcentrifuge tube containing 40 µl of a sterile 0.5 M EDTA solution. The whole blood was then centrifuged at 600 xg for 5 minutes at room temperature. The white layer of leukocytes, also referred to as the buffy coat, was then transferred to a new 2.0 ml microcentrifuge tube containing 2.0 ml RPMI 1640 media.

F. Human Buffy Coat Preparation

The human buffy coat was obtained from the Central Indiana Regional Blood Center (CIRBC) and shipped on ice. After receipt, the sealed end of the collection tube was snipped off with scissors to allow the buffy coat to drain from the blood bag to a 50 ml centrifuge tube.

G. Comet Assay

The comet slides for the fresh rat whole blood and the rat whole blood stored at 25ºC and 4ºC were prepared by adding 5 µl whole blood to 1000 µl of 1% low melting agarose (LMA) in phosphate buffered saline (PBS), pH 7.2, at 42ºC. This resulted in a final LMA concentration of 0.995%. The comet slides for the rat whole blood frozen at -80ºC were prepared by adding 5 µl whole blood to 500 µl of 1% LMA, resulting in a final LMA concentration of 0.990%. The comet slides for the fresh rat buffy coat and the rat buffy coat stored at 25ºC and 4ºC

were prepared by adding 5 µl buffy coat to 500 µl of 1% LMA, resulting in a final LMA concentration of 0.990%. The comet slides for the rat buffy coat frozen at -80ºC were prepared by adding 10 µl buffy coat to 500 µl of 1% LMA, resulting in a final LMA concentration of 0.980%. The comet slides for the fresh human buffy coat and the human buffy coat stored at 25ºC and 4ºC were prepared by adding 1 µl buffy coat to 1000 µl of 1% LMA, resulting in a final LMA concentration of 0.999%. The comet slides for the human buffy coat frozen at -80ºC were prepared by adding 1 µl buffy coat to 500 µl of 1% LMA, resulting in a final LMA concentration of 0.998%. The main concern in preparing the cell suspensions was to maintain a consistent cell number within the rat and human cell suspensions. The effect on the final LMA concentration was minimal and was not considered a great concern. After suspending the cells in agarose, 70 µl of the cell suspension was then pipetted into each well of the comet slides and placed in a refrigerator, 4ºC, for 40 minutes.

Once the agarose had cooled, a pre-chilled stock lysis solution (100 mM Na_2EDTA, 2.5 M NaCl, 10 mM Trizma Base, 1% Sodium Lauryl Sarcosinate, pH 10) was completed by adding 1% Triton X-100, 10% DMSO and 0.1 mM Deferoxamine Mesylate. The comet slides were then submerged in the completed lysis solution and placed in a refrigerator, 4ºC, for 1 hour.

After lysis, the comet slides were removed from the lysis solution, washed 3 times for 5 minutes each in a pre chilled fpg buffer solution (40mM HEPES, 0.1 M KCl, 0.5 mM Na_2EDTA, 0.2 mg/ml bovine serum albumin, pH 8.0) and allowed to dry in the dark at room temperature for 15 minutes. The slides were then

placed in a moist incubating chamber at 37ºC and the gel was covered with either 100 µl of fpg buffer or 100 µl of a 1:100 dilution of the fpg enzyme (0.8 mg protein/ml) in fpg buffer for 1 hour.

The slides were then slowly submerged into a pre chilled alkali buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH 13) and placed in a refrigerator, 4ºC, for 40 minutes to allow for DNA unwinding.

The slides were then transferred to an electrophoresis chamber filled with pre chilled alkali buffer and electrophoresed at 25 V (starting at 285 mA) for 30 minutes.

After electrophoresis was complete, the comet slides were washed in pre chilled Tris buffer (0.4 M Trizma HCl, pH 7.5) for 5 minutes and subsequently washed in pre chilled distilled water for 10 minutes. They were then allowed to dry overnight.

The following morning the slides were stained by placing 25 µl of a 20 ug/ml ethidium bromide in distilled water solution in each well and then covered with a coverglass. The comet slides were visualized using a Nikon Diaphot fluorescent microscope and Komet 4.0.4 software. A total of 150 cells were counted for each treatment group (50 cells per well performed in triplicate).

H. Storage of Whole Blood and Buffy Coat Samples

The same procedure was used in the cryopreservation/thawing of rat whole blood, rat buffy coat, and human buffy coat. After the comet slides were prepared for the fresh blood samples, 750 µl of whole blood was added to an equal volume of one of two freezing mixtures (RPMI 1640 with 10% DMSO or

20% glycerol) in a clear 2.0 ml microcentrifuge tube and inverted several times to ensure adequate mixing. Aliquots of 200 µl where then pipetted into 7 sterilized 2.0 ml screw cap microcentrifuge tubes, transferred to an isopropanol container, and then placed in a -80ºC freezer to be step frozen at a rate of -1ºC/minute. The frozen whole blood samples were thawed by submersion in a 42ºC water bath until there was no visible presence of ice. The comet assay was performed immediately there after as described above. A slight variation was also introduced at the 4 week time point to examine the effect of processing time after the thawing of the samples. In this case, the samples were allowed to incubate in a 37.5ºC water bath for 1 hour prior to the continuation with the comet assay.

The remaining rat whole blood and buffy coat samples were divided into two equal aliquots in a 1.5 ml microcentrifuge tube wrapped in aluminum foil and stored on the work bench at 25ºC or in the refrigerator at 4ºC. As for the human buffy coat, two aliquots of 5 ml were transferred to 15 ml centrifuge tubes wrapped in aluminum foil and stored on the work bench at 25ºC or in the refrigerator at 4ºC. In all studies, cell viability was consistently between 90-100% as determined by trypan blue exclusion.

I. Statistics

The data was analyzed by one-way ANOVA followed by the Holm-Sidak method for comparisons within each storage condition. The Student t-test was used to analyze comparisons between the different storage conditions. In all studies, treatment groups were considered significantly different if $p < 0.05$.

III. Results

A. Rat Whole Blood Storage and DNA Damage

The results for the storage of rat blood at 25ºC are shown in table 1. Oxidative damage was calculated by the difference between the buffer treated slides (representing DNA strand breaks only) and the slides treated with the bacterial fpg enzyme (representing DNA strand breaks and oxidative damage). Over the span of 48 hours, DNA strand breaks did not significantly increase compared to the fresh whole blood until the 48 hour time point (figure 8). However, there was a significant increase in oxidative damage at the 2 hour time point and a further significant increase did not occur until 48 hours after whole blood collection (figure 8).

The results for the storage of rat blood at 4ºC are shown in table 2. Over the span of 48 hours, DNA strand breaks did not significantly increase compared to the fresh whole blood, however, there was a significant decrease at 1 and 2 hours after whole blood collection (figure 9). There was a significant increase in oxidative damage 24 hours after whole blood collection and a further increase at 48 hours (figure 9).

The storage of rat whole blood at 25ºC compared to 4ºC showed a significant decrease in DNA strand breaks at the fresh and 2 hour time points and a significant increase at the 48 hour time point. Likewise, oxidative damage was significantly increased at the time points of 2, 24, and 48 hours.

The results for the storage of rat whole blood at -80ºC with the use of glycerol or DMSO as the cryoprotectant are shown in table 3 and table 4

respectively. Over the span of 4 weeks, there was not a significant change in DNA strand breaks or oxidative damage for either of the cryoprotectants used (figure 10 and 11).

The storage of rat whole blood at -80ºC with glycerol as the cryoprotectant compared to DMSO showed a significant increase in DNA strand breaks at 1, 3, and 4 weeks of storage. There was no significant difference in oxidative damage for the entire 4 weeks of storage.

When the whole blood samples cryopreserved for 4 weeks were allowed to incubate in a 37.5ºC water bath for 1 hour before the comet assay was performed, there was a significant increase in DNA strand breaks compared to the fresh whole blood for both cryoprotectants used. There was also a significant increase in oxidative damage, however, this only occurred in the whole blood frozen with DMSO as the cryoprotectant (figure 16).

B. Rat Buffy Coat Storage and DNA Damage

The results for the storage of rat buffy coat at 25ºC are shown in table 5. Over the span of 48 hours, DNA strand breaks did not significantly increase compared to the fresh buffy coat until the 24 hour time point and a further increase was observed at 48 hours after the buffy coat was prepared (figure 12). There was a significant increase in oxidative damage 24 hours after the buffy coat was prepared and this damage was significantly decreased at 48 hours (figure 12).

The results for the storage of rat buffy coat at 4ºC are shown in table 6. At the 1 and 2 hour time points there was a significant decrease in DNA strand

breaks compared to the fresh time point (figure 13). There was not a significant increase in oxidative damage up to 48 hours compared to the fresh time point, however, a significant increase in oxidative damage was observed at the 48 hour time point compared to 1 and 2 hour time point (figure 13).

The storage of rat buffy coat at 25ºC compared to 4ºC showed a significant increase in DNA strand breaks at the 24 and 48 hour time points. Oxidative damage was significantly increased at 2 and 24 hours.

The results for the storage of rat buffy coat at -80ºC with the use of glycerol and DMSO as cryoprotectants are shown in table 7 and table 8 respectively. Over the span of 4 weeks, there was not a significant change in DNA strand breaks or oxidative damage for either of the cryoprotectants used (figure 14 and 15).

The storage of a rat buffy coat at -80ºC with glycerol as the cryoprotectant compared to DMSO showed a no significant difference in DNA strand breaks or oxidative damage for the entire 4 weeks of storage.

When the buffy coat samples cryopreserved for 4 weeks were allowed to incubate in a 37.5ºC water bath for 1 hour before the comet assay was performed, there was also a significant increase in DNA strand breaks and oxidative damage in both the glycerol and DMSO cryoprotected buffy coats compared to the fresh buffy coat (figure 16).

C. Comparison of Rat Whole Blood vs. Buffy Coat Storage

When comparing the storage rat whole blood to a buffy coat at 25ºC a significant decrease in DNA strand breaks was observed at the fresh time point,

however, a significant increase was observed at 48 hours. A significant decrease in oxidative damage was observed at the fresh, 1 hour, and 24 hour time points.

Storage at 4ºC resulted in a significant decrease in DNA strand breaks at the fresh, 1, 2, and 48 hour time points when comparing rat whole blood to a buffy coat. Oxidative damage in rat whole blood was significantly lower at the fresh, 1, 2, and 24 hour time points compared to the buffy coat.

Storage at -80ºC with the use of the cryoprotectant glycerol did not show any significant differences in DNA strand breaks between rat whole blood and a buffy coat for the entire 4 weeks of storage. However, a significantly lower amount of oxidative damage in rat whole blood was observed at the 1, 2, and 4 week time points when compared to a buffy coat. Storage at -80ºC with the use of the cryoprotectant DMSO also did not show any significant differences in DNA strand breaks. However, a significantly lower amount of oxidative damage in rat whole blood was observed at the 2 week time point when compared to a buffy coat.

