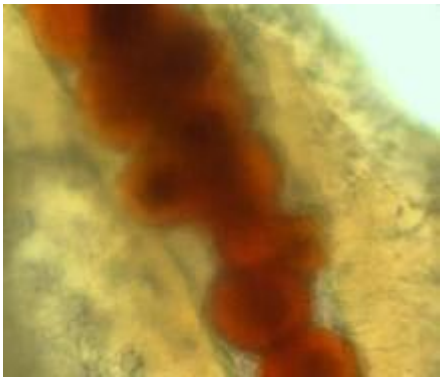
"Ferrous Promotion of Cross-Linking in Erythrocyte Membranes as it Pertains to Soft-Tissue Preservation"



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Abstract

Fossils provide important morphological and occasionally molecular evidence about ancient organisms (Schweitzer et al., 1997). Fossilization entails the mineral replacement of endogenous organic structures during which morphological and especially molecular components such as proteins are affected by physical, chemical, and environmental factors (Schweitzer et al., 1997 & 2006; Flannery et al., 1999;Rollo et al., 2002). This multivariate process determines the state of preservation fossil remains are found, which is highly variable (Schweitzer et al., 1997; Allison, 1988). The expectations for the preservation of endogenous biomolecules are extremely low: 100,000 years is the predicted survival limit until complete protein hydrolysis (Bada et al., 1999). However, excellently preserved tissue and cellular components from a 65+ million year old Tyrannosaurus *rex* was recovered, and conclusively identified collagen amino acid sequences from the de-mineralized tissue (Asara et al., 2007). These unique findings has forced a re-evaluation of the preservation limitations of the fossilization process (Schweitzer et al., 2007).

One mechanism proposed by Schweitzer et al. (2007) attempts to explain the preservation of soft tissue microstructures such as red blood cells and molecular constituents through free radical production initiated by oxidation of iron from hemoglobin breakdown. Free radicals are highly reactive and unstable molecules with an unpaired electron which can cause cross-linking of cell constituents such as proteins and lipids (Halliwell and Gutteridge, 1984; Magder, 2006). In order to elucidate this mechanism and its ability to improve cell preservation, I propose that raising the concentrations of Fe (II) may cause increased cross-linking in cell membranes.

Research design entails testing for increases in cross-linking through electrophoretic techniques of modern hemoglobin-free (ghost) erythrocytes after exposure to increasing concentrations of free iron. Increases in cross-linking of cell membrane would position iron as a participant in cell preservation. A decrease to no changes in cross-linking would imply that iron alone may not be part of the mechanism. Further studies should be conducted using free hemoglobin in addition to synergistic effects of hemoglobin and iron.

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Specific Aims:

Recent discoveries forced the scientific community to re-evaluate the preservational processes involved in fossilization (Schweitzer et al., 2007). In order to explain soft-tissue preservation and its variability in the fossil record, further studies are needed to elucidate how soft-tissues including cellular components have resisted degradation over (Schweitzer et al., 2008). Increased cross-linking has the potential for improved cell preservation by keeping cell structure intact and inhibiting proteolytic attack from microbes (Schweitzer et al., 2008*). One possible mechanism involves the iron mediated production of free radicals and the subsequent cross-linking of membrane constituents (Schweitzer et al., 2008*).

Aim #1: To prepare a membrane model system using resealed erythrocytes that have no internal contents (ghosts) exposed to varying free iron concentrations. The levels of free iron that will be used are from zero to levels used in previous studies, 0-100 μM (Buettner and Jurkiewicz, 1996; Vidrio et al., 2009). Environmental conditions include containment in physiological relevant buffer solution. Erythrocytes will be exposed for up to 24 hours for each treatment concentration of iron.

Aim #2: After the model system is set up, the free radical production will be measured and lipid peroxidation by-products will be detected. In order to ensure free radicals are being produced by the system and to quantify their levels per treatment level, High Performance Liquid Chromatography (HPLC) will be employed after the 24 hour incubation. To determine if lipid peroxidation is occurring within the system, detection of common lipid peroxidation products will also be detected with HPLC.

Aim #3: Subsequently, each erythrocyte treatment group will have membrane cross-linking evaluated by gel electrophoresis. Cell cross-linking will be evaluated using SDS-PAGE and dual staining of lipids and membrane proteins. Red blood cell membranes are well characterized and they are an ideal membrane model for this purpose due to the simplicity of their membrane components. Detection of cross-linking in membrane proteins only requires the analysis of a limited number of well-characterized protein

banding patterns in gel electrophoresis. Therefore they offer the opportunity to evaluate the effects of free radicals on membrane lipids and proteins (Gothoskar, 2004). Treatment groups will be compared with an untreated erthryocyte group not exposed to iron as a negative control. Cross-linking is expected to produce larger molecular weight (kDa) protein and lipid complexes; larger molecular weights correspond to slower migration rate and migration distances in polyacrylamide gels.

Background and Significance

Fossils have been aiding scientists in reconstructing the earth's biological and geological past for over one hundred years since the sophomoric years of paleontology and evolutionary biology. Fossils were originally used in the study of stratigraphy, the study of rock layers, because particular fossils reliably characterized certain geological layers and they were used to identify profitable sediment deposits such as coal or oil (Carroll, 1997). This consistent sequence of layering and biota, or total associated flora and fauna, provided the foundation for demonstrating the geological history of Earth and its timescale (Carroll, 1997).

Charles Darwin used the fossil record as major evidence for his theory of evolution and natural selection by demonstrating progressive changes in ancestral species' morphology occurring throughout time in direct relation to living species of today (Carroll, 1997). Moreover, evolutionary biology relies on fossils to construct phylogenies showing evolutionary relationships between organisms in addition to elucidating evolutionary mechanisms and pressures occurring within taxonomic groups across time (Carroll, 1997). Insight into community ecology is even possible in *Lagerstatten* biota, which are rock beds rich in fossil biota (Allison, 1988).

More specifically, fossils are invaluable clues to physiology and anatomy of ancient species, including metabolic rates and body composition. For example, an important discussion centers on the evolution of endothermy and feathers amidst non-avian dinosaurs and Mesozoic birds (Chiappe, 1995; Ruben, 1995). Furthermore, fossil evidence leaves clues to the ontogeny, or the developmental history of an organism to maturity as well as behaviors of ancient organisms. This includes predator versus scavenger lifestyles or even nesting behaviors (Towe and Urbanek, 1972; Martill and

Unwin, 1989; Norell et al., 1995; Schweitzer et al., 1997).

Analyses of fossil remains often employ gross anatomical comparisons to extant species. This also includes in-depth histological analyses of bone structures and dermal tissues such as skin or wing membranes (Martill and Unwin, 1989; Schweitzer et al., 1997). The level of information a fossil specimen can reveal, morphologically and histologically, is highly dependent on the condition in which it is found. Furthermore, the level of morphological preservation seen is dependent on a plethora of variables involved in the fossilization process.

The Fossilization Process

Fossils are the mineralized remains of a dead organism. Types of fossils can occur as molds, casts, petrifactions, which involves complete mineral replacement of organic material, and carbon films (Thompson, 1982). The primary mode of fossilization is permineralization and recrystallization, which involve the mineral replacement or overlay of endogenous, or original, organic material by minerals such as calcite. Furthermore the process of fossilization not only preserves the hard-parts of organisms such as the bones and shells of vertebrates and invertebrates, the majority of fossils, but also more fragile components such as leaves, bark, and in extremely rare instances originally proteinaceous soft-tissues such as dermal tissue are preserved in mineralized form (Thompson, 1982). Generally for hard part materials like bones, the original bone matrix and tissues are gradually replaced by inorganic minerals such as calcite that precipitate from the sediment that entombs it (Thompson, 1982). Finding soft tissue components like connective tissue, vascular structures, muscle, and cells is extremely rare due to innate decay processes (to be elucidated further on) and the fossilization process itself.

The process of becoming a fossil is ultimately a destructive one and destroys 99% of the remains of all organisms who have ever lived (Prothero, 2007). A 1% survival rate for remains seems to occur by luck. However, taphonomy, a sub-field of paleontology, studies the decay and fossilization process in dead, extant organisms (Allison, 1988). Taphonomical study attempts to address how and why fossilization occurs by elucidating the changes that occur post-mortem within an organism and the plethora of factors that interact to produce a fossil (Prothero, 2007). Taphonomic processes are an accumulative

term describing the decomposition as well as chemical and physical changes within a decaying organism (Prothero, 2007). Taphonomic study employs experiments with modern decaying animals that attempt to elucidate the decay of organic material and its ability to fossilize (Allison, 1988). Studies of archeological human remains in addition to the fossil record demonstrate that not only the paucity of fossils, but also the variable condition in which they are found is attributed to the variety of degradative actions that ensues after an organism dies (Prothero, 2007). They have shown that the preservational state of a fossil is dependent of the rate of decay of organic material and the rate of mineralization that is able to preserve the structural characteristics (Allison, 1988). The faster mineralization can occur before significant loss of original biological components, the better is the state of morphological and histological preservation(Allison, 1988).

The first stages in the fossilization process involve biological agents destroying much of the soft tissue components (Prothero, 2007; Raff et al., 2008). The organism's own cells undergo autolysis and necrosis after death, damaging the integrity of soft tissues such as the dermal layers or organs (Raff et al., 2008; Schweitzer et al., 2007). Bacteria and autolysis are thought to be the primary initiators of degradative processes by destruction by the use of lytic enzymes (Raff et al, 2008). Bacteria, fungi and insects collectively infiltrate and colonize the remains and decompose much of the organic material for metabolism, utilizing nitrogen and carbon sources such as amino acids, over the span of a few days to years, destroying endogenous organic material before it can be mineralized (Rollo et al., 2002; Prothero, 2007; Schweitzer et al., 2007).

Histological examinations have observed destruction by dissolution of bone material through fungal re-absorption of bone, bacterial nests in vascular channels and plant root invasion in archeological remains of ancient humans (Maat, 1993). Moreover, similar observations find losses in structural integrity resulting from microbes, plants and insects which cause "focal destruction", or tunneling, destroying vascular canals and Haversian systems in archeological human bones (Rollo et al., 2002). Haversian systems are the basic functional units of bone consisting of concentric layers of bone tissue around a canal bundle of blood vessels and nerves. These haversian systems appear to be targeted by microbes, plants, and insects because they provide points of infiltration and take residence there (Rollo et al. 2002). Thus studies of archeological remains show how

destructive and aggressive these relatively early processes can be.

In addition, scavengers often crush and fragment bones in addition to stripping flesh (Prothero, 2007). Modern day hyenas provide testament to this fact as their jaws are capable of crushing hard-parts such as bones. They often pulverize and scatter a majority of bones from carcasses (Prothero, 2007).

Furthermore, remains must endure physicochemical stresses such as temperature, pH, burial conditions, and erosion before, during and after burial (Schweitzer et al., 1997; Flanner et al., 1999; Rollo et al., 2002). These factors can affect the preservational state of bone material and any soft tissues that remain (Rollo et al., 2002). For example, lower temperatures are better suited for preserving remains: lower temperatures equates with lower rates of destruction by microbes and the improved stability of cellular components and molecular information such as DNA over long periods of time (Rollo et al., 2002; Maat, 1993). Higher temperatures are associated with increased bio-chemical degradation rates such as hydrolysis of proteins like collagen (Flanner et al, 1999)

Studies of peat bog mummies show that acidic conditions result in the dissolution of bone, exposing the remaining collagen tissue and cellular material to the environment (Flanner et al, 1999). In a few unique alkaline peat bogs, mitochondrial DNA has been retrieved from these sites that are estimated to be over 8000 years old (Flannery et al., 1999). Thus, pH can have a substantial effect on the chances of preservation for fossilization by affecting the protective bone mineral matrix.

Erosion by wind and water movement can also destroy remains. Before burial, exposure to the elements erodes organic and bone mineral material (Rollo et al., 2002) . After sediment is deposited on top of the remains over the years, the pressure of the layers and abrasive movement of soil particles wear down the tough outer original mineral matrix of bones causing cracks and fissures, which further expose bone to invasion by microbes and plant rootlets that breakdown of bone (Rollo et al., 2002). When the sediment layer is exposed later after thousands of years of erosion by wind, water and sediment upheaval, the elements further wear away whatever mineralized portions are left (Rollo et al., 2002).

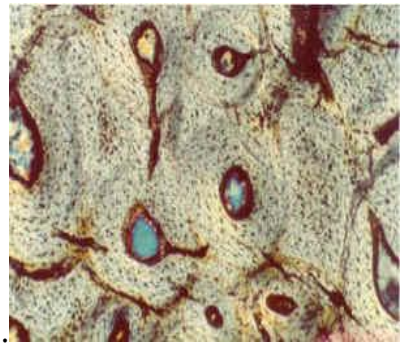
Another important physical parameter is the type of sediment in which the specimen is found, and the rate of deposition (Rollo et al., 2002; Flannery et al., 1999).