D. Human Buffy Coat Storage and DNA Damage

The results for the storage of a human buffy coat at 25ºC and 4ºC are shown in tables 9 and 10 respectively. Over the span of 48 hours, DNA strand breaks did not significantly increase for either storage temperature until the 48 hour time point (figures 17 and 18). Likewise, oxidative damage was not significantly increased until the 48 hour time point (figures 17 and 18).

The storage of a human buffy coat at 25ºC compared to 4ºC showed a significant increase in DNA strand breaks at the 24 and 48 hour time points. Oxidative damage in the human buffy stored at 25ºC was significantly greater at the 48 hour time point.

The results for the storage of a human buffy coat at -80ºC with the use of glycerol as the cryoprotectant are shown in table 11. Over the span of 4 weeks, there was a significant increase in DNA strand breaks at the 1 week time point compared to the fresh time point. The 1 week time point was also significantly greater than the 2 and 3 week time points (figure 19). There was no significant difference in oxidative damage for the entire 4 weeks of storage (figure 19).

The results for the storage of a human buffy coat at -80ºC with the use of DMSO as the cryoprotectant are shown in table 12. Over the span of 4 weeks, there was a significant increase in DNA strand breaks at the 1 day time point compared to the fresh time point. The 1 day time point was also significantly greater than the 4 week time point (figure 20). There was a significant increase in oxidative damage at the 3 week time point compared to the fresh time point. The 3 week time point was also significantly greater than the 1 day time point (figure 20).

The storage of a human buffy coat at -80ºC with glycerol as the cryoprotectant compared to DMSO showed a significant increase in DNA strand breaks at the 3 and 4 week time points. There was no significant difference in oxidative damage for the entire 4 weeks of storage.

Time Point	Treatment	Animal # 2764	Animal # 2765	Animal # 2766	Mean	SEM
Fresh	Buffer	0.15	0.13	0.14	0.14	0.01
	Fpg	0.23	0.33	0.32	0.29	0.03
	Oxidative Damage	0.08	0.20	0.18	0.15	0.04
1 hour	Buffer	0.15	0.12	0.20	0.16	0.02
	Fpg	0.30	0.28	0.42	0.33	0.04
	Oxidative Damage	0.15	0.16	0.22	0.18	0.02
2 hours	Buffer	0.15	0.14	0.14	0.14	0.00
	Fpg	0.61	0.83	0.59	0.68	0.08
	Oxidative Damage	0.46	0.69	0.45	0.53	0.08
24 hours	Buffer	0.50	0.90	0.44	0.61	0.14
	Fpg	1.05	1.31	0.99	1.12	0.10
	Oxidative Damage	0.55	0.41	0.55	0.50	0.05
48 hours	Buffer	7.29	6.31	5.72	6.44	0.46
	Fpg	8.06	7.36	6.92	7.45	0.33
	Oxidative Damage	0.77	1.05	1.20	1.01	0.13

Table 1. Storage of Rat Whole Blood at 25ºC

Tail moment values for the storage of rat whole blood at 25ºC.
Oxidative damage was assessed by the difference between buffy and fpg treated slides.

Time Point	Treatment	Animal # 2764	Animal # 2765	Animal # 2766	Mean	SEM
Fresh	Buffer	0.22	0.21	0.22	0.22	0.00
	Fpg	0.38	0.34	0.34	0.35	0.01
	Oxidative Damage	0.16	0.13	0.12	0.14	0.01
1 hour	Buffer	0.14	0.18	0.16	0.16	0.01
	Fpg	0.30	0.29	0.35	0.31	0.02
	Oxidative Damage	0.16	0.11	0.19	0.15	0.02
2 hours	Buffer	0.16	0.17	0.16	0.16	0.00
	Fpg	0.32	0.31	0.38	0.34	0.02
	Oxidative Damage	0.16	0.14	0.22	0.17	0.02
24 hours	Buffer	0.23	0.25	0.22	0.23	0.01
	Fpg	0.63	0.55	0.56	0.58	0.03
	Oxidative Damage	0.40	0.30	0.34	0.35	0.03
48 hours	Buffer	0.17	0.20	0.19	0.19	0.01
	Fpg	0.65	0.63	0.70	0.66	0.02
	Oxidative Damage	0.48	0.43	0.51	0.47	0.02

Table 2. Storage of Rat Whole Blood at 4ºC

Tail moment values for rat whole blood stored at 4ºC.
Oxidative damage was assessed by the difference between buffy and fpg treated slides.

Time Point	Treatment	Animal # 2764	# 2765	# 2766	# 2767	# 2768	# 2769	Mean	SEM
Fresh	Buffer	0.21	0.37	0.29	0.26	0.26	0.22	0.27	0.02
	Fpg	1.51	0.83	0.74	1.41	1.16	1.27	1.15	0.13
	Oxidative Damage	1.30	0.46	0.45	1.15	0.90	1.05	0.89	0.15
1 day	Buffer	0.63	0.55	0.31	0.24	0.23	0.24	0.37	0.07
	Fpg	2.58	1.78	1.04	0.86	0.95	1.35	1.43	0.27
	Oxidative Damage	1.95	1.23	0.73	0.62	0.72	1.11	1.06	0.20
1 week	Buffer	0.42	0.31	0.35	0.32	0.31	0.30	0.34	0.02
	Fpg	0.70	0.62	0.92	0.90	1.08	1.70	0.99	0.16
	Oxidative Damage	0.28	0.31	0.57	0.58	0.77	1.40	0.65	0.17
2 weeks	Buffer	0.45	0.30	0.36	0.30	0.40	0.31	0.35	0.03
	Fpg	1.40	0.76	1.20	1.35	1.29	1.48	1.25	0.10
	Oxidative Damage	0.95	0.46	0.84	1.05	0.89	1.17	0.89	0.10
3 weeks	Buffer	0.42	0.38	0.33	0.31	0.35	0.34	0.36	0.02
	Fpg	1.18	1.44	0.66	1.17	1.20	1.47	1.19	0.12
	Oxidative Damage	0.76	1.06	0.33	0.86	0.85	1.13	0.83	0.12
4 weeks	Buffer	0.30	0.35	0.34	0.34	0.38	0.36	0.35	0.01
	Fpg	1.17	0.93	1.27	1.04	1.02	1.25	1.11	0.06
	Oxidative Damage	0.87	0.58	0.93	0.70	0.64	0.89	0.77	0.06
4 weeks + 1 hour	Buffer				1.10	0.91	0.95	0.99	0.06
	Fpg				2.46	1.99	2.41	2.29	0.15
	Oxidative Damage				1.36	1.08	1.46	1.30	0.11

Table 3. Storage of Rat Whole Blood at -80ºC (glycerol)

Tail moment values for the storage of rat whole blood frozen with 10% glycerol at -80ºC. Oxidative damage was assessed by the difference between buffy and fpg treated slides.

Time Point	Treatment	Animal # 2764	# 2765	# 2766	# 2767	# 2768	# 2769	Mean	SEM
1 day	Buffer	0.61	0.51	0.28	0.16	0.17	0.21	0.32	0.08
	Fpg	1.86	0.92	0.62	0.86	1.01	0.99	1.04	0.17
	Oxidative Damage	1.25	0.41	0.34	0.70	0.84	0.78	0.72	0.13
1 week	Buffer	0.35	0.27	0.23	0.26	0.24	0.23	0.26	0.02
	Fpg	0.72	0.58	0.76	1.46	0.91	1.32	0.96	0.14
	Oxidative Damage	0.37	0.31	0.53	1.20	0.67	1.09	0.70	0.15
2 weeks	Buffer	0.42	0.25	0.32	0.25	0.27	0.22	0.29	0.03
	Fpg	0.81	0.50	1.20	1.01	1.16	1.35	1.01	0.13
	Oxidative Damage	0.39	0.25	0.88	0.76	0.89	1.13	0.72	0.14
3 weeks	Buffer	0.35	0.31	0.29	0.27	0.24	0.23	0.28	0.02
	Fpg	1.54	1.82	0.49	1.26	1.38	1.45	1.32	0.18
	Oxidative Damage	1.19	1.51	0.20	0.99	1.14	1.22	1.04	0.18
4 weeks	Buffer	0.26	0.33	0.29	0.23	0.22	0.25	0.26	0.02
	Fpg	1.25	1.35	0.66	1.10	1.29	1.02	1.11	0.10
	Oxidative Damage	0.99	1.02	0.37	0.87	1.07	0.77	0.85	0.11
4 weeks + 1 hour	Buffer				0.51	1.25	1.46	1.07	0.29
	Fpg				2.87	5.27	4.79	4.31	0.73
	Oxidative Damage				2.36	4.02	3.33	3.24	0.48

Table 4. Storage of Rat Whole Blood at -80ºC (DMSO)

Tail moment values for the storage of rat whole blood frozen with 5% DMSO at -80ºC. Oxidative damage was assessed by the difference between buffy and fpg treated slides. See Table 3 for the tail moment values of the fresh whole blood.

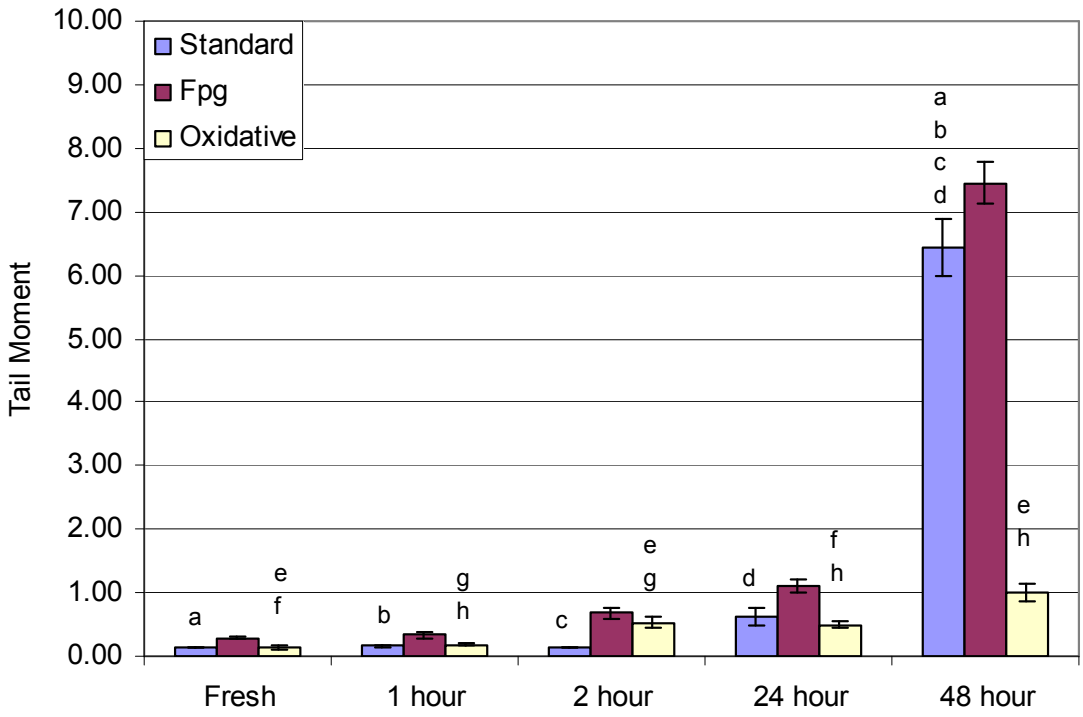

Figure 8. Storage of Rat Whole Blood at 25ºC

The comet assay was performed on fresh rat whole blood or rat whole blood stored on the bench at 25ºC for 1, 2, 24, or 48 hours. The bars labeled as "Standard" represent the slides treated with fpg buffer. The bars labeled as "fpg" represent the slides treated with the enzyme fpg. The bars labeled as "Oxidative" represent the difference between the buffer treated slides and the slides treated with fpg. The results are means ± SEM of 150 cells counted for n = 3 rats. Bars sharing a common letter are significantly different ($p < 0.05$) from one another in either the standard of the oxidative measurements according to a one way ANOVA followed by the Holm-Sidak method.

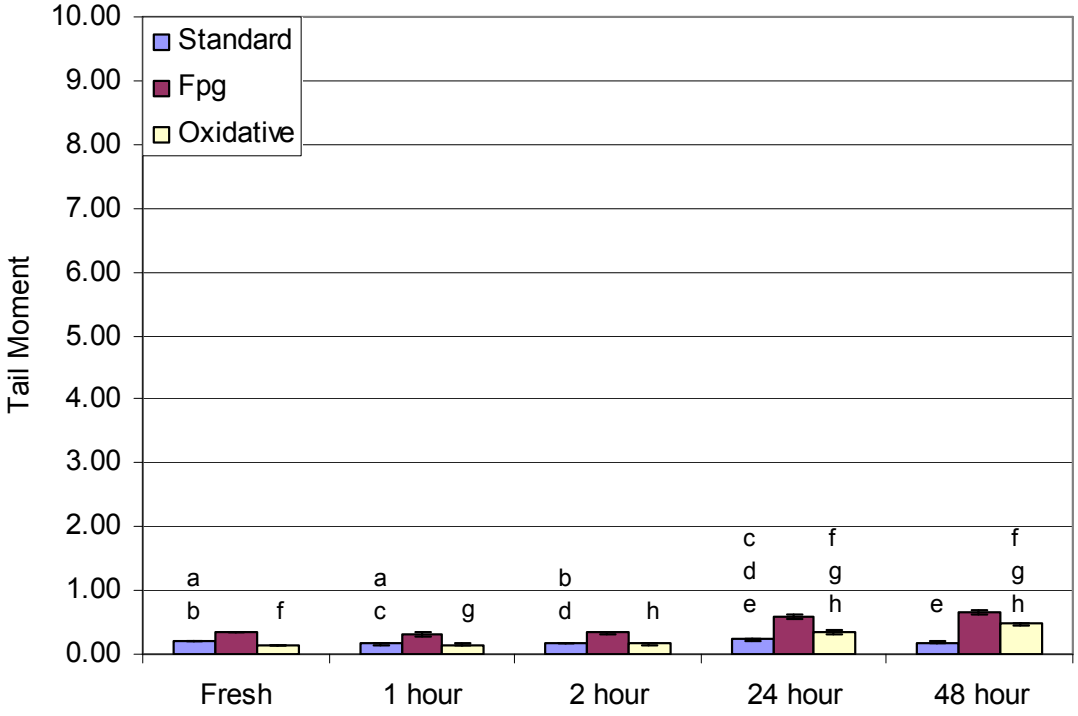

Figure 9. Storage of Rat Whole Blood at 4ºC

The comet assay was performed on fresh rat whole blood or rat whole blood stored in the refrigerator at 4ºC for 1, 2, 24, or 48 hours. The bars labeled as "Standard" represent the slides treated with fpg buffer. The bars labeled as "fpg" represent the slides treated with the enzyme fpg. The bars labeled as "Oxidative" represent the difference between the buffer treated slides and the slides treated with fpg. The results are means ± SEM of 150 cells counted for n = 3 rats. Bars sharing a common letter are significantly different ($p < 0.05$) from one another in either the standard of the oxidative measurements according to a one way ANOVA followed by the Holm-Sidak method.

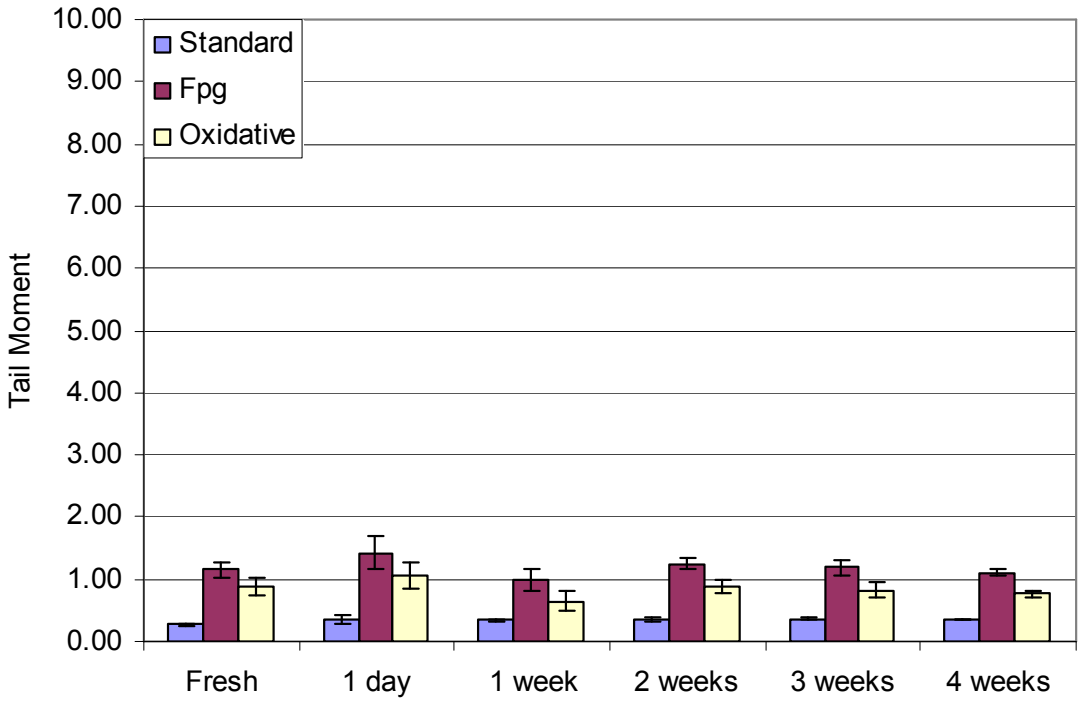

Figure 10. Storage of Rat Whole Blood at -80ºC (glycerol)

The comet assay was performed on fresh rat whole blood or rat whole blood cryopreserved with 10% glycerol in a -80ºC freezer for 1 day, 1 week, 2 weeks, 3 weeks, or 4 weeks. The bars labeled as "Standard" represent the slides treated with fpg buffer. The bars labeled as "fpg" represent the slides treated with the enzyme fpg. The bars labeled as "Oxidative" represent the difference between the buffer treated slides and the slides treated with fpg. The results are means ± SEM of 150 cells counted for n = 6 rats. No significant difference ($p < 0.05$) was observed according to a one way ANOVA.

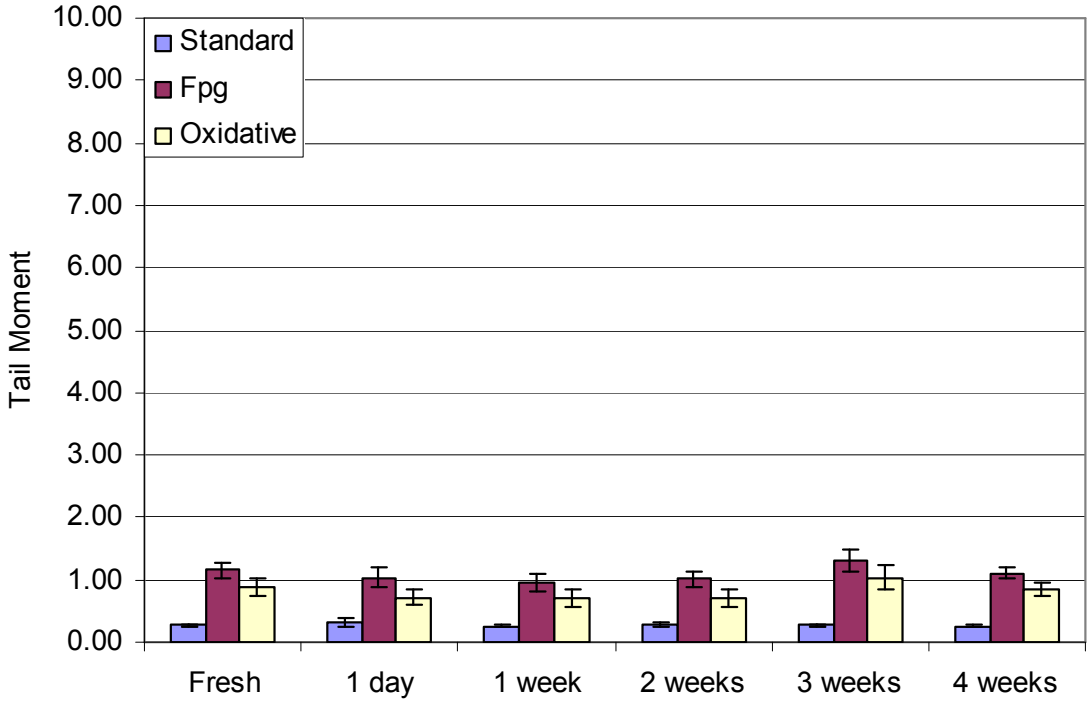

Figure 11. Storage of Rat Whole Blood at -80ºC (DMSO)

The comet assay was performed on fresh rat whole blood or rat whole blood cryopreserved with 5% DMSO in a -80ºC freezer for 1 day, 1 week, 2 weeks, 3 weeks, or 4 weeks. The bars labeled as “Standard” represent the slides treated with fpg buffer. The bars labeled as “fpg” represent the slides treated with the enzyme fpg. The bars labeled as “Oxidative” represent the difference between the buffer treated slides and the slides treated with fpg. The results are means ± SEM of 150 cells counted for n = 6 rats. No significant difference ($p < 0.05$) was observed according to a one way ANOVA.