Meta-analyses of fossils from different taxa buried in a variety of environments in addition to taphonomic experiments show that the burial environment significantly affects the preservation of remains (Allison, 1988; Schweitzer et al., 1997; Schweitzer et al., 2007). For example, remains deposited in marine sediments, sandstones and mudstones appear to have the best potential for preservation (Schweitzer, 2007). These sediments improve preservation potential by allowing water and decay products to percolate through the soil matrix, and preventing from pooling around the remains (Schweitzer et al., 1997). Also, the speed of burial is an important way to inhibit decay and rapid mineralization of organic material (Allison, 1988). Rapid burial promotes early mineralization of organic material before significant loss of endogenous organic material (Allison, 1988). Quick burial limits the access of microbes, fungi, and scavengers to the remains. Also, it limits the exposure of bones to the environment, while allowing minerals to precipitate or crystallize on the organic remains while they are intact (Allison, 1988). Rapid burial also inhibits aerobic decay by promoting anoxic conditions (Allison, 1988). Aerobic respiration by bacteria that require oxygen is more destructive due to its high redox potential (Allison, 1988). Altogether, sediment type, rapid burial and anoxic conditions inhibit the rate of decay and promote preservation. Promoting rapid mineralization likewise helps to inhibit degradation of morphological structures and the loss of important biomolecules like proteins (Allison, 1988).

These complex interactions of factors affect the level of preservation of morphological features. The fossilization process produces highly variable states of preservation in fossils due to varying rates of decay and mineralization as previously mentioned. And while some conditions such as low temperatures, anoxicity, rapid burial and mineralization help to promote and predict better states of morphological features, the variability of preservation within the fossil record is wide.

Poor morphological preservation results in fossil remains that are significantly altered leading to the loss of structural information and thus when mineralized, little to no preservation of finer structures such as vascular channels and tissue layers. While rare, fossils with excellent preservation of morphological features have been documented even since the beginnings of paleontology. Integumentary features such as feathers, mammal hair, scales, skin and keratinous claw sheaths have been well documented (Martill and

Unwin, 1989; Norell et al., 1995; Forster et al, 1996; Meng and Wyss, 1997). A pterosaur wing membrane examined by Martill and Unwin under SEM showed skin folds and separate dermal layers complete with structures resembling capillaries and spongy dermal tissue (Martill and Unwin, 1989). Other more fragile soft-tissues have also been documented such as sauropod embryos, muscle fibers, vascular channels complete with microstructures resembling red blood cells (Kellner, 1996; Forster et al., 1996; Sasso and Signore, 1998; Chiappe et al., 1998). Such fine structures are found in bone that has little morphological alteration due to mineral replacement of inorganic and organic bone matrix (see figure 1)(Allison, 1988).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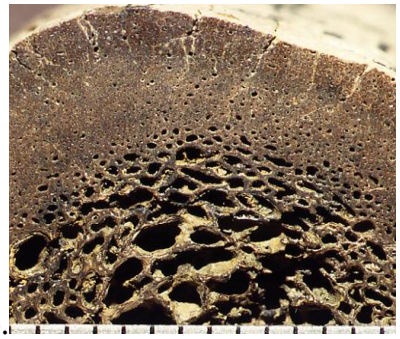


Figure 1: (a) Photomicrograph of dinosaur bone composed of phosphorite mineral that has replaced original bone mineral in addition to quartz that has precipitated in small spaces and replaced original organic material. *image courtesy of http://people.uncw.edu/dockal/GLY312/fossils/fossils.htm* (b) unaltered bone section. *image courtesy of http://www.geol.umd.edu/~tholtz/G104/lectures/104fossils.html*

The discovery of excellently preserved structures with little structural alteration has lead some to question whether excellent morphological preservation implies possible molecular preservation of proteins or even nucleic acids (Towe and Urbanek, 1972; Jong et al., 1974; Schweitzer et al., 1997 & 1999). Towe and Urbanek's electron microscopy studies revealed excellent morphological preservation of what appeared to be the structural protein, collagen, in the skeletal remains of a graptolite specimen over 443 million years old (1972). Their examination revealed morphology amazingly similar to modern day collagen which is constructed into layers of tight helical fibers with a central core and periodic banding between fiber bundles (Towe and Urbanek, 1972). This lead them to use high performance liquid chromatography to attempt to detect and quantify possible molecular preservation of amino acids. Their results showed low quantities of amino acids like glycine and proline, definitive amino acids like hydroxyproline and

hydroxylysine, characteristic of collagen composition, were not present. So while there was excellent morphological preservation, there was not conclusive molecular evidence to suggest the conservation of endogenous collagen amino acids. Morphological information alone is not enough to definitively determine the presence of original protein material (Schweitzer et al., 1997). Further testing for the presence of endogenous proteins involved the use of immunochemistry with positive preliminary results when using an enzyme-linked immunosorbent assay (ELISA) (Schweitzer et al., 1997 & 1999) . Further immunological studies by Schweitzer et al. (1997 & 1999) have shown immunochemical detection of beta-keratin, a structural protein of birds and reptiles, in a late cretaceous bird and the preliminary detection of collagen protein in the femoral bone of *Tyrannosaurus rex*. These studies advanced the case for possible preservation of original biomolecules like proteins in specimens displaying excellent morphological preservation with little structural alteration (Schweitzer et al., 2007).

Survival of Ancient Biomolecules

Retrieving ancient biomolecules like proteins, amino acids, and DNA, would prove invaluable as it offers scientists substantial evidence about the physiology and evolutionary relationships of ancient organisms (Schweitzer et al., 1997). However, the expectations surrounding the stability of proteins and nucleic acids are fairly low due to rates of decay and the number of previously mentioned environmental stressors on biomolecules (Bada et al., 1999). First, consider the onslaught of environmental processes previously mentioned that would contribute toward their degradation such as pH changes, UV exposure, and enzymatic degradation by microbes and fungi, to name a few. Compounding this are how proteins and DNA degrade over time without functioning biological machinery to maintain them (Bada et al., 1999).

The most coveted biomolecule, DNA, is also the most fragile: in a majority of environments DNA survival is estimated to be no more than 10.000 years with upper limits placed at 100,000 years (Bada et al., 1999). Rapid DNA chain breakage is cause by depurination, which involves the hydrolysis of guanine and adenine bonds (Bada et al., 1999). Hydrolysis caused by water and oxidation by oxygen are also key ways that DNA is damaged over time (Hoss, 2000).

Proteins are more stable than DNA and have longer survival limits with complete hydrolysis to amino acid constituents occurring around 100,000 years (Bada et al., 1999). While amino acid degradation is influenced by the same factors as DNA, generally, they compose far more of the tissue components and they can be used as indicators of biomolecular survival through racemization rates (Bada et al., 1999). Racemization is the process in which one enantiomer, or chiral form, of a molecules switches to its other enantiomer. Enantiomers come in two forms, D and L, that describe the symmetry of the molecule. Amino acids generally come in the L form, but over time predictably form ratios of both D and L in degrading matter (Bada et al., 1999). Racemization of amino acids occur in predictable patterns that have been used to aid those in search of biomolecular information and evaluate preservation (Bada et al., 1999). Certain amino acids can be used as indicators of age due to their predictable rates of racemization and ratios (Bada et al., 1999). The following amino acid racemization ratios are used as indicators: D:L aspartic>D:L alanine>D:L valine (Bada et al., 1999). These ratios of aspartic acid to alanine to valine also allow for evaluation of the state of preservation of material (Bada et al., 1999). Previous claims for the presence of amino acids in ancient material often show contamination from the environment, which is demonstrated by higher ratios of amino acids valine to alanine, for example, which stray from the above predicted patterns (Bada et al., 1999). Overall variation of amino acid racemization ratios demonstrate that remains are not closed system: exchange of free amino acids after hydrolysis of proteins with the environment contributes significantly to molecular information loss (Bada et al., 1999).