Time Point	Treatment	Animal # 2767	# 2768	# 2769	# 2770	# 2771	# 2772	Mean	SEM
Fresh	Buffer	0.31	0.32	0.29	0.30	0.28	0.27	0.30	0.01
	Fpg	0.84	1.28	1.39	1.28	0.97	1.44	1.20	0.10
	Oxidative Damage	0.53	0.96	1.10	0.98	0.69	1.17	0.91	0.10
1 hour	Buffer	0.43	0.29	0.24	0.19	0.16	0.21	0.25	0.04
	Fpg	1.05	1.32	1.44	0.84	0.88	1.01	1.09	0.10
	Oxidative Damage	0.62	1.03	1.20	0.65	0.72	0.80	0.84	0.09
2 hour	Buffer	0.52	0.30	0.22	0.19	0.23	0.18	0.27	0.05
	Fpg	1.15	0.96	1.15	0.95	0.78	1.00	1.00	0.06
	Oxidative Damage	0.63	0.66	0.93	0.76	0.55	0.82	0.73	0.06
24 hour	Buffer	2.45	0.96	0.41	0.78	1.05	1.16	1.14	0.28
	Fpg	5.70	4.64	3.77	7.73	8.50	6.36	6.12	0.73
	Oxidative Damage	3.25	3.68	3.36	6.95	7.45	5.20	4.98	0.76
48 hour	Buffer	1.35	1.60	1.28	3.20	2.87	3.09	2.23	0.37
	Fpg	2.49	2.92	3.62	6.43	4.83	4.55	4.14	0.59
	Oxidative Damage	1.14	1.32	2.34	3.23	1.96	1.46	1.91	0.32

Table 5. Storage of Rat Buffy Coat at 25ºC

Tail moment values for the storage of rat buffy coat at 25ºC.
Oxidative damage was assessed by the difference between buffy and fpg treated slides.

Time Point	Treatment	Animal # 2767	# 2768	# 2769	# 2770	# 2771	# 2772	Mean	SEM
1 hour	Buffer	0.28	0.25	0.21	0.23	0.21	0.24	0.24	0.01
	Fpg	0.81	0.97	1.06	0.91	0.87	0.85	0.91	0.04
	Oxidative Damage	0.53	0.72	0.85	0.68	0.66	0.61	0.68	0.04
2 hour	Buffer	0.25	0.21	0.19	0.17	0.20	0.20	0.20	0.01
	Fpg	0.58	0.79	0.68	0.78	0.77	0.85	0.74	0.04
	Oxidative Damage	0.33	0.58	0.49	0.61	0.57	0.65	0.54	0.05
24 hour	Buffer	0.35	0.28	0.20	0.28	0.26	0.27	0.27	0.02
	Fpg	1.13	1.05	1.30	1.52	1.14	2.02	1.36	0.15
	Oxidative Damage	0.78	0.77	1.10	1.24	0.88	1.75	1.09	0.15
48 hour	Buffer	0.29	0.28	0.32	0.27	0.32	0.29	0.30	0.01
	Fpg	1.07	1.32	1.48	2.39	1.01	2.75	1.67	0.30
	Oxidative Damage	0.78	1.04	1.16	2.12	0.69	2.46	1.38	0.30

Table 6. Storage of Rat Buffy Coat at 4ºC

Tail moment values for the storage of rat buffy coat at 4ºC.
Oxidative damage was assessed by the difference between buffy and fpg treated slides.
See Table 5 for the tail moment values of the fresh buffy coat.

Time Point	Treatment	Animal # 2767	# 2768	# 2769	# 2770	# 2771	# 2772	Mean	SEM
1 day	Buffer	0.42	0.36	0.31	0.27	0.19	0.26	0.30	0.03
	Fpg	1.31	1.10	1.23	2.14	1.82	0.98	1.43	0.18
	Oxidative Damage	0.89	0.74	0.92	1.87	1.63	0.72	1.13	0.20
1 week	Buffer	0.34	0.37	0.30	0.34	0.28	0.33	0.33	0.01
	Fpg	1.71	1.56	1.86	1.24	1.31	1.32	1.50	0.10
	Oxidative Damage	1.37	1.19	1.56	0.90	1.03	0.99	1.17	0.10
2 weeks	Buffer	0.39	0.26	0.27	0.31	0.28	0.30	0.30	0.02
	Fpg	1.28	1.22	1.79	2.08	1.77	1.84	1.66	0.14
	Oxidative Damage	0.89	0.96	1.52	1.77	1.49	1.54	1.36	0.14
3 weeks	Buffer	0.35	0.31	0.35	0.25	0.31	0.32	0.32	0.02
	Fpg	1.27	1.56	1.85	1.25	1.21	1.50	1.44	0.10
	Oxidative Damage	0.92	1.25	1.50	1.00	0.90	1.18	1.13	0.09
4 weeks	Buffer	0.38	0.36	0.33	0.22	0.21	0.23	0.29	0.03
	Fpg	1.19	1.36	1.69	1.06	1.24	1.26	1.30	0.09
	Oxidative Damage	0.81	1.00	1.36	0.84	1.03	1.03	1.01	0.08
4 weeks + 1 hour	Buffer				2.05	1.24	1.47	1.59	0.24
	Fpg				3.72	2.81	3.07	3.20	0.27
	Oxidative Damage				1.67	1.57	1.60	1.61	0.03

Table 7. Storage of Rat Buffy Coat at -80ºC (glycerol)

Tail moment values for the storage of rat buffy coat frozen with glycerol at -80ºC.
Oxidative damage was assessed by the difference between buffy and fpg treated slides.
See Table 5 for the tail moment values of the fresh buffy coat.

Time Point	Treatment	Animal # 2767	# 2768	# 2769	# 2770	# 2771	# 2772	Mean	SEM
1 day	Buffer	0.32	0.27	0.32	0.20	0.24	0.21	0.26	0.02
	Fpg	1.15	1.10	1.23	1.54	0.98	1.16	1.19	0.08
	Oxidative Damage	0.83	0.83	0.91	1.34	0.74	0.95	0.93	0.09
1 week	Buffer	0.39	0.27	0.28	0.25	0.28	0.21	0.28	0.02
	Fpg	1.33	1.16	1.80	1.02	0.98	0.82	1.19	0.14
	Oxidative Damage	0.94	0.89	1.52	0.77	0.70	0.61	0.91	0.13
2 weeks	Buffer	0.33	0.28	0.30	0.25	0.24	0.26	0.28	0.01
	Fpg	1.35	1.17	1.62	1.70	1.30	1.77	1.49	0.10
	Oxidative Damage	1.02	0.89	1.32	1.45	1.06	1.51	1.21	0.10
3 weeks	Buffer	0.41	0.24	0.26	0.22	0.23	0.19	0.26	0.03
	Fpg	1.14	1.21	1.74	1.01	1.03	1.37	1.25	0.11
	Oxidative Damage	0.73	0.97	1.48	0.79	0.80	1.18	0.99	0.12
4 weeks	Buffer	0.36	0.31	0.42	0.25	0.22	0.19	0.29	0.04
	Fpg	1.06	1.15	1.57	1.26	1.22	1.36	1.27	0.07
	Oxidative Damage	0.70	0.84	1.15	1.01	1.00	1.17	0.98	0.07
4 weeks + 1 hour	Buffer				1.20	1.40	0.96	1.19	0.13
	Fpg				2.57	2.89	2.34	2.60	0.16
	Oxidative Damage				1.37	1.49	1.38	1.41	0.04

Table 8. Storage of Rat Buffy Coat at -80ºC (DMSO)

Tail moment values for the storage of rat buffy coat frozen with DMSO at -80ºC.
Oxidative damage was assessed by the difference between buffy and fpg treated slides.
See Table 5 for the tail moment values of the fresh buffy coat.

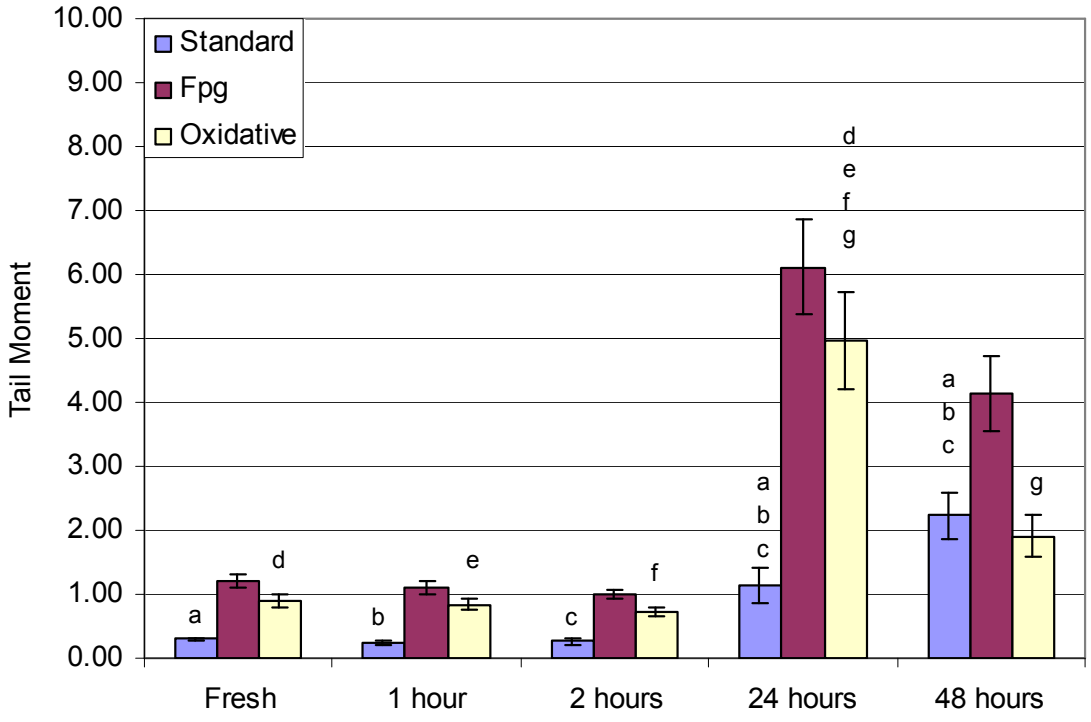

Figure 12. Storage of Rat Buffy Coat at 25ºC

The comet assay was performed on a fresh rat buffy coat or a rat buffy coat stored on the bench at 25ºC for 1, 2, 24, or 48 hours. The bars labeled as "Standard" represent the slides treated with fpg buffer. The bars labeled as "fpg" represent the slides treated with the enzyme fpg. The bars labeled as "Oxidative" represent the difference between the buffer treated slides and the slides treated with fpg. The results are means ± SEM of 150 cells counted for n = 6 rats. Bars sharing a common letter are significantly different ($p < 0.05$) from one another in either the standard of the oxidative measurements according to a one way ANOVA followed by the Holm-Sidak method.