The only way to halt information loss from ancient specimens is thought to be through rapid mineralization of bone and tissues as previously mentioned; however, this ultimately replaces organic material and often alters any molecular information that could be retrieved (Allison, 1988). Therefore innate protein and DNA degradation rates coupled with environmental factors provide for a low chance of survival of endogenous biomolecules without alteration (Bada et al., 1999).

Defying Expectations: Case for Endogenous Molecular Information

Up until recently, little conclusive evidence bolstered the case for the ability of

the fossil record to preserve molecular information from fossil material such as bones (Schweitzer et al., 1997). However, this changed in 1997 when Dr. Mary H. Schweitzer et al. uncovered an exceptionally well-preserved *Tyrannosaurus rex* specimen. It showed excellent morphological preservation with little signs of mineral precipitation in the cavities of the femoral bone or mineral replacement of hard bone material (Schweitzer et al., 1997). Believing it could be likely that such a well-preserved specimen could harbor endogenous molecular information, they decalcified the bone to remove the mineral material (Schweitzer et al., 1997). Demineralization revealed soft, pliable tissues and what appeared to be matrices of collagen fibers (see figure 4). Cellular components resembling red blood cells were also found within the hollow vascular channels (Schweitzer et al., 2005).

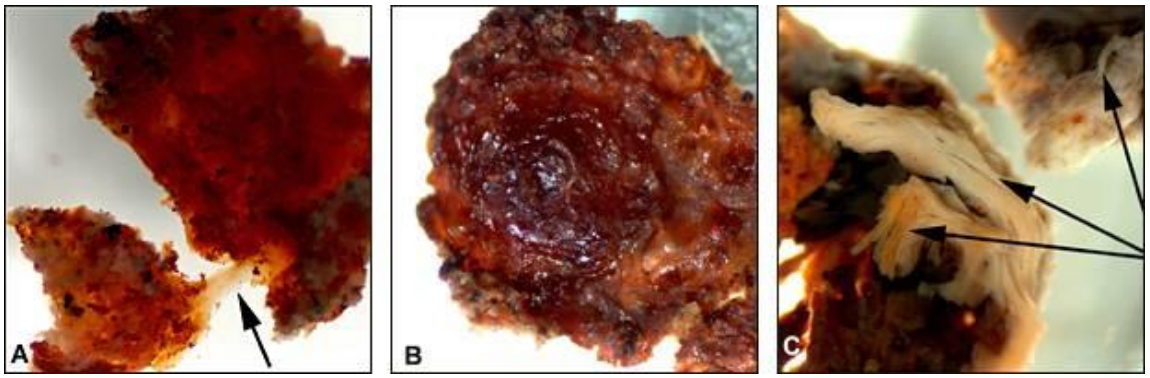


Figure 4: tissues that lined the marrow cavity of femur of *Tyrannosaurus rex* specimen found in Hell's Creek, Montana. A) the tissue is stretchy and resilient. When pulled, it can return to its original shape. B) Same tissue piece after air drying. C) Fibers that characterized much of the tissue. (Schweitzer et al., 2005)

This defies previous scientific ideas on the stability and preservation of soft-tissues over time. As Schweitzer succinctly stated, " If you take a blood sample and you stick it on a shelf, you have nothing recognizable in about a week. So why would there be anything left in dinosaurs?" *Further examinations were conducted to conclusively identify these soft-tissue components by mass spectrometry analysis for amino acids (Schweitzer et al., 2005).

In 2007, using mass spectrometry, Schweitzer et al. were able to obtain amino acid sequences from this 65+ million year old specimen. These sequences showed remarkable compatibility with collagen type α1(I) which is unique to modern day birds

like chickens (Schweitzer et al., 2007). These findings stimulated discussion of not only the preservation process, but also the possible mechanisms allowing for the conservation of soft tissues and its molecular constituents.

Most taphonomic models proposed can only explain cases of preservation for morphological features (Schweitzer et al., 2007). As mentioned previously, many natural factors operate together to quickly destroy tissues and molecular information (Schweitzer et al., 2007). So it has been of great interest to be able to explain what mechanisms can explain the presence of soft-tissues. Futhermore, variability in the fossil record could be elucidated if such biogeochemical mechanisms were investigated in order to explain why collagen protein and pliable cellular structures can be recovered from a 65 million year old dinosaur material and not more recent archeological human remains less than a hundred thousand years old (Bada et al., 1999; Schweitzer et al., 2007).

One mechanism offered by Schweitzer et al. (2007) proposes that certain chain reactions involving the breakdown of the globular proteins hemoglobin and myoglobin after death can stabilize soft tissue via cross-linking of proteins. These proteins both contain iron which readily initiates free radical production (Halliwell and Gutteridge, 1984). Cross-linking is due to the interaction of free radicals produced from the oxidation of exposed iron with cell membrane constituents like lipids and proteins (Schweitzer et al., 2008). Cross-linking makes membranes more stable by inhibiting access of cellular components to degradation. By inhibiting degradation of the protective membrane, preservation of cell structure and its inner contents are improved. The role of free radical in the body has been extensively studied from the 1980's (Halliwell and Gutteridge, 1984).

Free Radicals and Reactive Oxygen Species

All living organisms require energy to live and carry out essential processes at the cellular and organismal level. Most organisms are able to convert energy locked within organic compounds such as glucose into a useable, chemical form of energy such as ATP through oxidation (Magder, 2006). If a substance has been oxidized this means that it has lost electrons. Alternatively, an oxidizing agent retrieves an electron or a proton from a substance, and can add oxygen as well (Magder, 2006). O_2 acts as an oxidizing agent

during cellular respiration, the process of aerobic energy production in cells. This process reduces ATP and NAD^+, the energy currency of the cell. It also drives the chemi-osmotic machinery of the electron transport chain, with O_2 acting as the final electron acceptor (Magder, 2006).

However, the high oxidative potential of O_2 also makes it injurious and toxic (Magder, 2006). In physical processes, oxygen causes rust and it is essential for the combustion process (Magder, 2006). Physiologically speaking, organisms that have adapted to living in an oxygen rich environment have developed anti-oxidant mechanisms to endure the deleterious effects of O_2. Free radicals by definition are substances that have electrons that are unpaired and exist independently, not in a transient state (Magder, 2006). Oxygen's reactivity is due in part because it is a radical with two unpaired electrons (Magder, 2006). However, oxygen is roughly stable in this form because it must take one electron at a time into its orbital (Magder, 2006). Oxygen derived radicals include superoxide ($O_2\bullet^-$), which is more reactive than O_2, but not as reactive as other radical species such as the hydroxyl radical, $OH\bullet$, produced from H_2O_2 (Magder, 2006). Superoxide ($O_2\bullet^-$) can further react with H+ or HO_2 to form H_2O_2, or become oxidized to O_2 (Magder, 2006). The important aspect of free radicals is that they tend to propagate other free radicals, unstable compounds, and reactive oxygen species (ROS) (Magder, 2006). Reactive oxygen species is a collective term that defines chemicals that participate in "radical type reactions" involving the gain or loss of electrons (Magder, 2006). This includes radicals themselves and compounds that do not have unpaired electrons such as H_2O_2 and ozone (O_3) (Magden, 2006).

Sources of Free Radicals and ROS

Under normal physiological conditions, free radicals and ROS are produced from multiple processes within cells and the body at large (Magder, 2006). Normally functioning metabolism is a major source of superoxide, produced during cellular respiration in mitochondria; a previous study estimated 1-3% of O_2 is reduced to $O_2\bullet^-$ during this process (Magder, 2006). Moreover, enzymes are an additional source of $O_2\bullet^-$ in the body during pathological as well as normal functioning (Magder, 2006). Xanthine oxidase, a dehydrogenase, which is found in the cytosol of multiple tissues and

circulating blood, under pathological conditions such as sepsis, can act as an oxidase and produce $O_2\bullet$- (Magder, 2006). Some enzymes such as cytochrome P450 produce $O_2\bullet$- typically during normal enzymatic activity as a side reaction (Magder, 2006). Other enzymes function to control the propagation of reactive molecules such as superoxide by converting them to the more stable H_2O_2 (Magder, 2006). Moreover, cells contain peroxisomes within their cytosol; these are organelles that compartmentalize enzymes that produce and degrade H_2O_2 to water as a part of detoxification processes and lipid breakdown (Radwanski, 2008).

However, recent studies also demonstrate an alternative view of ROS as an integral part of cell signaling and daily rhythms (Magder, 2006; Grover et al, 2009). Since superoxides are normally produced within mitochondria during cellular respiration and then converted to H_2O_2, a recent study supported the role of H_2O_2 as an integral cell signaling molecule regulating circadian rhythms within the cell (Grover et al, 2009). Because most organisms live in an oxygenated environment, cells deal with ROS and free radicals on a day to day basis as a part of normal and pathological functioning (Magder, 2006).

Physiological Effects of Free Radicals

The presence of free radicals and their reactive counterparts, ROS, cause oxidative stress. Significant levels of reactive species, such as peroxides or hydroxyl radicals, is a general indicator of cell distress: they are present during inflammation of tissues, necrosis, septic shock, as well as general tissue damage and injury (Negre-Salvayre et al, 2008; Vidrio et al., 2009). Also, they are believed to contribute to the ageing process by accelerating signs of tissue damage, and are also implicated in Alzheimer's disease leading to a build up of protein aggregates, resistant to degradation by the body (Negre-Salvayre et al, 2008). Radical species show no discrimination between molecular sites of attack and they are random in nature, showing no affinity for particular molecular sites (Vidrio et al., 2009).

Cell membranes help to protect the cell's organelles, proteins, and nucleic acids from chemical modification and damage by oxygen intermediates such as H_2O_2, $O_2\bullet$- , and OH•. The cell membrane is composed of a phospholipid bilayer, with a hydrophobic

interior and hydrophilic interior, which varies in composition of saturated and unsaturated fatty acids, glycolipids, lipid proteins and steroids such as cholesterol (Radwanski, 2008). Acting as the cell's barrier, the membrane compartmentalizes cell contents and limits molecular traffic to protect important biomolecules, such as DNA (Smith and Murphy, 2008) . Since cell membranes act primarily as protective barriers they are the first point of attack by free radicals and ROS (Smith and Murphy, 2008).

Because cell membranes are generally the first site of attack by radical and ROS, in order to study the effects of oxidative stress on cells many biomedical studies have investigated free radical interaction with varying membrane constituents, mainly fatty acids and membrane proteins (Gutteridge, 1995; Negre-Salvayre et al, 2008; Vidrio et al., 2009). Free radical oxidation of polyunsaturated fatty acids in cell membranes is known as lipid peroxidation (Gutteridge, 1995). This is the main mechanism by which free radicals attack cell membranes (Gutteridge, 1995; Negre-Salvayre et al, 2008). Lipid peroxidation is a chain reaction of events that result in an enormous variety of end products, which are markers of oxidative stress (Gutteridge, 1995). Initiation of lipid peroxidation involves the abstraction of H+ from a methyl group on a fatty acid chain by radicals like OH• and $HO_2^{\bullet -}$ (Gutteridge, 1995). This leaves an unpaired electron on the carbon that then undergoes rearrangement, which after combining with O_2 forms a peroxyl radical (Gutteridge, 1995). The peroxyl radical then starts a chain reaction that abstracts H+ from another fatty acid chain (Gutteridge, 1995). Cessation of this chain reaction requires intervention by compounds such as antioxidants (Gutteridge, 1995).

Lipid peroxidation produces cyclic peroxides, epoxides, and other derivatives whose decomposition result in the formation of reactive carbonyl compounds (RCC), ketones, and alkenes (Negre-Salvayre et al., 2008). RCCs such as aldehydes, dicarbonyls, and malondialdehyde (MDA), can chemically modify proteins (Negre-Salvayre et al, 2008). Modification entails irreversible, intermolecular and intramolecular cross-linking; production of dysfunctional proteins by dimerization between sulfide groups in polypeptides and the formation of adducts (Gutteridge, 1995; Dunne et al, 2006; Negre-Salvayre et al., 2008). Adducts are the products of the addition of two or more molecules resulting in a distinct reaction product (Negre-Salvayre et al., 2008). Free radicals and ROS have been implicated in the production of protein adducts in Alzheimer's disease,

which are highly resistant to normal degradation by enzymes like lysozymes (Negre-Salvayre et al, 2008). Lipid peroxidation also results in alterations in cell membrane fluidity and shape (Khalid and Ashraf, 1993). In summation, free radicals and ROS like H_2O_2 are toxic to cells because of these chemical modifications they induce with OH• being the most extensively studied because it is thought to be the most deleterious culprit of oxidative damage (Gutteridge, 1995; Negre-Salvayre et al., 2008; Vidrio et al., 2009).