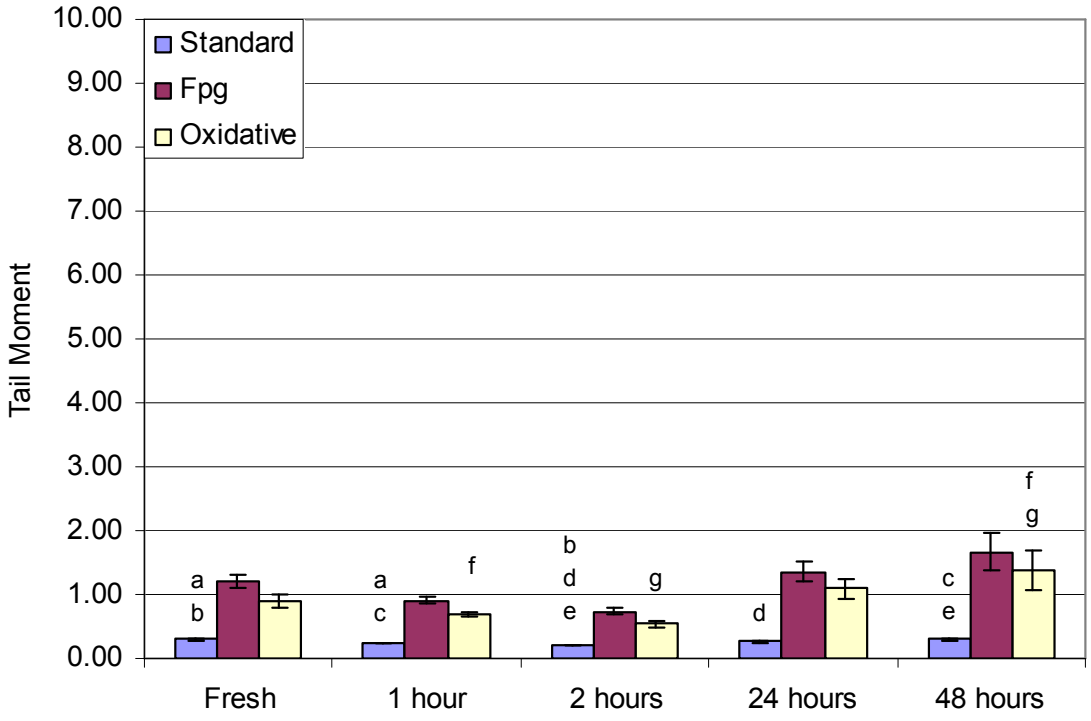

Figure 13. Storage of Rat Buffy Coat at 4ºC

The comet assay was performed on a fresh rat buffy coat or a rat buffy coat stored in the refrigerator 4ºC for 1, 2, 24, or 48 hours. The bars labeled as "Standard" represent the slides treated with fpg buffer. The bars labeled as "fpg" represent the slides treated with the enzyme fpg. The bars labeled as "Oxidative" represent the difference between the buffer treated slides and the slides treated with fpg. The results are means ± SEM of 150 cells counted for n = 6 rats. Bars sharing a common letter are significantly different ($p < 0.05$) from one another in either the standard of the oxidative measurements according to a one way ANOVA followed by the Holm-Sidak method.

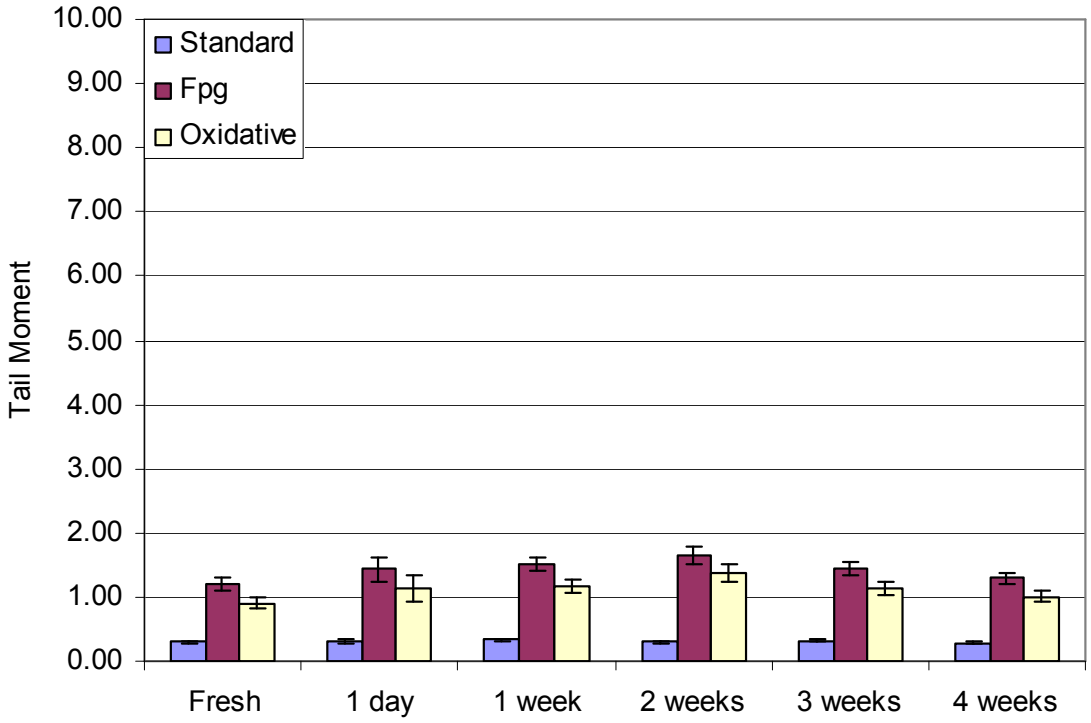

Figure 14. Storage of Rat Buffy Coat at -80ºC (glycerol)

The comet assay was performed on a fresh rat buffy coat or a rat buffy coat cryopreserved with 10% glycerol in a -80ºC freezer for 1 day, 1 week, 2 weeks, 3 weeks, or 4 weeks. The bars labeled as “Standard” represent the slides treated with fpg buffer. The bars labeled as “fpg” represent the slides treated with the enzyme fpg. The bars labeled as “Oxidative” represent the difference between the buffer treated slides and the slides treated with fpg. The results are means ± SEM of 150 cells counted for n = 6 rats. No significant difference ($p < 0.05$) was observed according to a one way ANOVA.

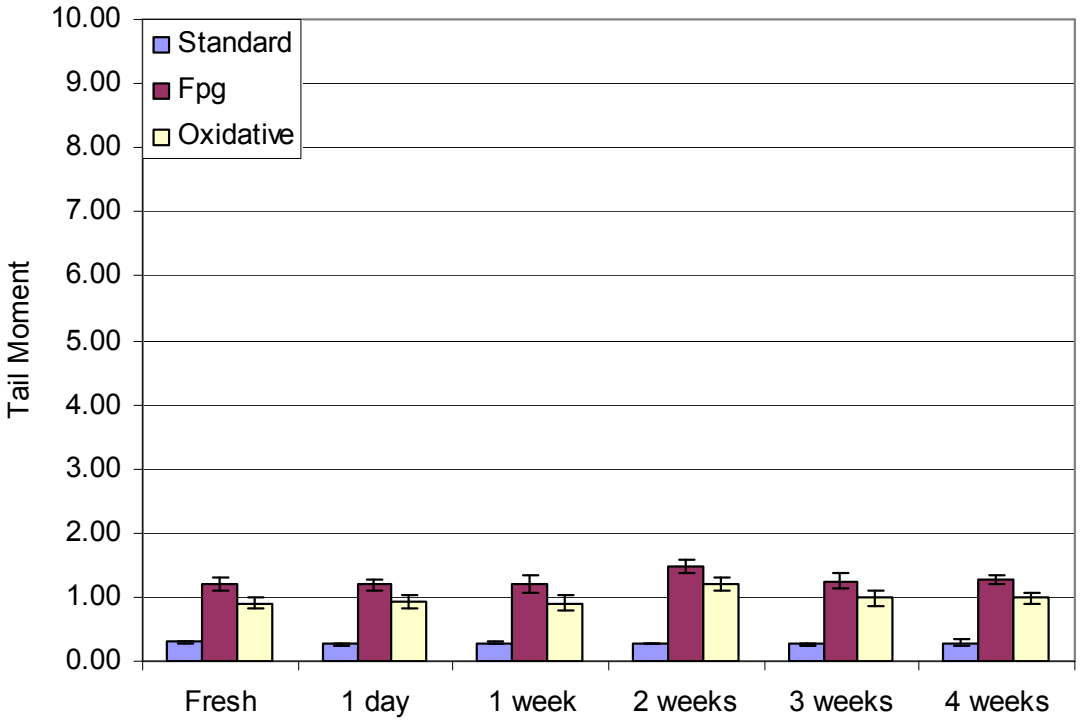

Figure 15. Storage of Rat Buffy Coat at -80ºC (DMSO)

The comet assay was performed on a fresh rat buffy coat or a rat buffy coat cryopreserved with 5% DMSO in a -80ºC freezer for 1 day, 1 week, 2 weeks, 3 weeks, or 4 weeks. The bars labeled as “Standard” represent the slides treated with fpg buffer. The bars labeled as “fpg” represent the slides treated with the enzyme fpg. The bars labeled as “Oxidative” represent the difference between the buffer treated slides and the slides treated with fpg. The results are means ± SEM of 150 cells counted for n = 6 rats. No significant difference ($p < 0.05$) was observed according to a one way ANOVA.