Iron and Free Radical Formation

Iron (Fe) is a transition metal whose ions readily enters into electron transfers with oxygen (Gutteridge, 1995; Vidrio et al., 2009). Most notably in the red blood cells, Fe binds and transports oxygen in the blood. Fe is an essential micronutrient for most species, but it can also be toxic: excess or unbound iron can lead to oxidative damage particularly in erythrocytes (Rice-Evans and Baysal, 1987; Gutteridge, 1995; Vidrio et al., 2009). This can come from exposure to Fe atoms from the environment, or breakdown of hemoglobin containing erythrocytes or other iron containing metalloproteins such as myoglobin in muscle tissue (Rice-Evans and Baysal, 1987; Vidrio et al., 2009).

This toxicity is attributed to in part by its ability to mediate the formation of free radicals and ROS because it acts as a catalyst (Rice-Evans and Baysal, 1987; Gutteridge, 1995; Vidrio et al., 2009). In the presence of H_2O_2, oxygen and $O_2^{\bullet-}$, Fe^{2+} leads to the formation of OH•, $O_2^{\bullet-}$,OH- and HOOH (Gutteridge, 1995;Vidrio et al., 2009) (R1-3).

$$Fe(II) + HOOH \rightarrow Fe(III) + \bullet OH + OH^- \quad (R1)$$

$$Fe(II) + O_2 \rightarrow Fe(III) + O_2^- \quad (R2)$$

$$Fe(II) + O_2^- + 2H^+ \rightarrow Fe(III) + HOOH \quad (R3)$$

Biological samples contain variable amounts of peroxidized material that iron can react with (Negre-Salvayre et al, 2008). Peroxides are organic compounds that contain the functional group, ROOH. The O-O bond readily breaks to form free radicals. In cell membranes, Fe can catalyze the formation of radicals by reacting with lipid peroxides found in fatty acid chains to produce alkoxyl and peroxyl radicals (R4) (Halliwell and Gutteridge, 1984; Gutteridge, 1995):

$$LOOH \rightarrow LO\bullet + LO_2\bullet + H_2O \quad (R4)$$

Iron is primarily found associated with metalloproteins such as hemoglobin, which is involved in the transport of oxygen in red blood cells (erythrocytes) through the vascular system. Hemoglobin is a tetramer protein, each intimately associated with a heme prosthetic group (figure 3a). Heme is a heterocyclic ring with an Fe ion in the center (figure 3b).

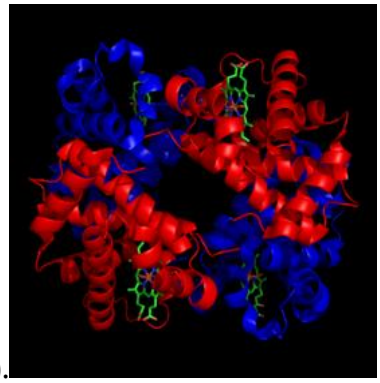
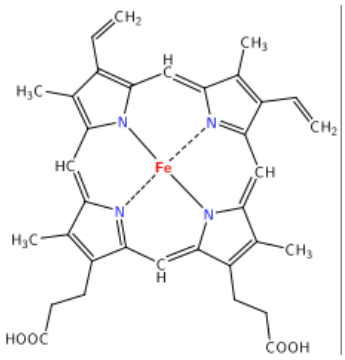
a).  b.) 

Figure 3: a) Human hemoglobin protein. Heme group is highlighted in green, and polypeptide subunits in red and blue. b) Molecular structure of heme group with Fe ion in the center of ring. *Images courtesy of http://en.wikipedia.org/wiki/Hemoglobin*

Erythrocytes have to deal with oxidative stress on a daily basis due to the reduction-oxidation cycling between iron and O_2 in the heme groups (Halliwell and Gutteridge, 1984). Fe cycles between Fe^{2+} and Fe^{3+} after oxygen binds. Breakdown of hemoglobin after death introduces protein-bound and unbound Fe. As shown previously, iron is a common mediator of oxidative stress for cells by producing free radicals, like OH• (Gutteridge, 1995; Dunne et al., 2006; Vidrio et al., 2009). Iron also readily degrades lipid peroxides and ROS that would be present even under normal conditions. Furthermore, after the death of an organism there would be an obvious lack of antioxidant defenses and other protective measures (Negre-Salvayre et al., 2008). Post mortem, the breakdown of hemoglobin would allow Fe to become readily available for free radical formation. As previously mentioned free radical interaction with the fatty acid chains in cell membranes initiates a chain reaction producing a variety of reactive compounds (Gutteridge, 1995; Negre-Salvayre et al., 2008). This cascade causes chemical modifications of lipids and proteins; one of those being irreversible cross-linking of proteins as well as lipids (Khalid and Ashraf, 1993; Negre-Salvayre et al., 2008). Free radical polymerization has been used in the production of bio- and industrial

polymers.

Proposal

So the question remains whether Schweitzer's (2008) proposal can be a viable mechanism for soft-tissue preservation. Can hemoglobin breakdown leading to the exposure of iron mediate cross-linking of cell membranes and lead to improved stability? I propose to test this hypothesis by specifically by looking at the role of iron mediated cross-linking as it pertains to soft-tissue preservation. *Therefore I propose that raising Fe (II) levels may cause increased cross linking in cell membranes that would inhibit cell degradation and increase the chances for preservation.* I want to utilize resealed erythrocytes as a well-characterized membrane model that could test this question by exposing them to varying concentrations of iron. Resources and time often limit experimentation in long term, real life conditions, so small scale experimentation can help illuminate the preservational mechanism by using a simple membrane model to examine the relationship between iron and cross-linking in soft-tissues. This question is important to pursue because it is essential to re-evaluate the preservational processes that would lead to these rare occurrences in the fossil record.

Research Design and Methods

Aim #1: Preparation of Membrane Model and Iron Treatment Groups

My experimental construct necessitates the use of a membrane model exposed to varying concentrations of iron. Using erythrocyte ghosts, which are red blood cells with no internal contents, as a membrane model is ideal for two reasons. First, erythrocytes have been extensively studied due to the simplicity of their membrane components so the detection of cross-linking in membrane proteins only requires the analysis of a limited number of proteins in gel electrophoresis (Radwanski, 2008; Tobiason, personal communication). Therefore they offer straightforward opportunity to evaluate the effects of free radicals on membrane constituents (Gothoskar, 2004). Secondly, in order to specifically look at iron's role in soft-tissue preservation, my study is able to solely investigate iron's role by removing hemoglobin, which could obfuscate the interpretation of results. Subsequent results from this experiment can be used as a comparison to future studies involving varying levels of hemoglobin and its breakdown products.