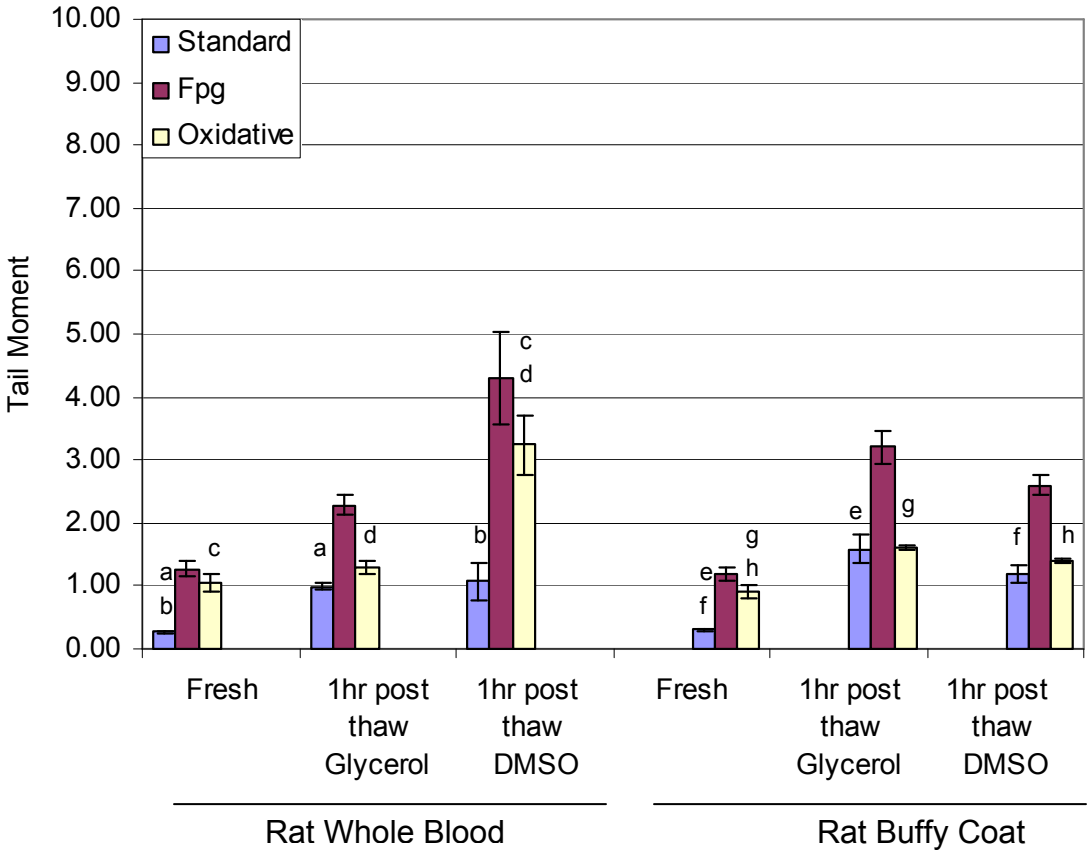

Figure 16. 1 Hour Post Sample Thaw

The comet assay was performed on fresh rat whole blood or a rat buffy coat. After cryopreservation for 4 weeks with either 10% glycerol or 5% DMSO, the comet assay was performed 1 hour post sample thaw. The bars labeled as "Standard" represent the slides treated with fpg buffer. The bars labeled as "fpg" represent the slides treated with the enzyme fpg. The bars labeled as "Oxidative" represent the difference between the buffer treated slides and the slides treated with fpg. The results are means ± SEM of 150 cells counted for n = 6 rats for the fresh time point and n = 3 rats for the 1 hour post thaw time point. Bars sharing a common letter are significantly different ($p < 0.05$) from one another in either the standard of the oxidative measurements according to a one way ANOVA followed by the Holm-Sidak method. No comparison was made between the rat whole blood and rat buffy coat.

Time Point	Treatment	Human # 1	Human # 2	Human # 3	Mean	SEM
Fresh**	Buffer	0.17	0.20	0.20	0.19	0.01
	Fpg	0.43	0.37	0.49	0.43	0.03
	Oxidative Damage	0.26	0.17	0.29	0.24	0.04
24 hour	Buffer	0.75	0.48	0.37	0.53	0.11
	Fpg	0.96	0.78	0.60	0.78	0.10
	Oxidative Damage	0.21	0.30	0.23	0.25	0.03
48 hour	Buffer	2.32	1.51	1.75	1.86	0.24
	Fpg	4.46	3.32	3.56	3.78	0.35
	Oxidative Damage	2.14	1.81	1.81	1.92	0.11

Table 9. Storage of Human Buffy Coat at 25ºC

Tail moment values for the storage of human buffy coat at 25ºC.
Oxidative damage was assessed by the difference between buffy and fpg treated slides.
**Comet assay performed immediately upon receipt from CIRBC.

Time Point	Treatment	Human # 1	Human # 2	Human # 3	Mean	SEM
24 hour	Buffer	0.16	0.18	0.21	0.18	0.01
	Fpg	0.37	0.44	0.53	0.45	0.05
	Oxidative Damage	0.21	0.26	0.32	0.26	0.03
48 hour	Buffer	0.25	0.27	0.30	0.27	0.01
	Fpg	1.28	1.56	1.28	1.37	0.09
	Oxidative Damage	1.03	1.29	0.98	1.10	0.10

Table 10. Storage of Human Buffy Coat at 4ºC

Tail moment values for the storage of human buffy coat at 4ºC.
Oxidative damage was assessed by the difference between buffy and fpg treated slides.
See Table 9 for the tail moment values of the fresh buffy coat.

Time Point	Treatment	Human # 1	Human # 2	Human # 3	Mean	SEM
1 day	Buffer	0.66	0.50	0.59	0.58	0.05
	Fpg	1.80	1.20	1.21	1.40	0.20
	Oxidative Damage	1.14	0.70	0.62	0.82	0.16
1 week	Buffer	1.34	0.77	0.65	0.92	0.21
	Fpg	2.31	1.31	1.50	1.71	0.31
	Oxidative Damage	0.97	0.54	0.85	0.79	0.13
2 weeks	Buffer	0.36	0.35	0.23	0.31	0.04
	Fpg	2.67	1.59	1.63	1.96	0.35
	Oxidative Damage	2.31	1.24	1.40	1.65	0.33
3 weeks	Buffer	0.44	0.31	0.30	0.35	0.05
	Fpg	1.78	1.07	3.04	1.96	0.58
	Oxidative Damage	1.34	0.76	2.74	1.61	0.59
4 weeks	Buffer	0.58	0.52	0.46	0.52	0.03
	Fpg	1.42	1.81	2.31	1.85	0.26
	Oxidative Damage	0.84	1.29	1.85	1.33	0.29

Table 11. Storage of Human Buffy Coat at -80ºC (glycerol)

Tail moment values for the storage of human buffy coat frozen with glycerol at -80ºC. Oxidative damage was assessed by the difference between buffy and fpg treated slides. See Table 9 for the tail moment values of the fresh buffy coat.

Time Point	Treatment	Human # 1	Human # 2	Human # 3	Mean	SEM
1 day	Buffer	0.51	0.38	0.28	0.39	0.07
	Fpg	0.78	0.88	0.63	0.76	0.07
	Oxidative Damage	0.27	0.50	0.35	0.37	0.07
1 week	Buffer	0.35	0.44	0.28	0.36	0.05
	Fpg	1.52	1.27	1.07	1.29	0.13
	Oxidative Damage	1.17	0.83	0.79	0.93	0.12
2 weeks	Buffer	0.23	0.29	0.20	0.24	0.03
	Fpg	1.15	1.04	1.13	1.11	0.03
	Oxidative Damage	0.92	0.75	0.93	0.87	0.06
3 weeks	Buffer	0.21	0.21	0.21	0.21	0.00
	Fpg	1.29	1.10	2.08	1.49	0.30
	Oxidative Damage	1.08	0.89	1.87	1.28	0.30
4 weeks	Buffer	0.18	0.18	0.24	0.20	0.02
	Fpg	1.11	0.83	1.74	1.23	0.27
	Oxidative Damage	0.93	0.65	1.50	1.03	0.25

Table 12. Storage of Human Buffy Coat at -80ºC (DMSO)

Tail moment values for the storage of human buffy coat frozen with DMSO at -80ºC. Oxidative damage was assessed by the difference between buffy and fpg treated slides. See Table 9 for the tail moment values of the fresh buffy coat.

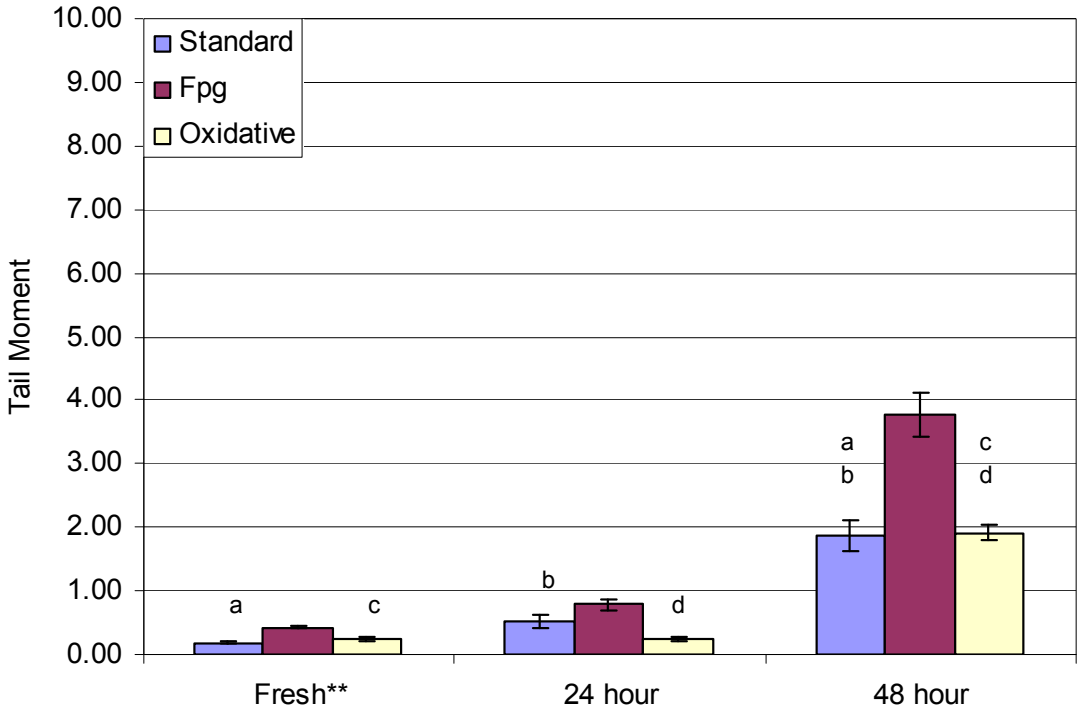

Figure 17. Storage of Human Buffy Coat at 25ºC

The comet assay was performed on a fresh human buffy coat or a human buffy coat stored on the bench at 25ºC for 24 or 48 hours. The bars labeled as "Standard" represent the slides treated with fpg buffer. The bars labeled as "fpg" represent the slides treated with the enzyme fpg. The bars labeled as "Oxidative" represent the difference between the buffer treated slides and the slides treated with fpg. The results are means ± SEM of 150 cells counted for n = 3 humans. Bars sharing a common letter are significantly different ($p < 0.05$) from one another in either the standard of the oxidative measurements according to a one way ANOVA followed by the Holm-Sidak method.
**Comet assay performed immediately upon receipt from CIRBC.