1a) Ghost cell preparation

The procedure is adapted from Derkham and Harding's (2002) protocol, which calls for the extraction of freshly drawn blood from a pig subject followed by centrifugation, washing the cells, hypotonic lysing, separation of hemoglobin and resealing the membranes by restoring isotonic conditions. Freshly drawn sheep blood will be collected in vacuum tubes and stored at 4°C with heparin (5mg/100 ml) to prevent coagulation during sampling (Kennedy et al, 1986). Blood will first be centrifuged at 1000g for 10 minutes to remove plasma and white blood cells from erthryocytes. Isolated erythrocytes will then be re-suspended in an isotonic HEPES buffer with a pH of 7.4. In order to release internal contents, packed erythrocytes are to be vortexed gently and lysed within a hypo-osmotic buffer (15 mM Pipes and 0.1 M EDTA) with a pH of 6.

In order to ensure the ghost cells are hemoglobin-free, the samples will be filtered using gel filtration chromatography as outlined in Durham and Harding's protocol (2002). Samples will be loaded into a Sepharose 2B size-exclusion column which is a cross-linked agarose beads with a broad fraction range of 70K-40,000 K. The column temperature will be sustained at 0°C via a cooling jacket to prevent resealing. HEPES will be used to elute column. To produce a calibration curve, the molecular weight standard, Fluka-69883, with a molecular weight range of 6500-660,000K, will be purchased from Sigma-Aldrich and prepared by company instruction (Amersham,1998). Based on elution volumes from re-sealing protocol from Derham and Harding (2002) and molecular weights, ghost cell fraction should elute with a void volume of approximately 70 ml; hemoglobin band should elute 130 mls later. Fractions collected will be analyzed using UV-spectrometry with a λmax of 280 nm to confirm fraction identity (Amersham, 1998). Ghost cell fraction should be white to colorless indicating hemoglobin free nature. Cell fractions will be pooled and washed with chilled isotonic HEPES buffer five times to remove hypo-osmotic buffer solution.

To reseal ghost cells, packed cells in isotonic HEPES buffer will be shaken and incubated in water bath at 37°C for 30 minutes. Tubes will then be centrifuged at 10,000 g, mixed and washed three times in HEPES buffer, pH 7 (20 mM Hepes and 145 mM NaCl) and transferred to media described below at 50% hematocrit levels. All dH_2O used in preparation of solutions will be treated with Chelex-100 molecular biology grade resin

from BioRad Laboratories to chelate metal species that could contaminate cell treatment solutions. Specifically, more iron in solution that is unaccounted for would alter the expected concentration levels. Trace amounts of iron would also contaminate the negative control, which should contain no iron.

1b) Iron Treatment and Controls

By using a range of concentrations, the role of iron as a proposed mechanism for cellular preservation can be evaluated. If iron mediates a preservational mechanism, then exposing cellular components such as ghost erythrocytes to varying concentrations can experimentally assess the relationship between iron, free radical production and cell membrane cross-linking. Moreover, it provides insight into base-line concentrations of available iron required to improve preservation potential of cells such as erythrocytes.

Relevant iron levels were chosen based on previous studies that examined free radical chemistry in vitro. Buettner and Jurkiewicz (1996) found that iron concentrations in the range of 1-10 μmol participate in iron oxidation pathways. Studies on oxidative damage from free radical production in physiological buffers commonly use 100 μmol iron (Arai et al., 2005; Vidrio et al.,2009). Therefore, a range of iron concentrations (1μmol, 5μmol, 10μmol, 25μmol, 50μmol, and 100 μmol) will be prepared from ferrous sulfate (99.9+%) purchased from Sigma and then dissolved in an 0.9% saline solution (Starke and Farber, 1985; Vidrio et al., 2009). All iron salt solutions will be freshly prepared the same day to limit the exposure of iron to air and contamination from mishandling of solutions. In 150-ml acid-washed Teflon FEP bottles wrapped in aluminum foil, each treatment solution of Fe (II) will be added to 15 ml of ghost suspension. Acid washing thoroughly cleans the Teflon, removing chemical contaminants, and the aluminum limits exposure of the cell suspension to light. HOOH in 1.0 mM will be added to catalyze the production of hydroxyl radicals (R1) (Vidrio et al., 2009).

$$Fe(II) + HOOH \rightarrow Fe(III) + \bullet OH + OH^- \quad (R1)$$

Transition metals like Fe (II) are known to react with dissolved O_2 to produce superoxides (R2), which react with a reduced metal ion to form HOOH (R3) that participates in R1 (Vidrio et al., 2009).

$Fe\ (II) + O_2 \rightarrow Fe\ (III) + O_2^-$ (R2)

$Fe\ (II) + O_2^- + 2H^+ \rightarrow Fe\ (III) + HOOH$ (R3)

However, HOOH is a frequent occurrence in cells that converts other radicals and ROS to this more stable form (Gutteridge, 1993; Magder, 2006). In tissue post-mortem, the exposure of cells to aerobic conditions and to oxidative species that build during autolysis, necrosis, and general cell stress would no doubt provide iron ions plenty oxygen species with which to react (Gutteridge, 1994; Magder, 2006; Dunne et al, 2006; Schweitzer et al., 2008). Therefore using HOOH (instead of a complex mixture of oxygen species or using a basal amount of oxygen in solution) is required to provide a simple starting point for the production of hydroxyl radical, which is the most commonly cited damaging product of iron auto-oxidation (Gutteridge, 1994; Vidrio et al., 2009). The Teflon bottles that contain the cell suspension with iron will be incubated at room temperature and shaken gently for 24 hours in the dark (Vidrio et al., 2009).

A negative control containing resealed ghost erythrocytes with no added iron but hydrogen peroxide, and a negative control with no iron or hydrogen peroxide under conditions delineated below will be used for comparison purposes. The negative control with peroxide but no iron will help to clarify if metal contamination or other oxidizing reactions have occurred without the addition of catalytic iron. The other negative control that contains only resealed ghosts that will be incubated under the same conditions will likewise reveal possible any confounding effects. Four replicates per treatment level will used for statistical analysis as described later.

Preparation of resealed ghosts is a standard procedure that is straightforward. There should be no difficulty in their preparation. Preparation of iron solutions could encounter problems due to mishandling of samples that would result in exposure to the environment particularly air and light. Solutions will only be prepared and handled by trained individuals.

Aim #2: After the model system is set up, the free radical production will be measured and lipid peroxidation by-products will be detected. In order to ensure free radicals are being produced by each treatment and to quantify their levels, High Performance Liquid Chromatography (HPLC) will be employed after the 24 hour

incubation. To determine if lipid peroxidation is occurring within the system, detection of common lipid peroxidation products will also be detected with HPLC.

2a) Measuring OH Production with High-Performance Liquid Chromatography

Benzoate is a free radical scavenger used to detect •OH in biological samples (Gutteridge, 1987). Benzoate acts as a detector molecule that produces highly fluorescent derivatives through at least three radical mechanisms which result in the formation of phenol, hydroxylation of benzoate, and production of thiobarbituric-acid reactive material (Gutteridge, 1987). The main mechanism involves the hydroxylation of benzoate to hydroxybenzoates by an addition reaction that are highly fluorescent, and can be detected with spectrometry when it is used in conjunction with HPLC (Gutteridge, 1987). This procedure has been commonly used since the 1980's as a straightforward way to detect and quantify •OH production in solution via the measurement of p-hydroxybenzoate (p-HBA), which is a stable product (R5) (Gutteridge, 1987; Vidrio et al., 2009).