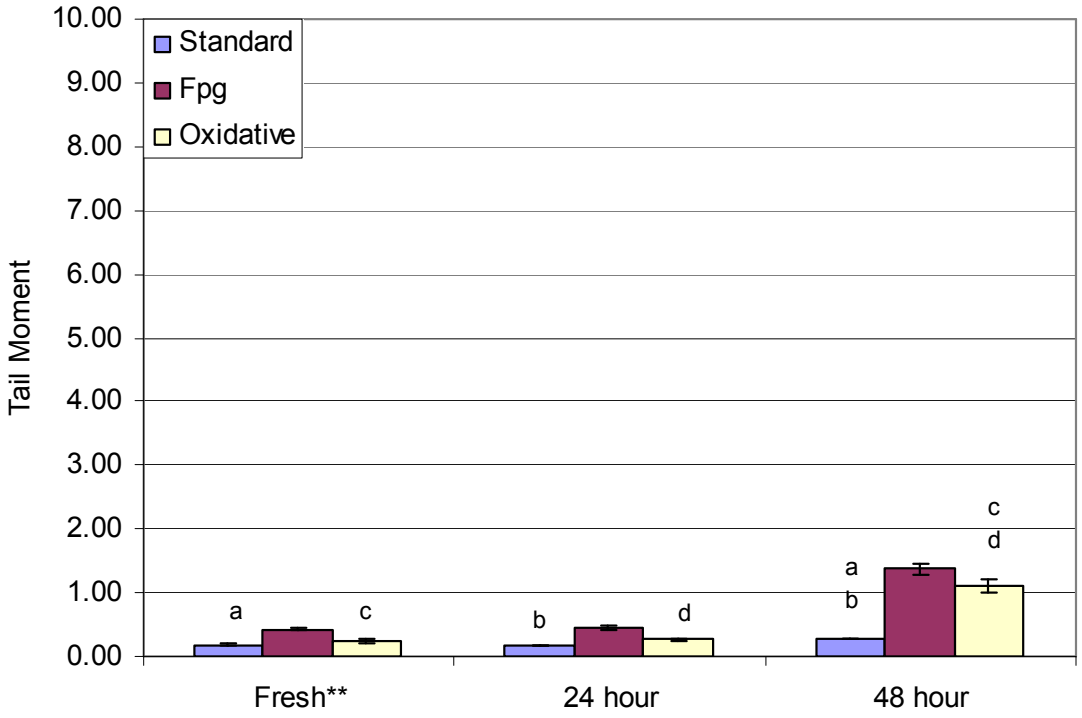

Figure 18. Storage of Human Buffy Coat at 4ºC

The comet assay was performed on a fresh human buffy coat or a human buffy coat stored in the refrigerator at 4ºC for 24 or 48 hours. The bars labeled as "Standard" represent the slides treated with fpg buffer. The bars labeled as "fpg" represent the slides treated with the enzyme fpg. The bars labeled as "Oxidative" represent the difference between the buffer treated slides and the slides treated with fpg. The results are means ± SEM of 150 cells counted for n = 3 humans. Bars sharing a common letter are significantly different ($p < 0.05$) from one another in either the standard of the oxidative measurements according to a one way ANOVA followed by the Holm-Sidak method.
**Comet assay performed immediately upon receipt from CIRBC.

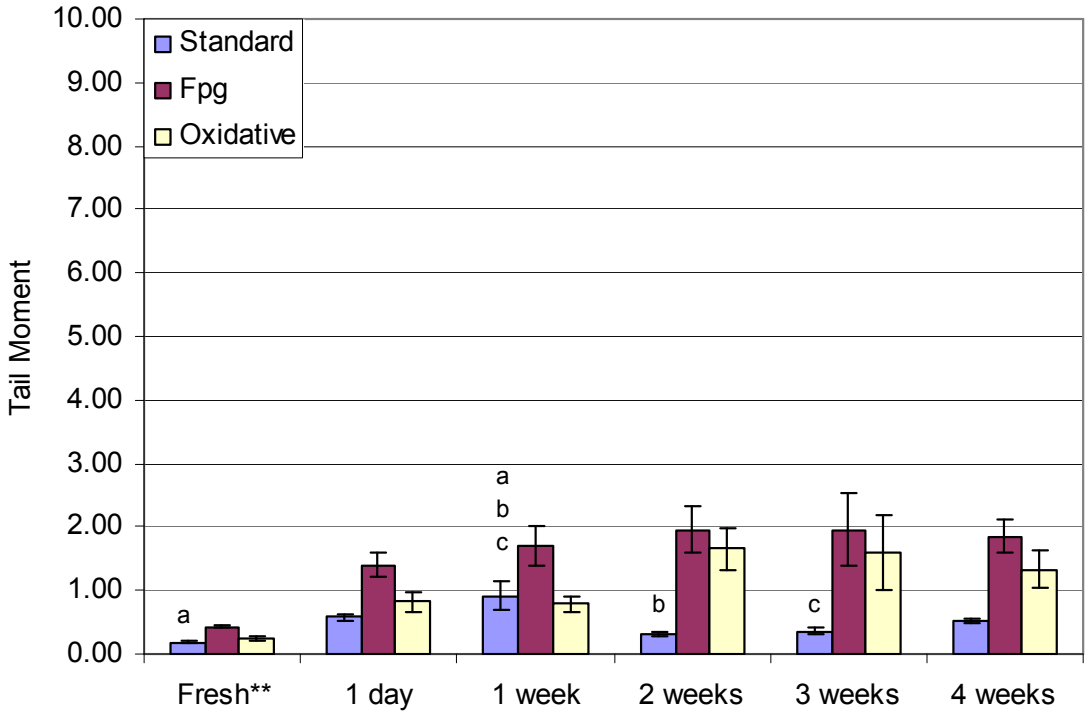

Figure 19. Storage of Human Buffy Coat at -80ºC (glycerol)

The comet assay was performed on a fresh human buffy coat or a human buffy coat cryopreserved with 10% glycerol in a -80ºC freezer for 1 day, 1 week, 2 weeks, 3 weeks, or 4 weeks. The bars labeled as "Standard" represent the slides treated with fpg buffer. The bars labeled as "fpg" represent the slides treated with the enzyme fpg. The bars labeled as "Oxidative" represent the difference between the buffer treated slides and the slides treated with fpg. The results are means ± SEM of 150 cells counted for n = 3 humans. Bars sharing a common letter are significantly different ($p < 0.05$) from one another in either the standard of the oxidative measurements according to a one way ANOVA followed by the Holm-Sidak method.
**Comet assay performed immediately upon receipt from CIRBC.

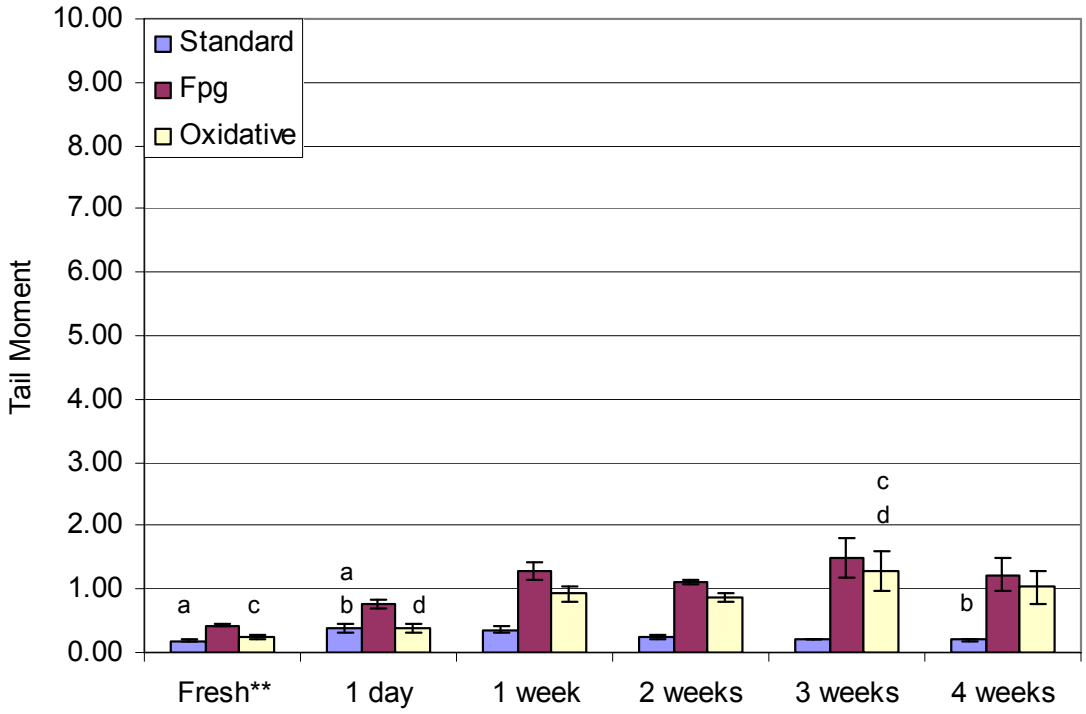

Figure 20. Storage of Human Buffy Coat at -80ºC (DMSO)

The comet assay was performed on a fresh human buffy coat or a human buffy coat cryopreserved with 5% DMSO in a -80ºC freezer for 1 day, 1 week, 2 weeks, 3 weeks, or 4 weeks. The bars labeled as "Standard" represent the slides treated with fpg buffer. The bars labeled as "fpg" represent the slides treated with the enzyme fpg. The bars labeled as "Oxidative" represent the difference between the buffer treated slides and the slides treated with fpg. The results are means ± SEM of 150 cells counted for n = 3 humans. Bars sharing a common letter are significantly different ($p < 0.05$) from one another in either the standard of the oxidative measurements according to a one way ANOVA followed by the Holm-Sidak method.
**Comet assay performed immediately upon receipt from CIRBC.

IV. Discussion

The human body is constantly under attack by chemical agents that can cause DNA damage by non-oxidative and oxidative mechanisms, which can cause initiation and begin the process of carcinogenesis. The comet assay is a useful tool for monitoring individuals who may be at risk of DNA damage beyond the body's natural defense and repair processes. Leukocytes in blood samples provide a means of obtaining cells for the use of such monitoring. Instances may arise when samples must be stored for later analysis. It is important to understand the effects, if any, that storage conditions could have on the detection of oxidative DNA damage.

No published work relating the effect of storage conditions on oxidative damage in whole blood was found. Research relating storage conditions to DNA strand breaks in whole blood has been published. Anderson et al. [5] reported no biologically meaningful changes in DNA strand breaks (tail moment) when human blood was stored for up to 4 days at room temperature (exact temperature was not provided) or 4ºC. Narayanan et al. [77] reported an increase in DNA strand breaks when human blood was stored for 24 and 48 hours at both room temperature (exact temperature was not provided) and 4ºC. Narayanan et al. also reported that red blood cell contamination of human lymphocytes increased DNA strand breaks 10-fold when compared to isolated lymphocytes which appears to be inconsistent with other data presented within their paper. The discrepancy between the two groups could have been a result of slight differences in the comet assay protocol. The former group allowed the

DNA to unwind for 20 minutes and electrophoresis was performed for 20 minutes while the later group performed the DNA unwinding and electrophoresis steps for 40 minutes and 30 minutes, respectively. This could account for the discrepancy between the two groups because increasing the time in both the unwinding and electrophoresis steps will increase the sensitivity of the assay [40]. Chuang et al. [18] reported no change in DNA strand breakage in human and rat blood stored at 4ºC for up to 4 hours. However, this group also used a less sensitive method by allowing the DNA to unwind for 15 minutes and performing the electrophoresis for 20 minutes. Due to the protocol variability and the discrepancy between the groups, it is important to reevaluate the effect of storage conditions on DNA strand breaks and also include information on whether storage affects oxidative damage.

The results of this study suggest that when storing rat whole blood at 25ºC (room temperature) the comet assay should be performed within 2 hours of blood collection for the evaluation of DNA strand breaks and within 1 hour for the evaluation of oxidative DNA damage. Although DNA strand breaks showed no significant difference at 24 hours compared to the fresh time point within the scope of the ANOVA, the increase was over 4-fold and could be considered biologically significant. The results at this storage temperature are in contrast to Anderson et al. and in partial support of Narayanan et al. Narayanan et al. observed an increase in DNA strand breaks when human blood was stored for 24 and 48 hours at room temperature, however, they also observed an increase in lymphocyte DNA strand breaks when these cells were assayed in whole blood.

Experiments with whole blood in this paper did not show any such DNA damage. This discrepancy could be due to the nature of the lysis solution used in this study. In addition to the lysis solution used by Narayanan et al., the lysis solution used in this study also contained 1% sodium lauryl sarcosinate, 10% DMSO, and 0.1 mM deferoxamine mesylate. DMSO is an antioxidant and a radical scavenger and was used to inhibit additional DNA damage by reducing reactive oxygen species or binding free radicals released by granulocytes (neutrophils, basophils, eosinophils) during the lysis step. Deferoxiamine mesylate is an iron chelating agent that was used to bind iron as a result of red blood cell lysis and prevent a Fenton type reaction from occuring.

The data also suggest that if rat whole blood is to be stored at 4ºC the time of storage may be increased up to 48 hours for the evaluation of DNA strand breaks and up to 2 hours for the evaluation of oxidative damage. These results are consistent with Chuang et al. who reported no increase in DNA strand breaks when rat blood was stored at 4ºC for up to 4 hours. It is, however, in contrast to Narayanan et al. who reported that human blood can not be stored for 24 hours or more even at 4ºC. There is no clear explanation for this discrepancy but perhaps the differences lie in the preparation of the whole blood samples. In these experiments there was no manipulation of whole blood samples other than addition of an anticoagulant. However, in the experiments performed by Narayanan et al., whole blood samples were diluted with media supplemented with 10% FCS (fetal calf serum), pelleted, and washed with PBS before the comet assay or storage took place.

In the event that rat blood should require long term storage prior to analysis, it should be frozen at -80ºC with the use of either glycerol or DMSO as the cryoprotectant. Under these storage conditions there was no observable increase in DNA strand breaks or oxidative damage for up to 4 weeks in storage. These results are consistent with Chuang et al. who reported no increase in DNA strand breaks when rat blood was frozen with DMSO and stored for 5 weeks. They are also similar to findings presented by Duthie et al. [25] who reported no increase in DNA strand breaks or oxidative damage in isolated human lymphocytes frozen with DMSO and stored for 2 months. As for the comparison with the use of the cryoprotectants glycerol and DMSO there appears to be no biological difference between the two although 3 of the 5 storage time points revealed a statistically significant difference with the samples frozen with glycerol exhibiting a slightly larger amount of DNA stand breaks.

Surprisingly, no published work relating the effect of storage conditions on DNA strand breaks or oxidative damage in a buffy coat was found. Never the less, this study used a rat buffy coat in attempts to reveal any impact that red blood cells could have on blood storage and to be consistent with the whole blood studies with respect to the cell populations under investigation.

The results of the present investigation suggest that when storing a rat buffy coat at 25ºC (room temperature) the comet assay should be performed within 2 hours for the evaluation of DNA strand breaks and oxidative damage. These results are similar to the storage of rat whole blood except the onset of oxidative damage was delayed by at least one hour.

Storage of a rat buffy coat at 4ºC suggests that the comet assay should be performed within 48 hours for the evaluation of DNA strand breaks and oxidative damage. As was the case with storage at 25ºC, the onset of oxidative damage in the buffy coat was delayed by at least 24 hours when compared to whole blood.

Long term storage of the rat buffy coat frozen with either glycerol or DMSO showed similar results as rat whole blood. There was no observable increase in DNA strand breaks or oxidative damage for up to 4 weeks of storage. There was also no difference between the use of glycerol or DMSO as the cryoprotectant.

In all of the cryopreservation studies red blood cell lysis occurred after thawing with both of the cryoprotectants used. This was determined by adding 1 ml of PBS to the remaining aliquots, centrifuging the aliquots at 600 xg for 5 minutes, and observing the color of the supernatant. Red blood cell lysis did not have a significant impact on DNA strand breaks or oxidative damage when the comet assay was performed within minutes of thawing, even while the cells underwent the 40 minute cooling process to allow the agarose to solidify. One possible explanation is that the white blood cells where diluted by a factor of 200 in the agarose. This would greatly reduce their chances of coming into contact with a harmful agent or being activated as a result of the hemolysis. However, when the white blood cells were allowed to incubate in the lysed red blood cells for 1 hour prior to the comet assay there was a significant increase in DNA strand breaks and oxidative damage which occurred in the whole blood samples as well

as the buffy coat samples. This may be expected considering the buffy coat still contains a limited number of red blood cells.

Studies involving storage of a human buffy coat produced similar results compared to the storage of a rat buffy coat. However, because the human buffy coat was collected, processed, and delivered by a local blood bank it was not possible to accurately assess the storage time points of 1 and 2 hours after blood collection. Furthermore, the fresh time point reflects the performance of the comet assay upon the arrival of the buffy coat. With that being said, when storing a human buffy coat at 25ºC the comet assay should be performed in less than 24 hours for the evaluation of DNA strand breaks and oxidative damage. Although DNA strand breaks showed no significant difference at 24 hours compared to the fresh time point within the scope of the ANOVA, the increase was nearly 3-fold and could be considered biologically significant.
Since the integrity of the non fpg treated slides were compromised by the storage, oxidative damage may be underestimated. Therefore, storage for 24 hours could be considered a non viable time of storage for assessing oxidative damage.

Similar to the findings involving rat whole blood and rat buffy storage at 4ºC, the human buffy coat was able to be stored for up to 48 hours with no biologically significant increase in DNA strand breaks. However, at 48 hours of storage there was a significant increase in oxidative damage.

Long term storage of the human buffy coat frozen with the cryoprotectants glycerol or DMSO did not show the same degree of consistency within the 4

week study. In regards to the human buffy stored with glycerol as the cryoprotectant, DNA strand breaks were consistently higher for the frozen samples as compared to the fresh buffy coat although only the one week time point resulted in a significant increase. Oxidative damage was also increased, but none of the time points showed significance due to the variability between buffy coat samples and a small n. In regards to the human buffy coat stored with DMSO as the cryoprotectant, DNA strand breaks were more consistent with the fresh time point and only the 1 day time point showed a significant increase. However, oxidative damage appeared to increase in the frozen samples with the time point of 3 weeks showing significance. In comparison, there did not appear to be any biologically significant difference between the two cryoprotectants used regarding DNA strand breaks or oxidative damage. One possible explanation for the lack of consistency within the 4 week study could be attributed to the small group size. Perhaps increasing the n would compensate for this. Another could be attributed to the manner in which the human buffy coat was processed and stored by the Central Indiana Regional Blood Center prior to delivery.

In conclusion, the use of whole blood or a buffy coat are viable options for detecting strand breaks as well as oxidative damage to the DNA of white blood cells. Processing time is limited if the whole blood or buffy coat samples are kept at 25ºC or 4ºC. However, if cryopreserved using glycerol or DMSO, the samples may be stored for at least 4 weeks without DNA strand breaks or oxidative damage deviating significantly from the fresh samples. Promptness should be a concern following sample thawing and the comet assay should be performed

immediately thereafter. It would be interesting to investigate the effects of storage times between 2 and 24 hours at both the temperatures of 25ºC and 4ºC. It would also be interesting to investigate the role that antioxidants could play in delaying the effects of oxidative damage but these questions have yet to be addressed.

V. References

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Curriculum Vitae

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EDUCATION:

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Mentor: James E. Klaunig, Ph.D.
Indiana University
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B.S. in Chemistry (2003)
Purdue University
Fort Wayne, Indiana

EMPLOYMENT HISTORY:

Student Research Technician (2004 – 2006)
Indiana University School of Medicine
Indianapolis, Indiana

Responsibilities
- cell culture
- immunohistochemistry
- assays to detect protein content, antioxidant capacity, and DNA damage
- small animal dosing, surgeries, and tissue collection

Distribution Technician (2003 – 2004)
Central Indiana Regional Blood Center
Indianapolis, Indiana

Responsibilities
- receive orders for biological products and testing services
- process, pack and distribute products to customers
- leukodepletion of products
- QC sampling and pH testing on platelet products

Undergraduate Research Assistant (2002 – 2003)
Mentor: Ronald S. Friedman, Ph.D.
Purdue University
Fort Wayne, Indiana

Responsibilities
- calculate the resonance energies of excited and bound electronic states
- analyze data and present results to my mentor

Hospital Services Technician (2001 – 2003)
American Red Cross
Fort Wayne, Indiana

Responsibilities
- provide customer assistance in ordering blood products
- process, pack and distribute blood products
- monitor and maintain temperatures of blood products

Mobile Unit Assistant (1998 – 2000)
American Red Cross
Fort Wayne, Indiana

Responsibilities
- transportation of supplies, equipment and blood products to and from mobile sites
- on site processing of blood donations
- assist phlebotomists in donor relative activities

SOCIETY MEMBERSHIPS:

American Chemical Society (2003 – Present)