Benzoate + •OH --> p-HBA + other products (R5)

Using a previously established protocol by Vidrio et al. (2009), 10 mM of benzoate will be prepared from sodium benzoate (A.C.S.). At the end of the incubation period, samples from each treatment concentration and both controls will be homogenized and centrifuged; the supernatant will be quickly added to the benzoate solution in auto-sampler vials under dark conditions ready for immediate HPLC analysis.

The HPLC apparatus entails a LC10-AT pump, and a C-18 reverse-phase column (250 x 3 mm; 5 μm beads) with a SPD-10AV UV-visible detector with $\lambda = 256$ nm (Vidrio et al., 2009). Eluent is composed of 70% filtered, $_dH_20$ and 30% CH_3CN with pH adjusted to pH=2 with $HClO_4$, and a flow rate of 0.60 ml min $^{-1}$. A calibration curve will be obtained using p-HBA standards made in the ghost suspension fluid used during the reaction (Vidrio et al., 2009). Calibration will be performed on the day of each experiment. Concentration of •OH will be determined using the following equation from established by Jung et al (2006):

$$[OH] = [p\text{-}HBA] / (Y_{p\text{-}HBA} \text{ X } \#\#\#_{BA}) \quad (R5)$$

[p-HBA] stands for the measured concentration of p-hydroxybenzoic acid; Y_{pHBA} is the molar yield of p-hydroxybenzoic acid produced from the reaction of benzoate with •OH

in buffer without cells. In addition, spectra will be compared to reference spectra from previously established HPLC quantification protocols (Gutteridge, 1987).

2b) Detection of Lipid Peroxidation

Lipid peroxidation and its derivatives should not be assumed simply from detecting the presence of •OH. Therefore, detection of lipid peroxidation through the presence of its varied products is required (Gutteridge, 1995). Detection of lipid peroxidation is also important because this pathway provides evidence that free radicals are interacting with the cell membrane (Schweitzer et al., 2008). Lipid peroxidation in each treatment group can be compared to the amount of free radical production, and alterations in proteins and lipids seen in electrophoresis. There is a multitude of techniques that test for lipid peroxidation products such as aldehydes, ketones, lipid peroxides, and diene conjugation of fatty acids (Gutteridge, 1995). Erythrocyte membranes are rich in polyunsaturated fatty acids, so they are susceptible to lipid peroxidation (Lykkesfeldt, 2001). Also, other fluorescent assays measure aldehydes by the formation of fluorescent products; however, these assays introduce complex reaction products and side reactions due to derivatization making it difficult to assess whether fluorescent products are actually from lipid peroxidation (Gutteridge, 1995).

Lipid hydroperoxides (LOOH) and its moieties such as its hydroxy derivative (LOH), are the main stable products of lipid peroxidation (Browne and Armstrong, 2000). HPLC allows for an accurate depiction of LOOH levels without derivatization (Browne and Armstrong, 2000). HPLC allows for the separation of LOOHs and LOHs without extensive manipulation. Both conjugated dienes of LOOH and LOH absorb strongly near 234 nm (Browne and Armstrong, 2000). Reverse-phase HPLC as been shown in previous studies to provide an accurate representation of LOOH and its moieties with improved resolution (Browne and Armstrong, 2000).

For this study, the HPLC protocol will be adapted from Browne and Armstrong (2000). All reagents used will be HPLC grade of the highest quality. Autoxidation will be reduced during sample handling by removing dissolved oxygen from solvents involved in extraction and elution. This will be accomplished by placing solvents under reduced pressure, solvent will be ultrasonicated, and then there will be 15 minutes of bubbling

with helium (Browne and Armstrong, 2000).

A portion of each erythrocyte treatment sample will be washed with phosphate-buffered saline. A 40-μL sample will be diluted with 100 μL of H20, and then mixed with 20-μL of 2.5 mmol/L butylated hydroxytoluene (BHT) in ethanol, 40-μL of sodium dodecyl sulfate, and 600 μL of thiobarbituric acid (TBA) reagent diluted 1:1 with 200 mL/L acetic acid (pH 3.5). The sample will be heated to 95°C for an hour and cooled. An additional 200 μL of H2O and 1000 μL of butanol-pyridine (15:1) will be added. The centrifuge tube will be vortexed vigorously, and the organic layer will be separated via centrifugation at 16 000g. Sample injections will be run at 1 mL/ min. Calibration curves will constructed with 2.5-100 μmol/L of tetramethoxypropane. An automated HPLC gradient system with a autosampler (4 C), gradient pump, vacuum degasser, and fluorescence detector (excitation, 515 nm; emission 553 nm) will be employed. A C8 column with a particle size of 5 μm and 150 x 4.6 nm sized column will be fitted with a guard column. Mobile phases are composed of 300 mL/L methanol in 50 mmol/L potassium dihydrogen phosphate buffer (pH 7.0) for phase A, and 800 mL/L of methanol in same phosphate buffer. Sodium azide was added to mobile phases to prevent bacterial growth. A reagent blank will be run along with samples.

As the iron concentration rises from 0-100 μM, the free radical levels would be expected to rise (Gutteridge, 1995). This positive trend would be expected because there is more iron to catalyze with HOOH and any dissolved oxygen to produce more free radicals. I have not found previous studies to quantify lipid peroxidation products in relation to free radical levels. I would expect that lipid hydroperoxide levels that will be detected during HPLC analysis would rise. However, lipid peroxidation is a chain reaction occurring with the cell membrane consituents, and there are a set number of consituents from which to produce lipid hydroperoxides. Increasing free radical production perhaps only increases the rate at which this achieved not the levels of hydroperoxides. Also, HPLC analysis of lipid hydroperoxides is only examining one by-product, and although it is a primary by-product.

The HOOH but no iron should not produce free radicals, nor should lipid hydroperoxides be detected in the sample. If there is no iron contamination of the solution, there should be no production of free radicals if the solution was prepped and

stored correctly. Also, it should show no signs of lipid peroxidation because it contains no cells. The second negative control contains only ghost cells with no reagents added. I would expect to see little to no free radical production, and little to no lipid hydroperoxides. However, cell membranes in living organisms, particularly red blood cells, normally contain small levels of lipid peroxides in them, but they may below the detectable limits using HPLC (Gutteridge, 1995).

Furthermore, another issue are the detection limits and detection of differences between treatments of free radicals and lipid hydroperoxides. The concentrations of iron used in this study were chosen from previously established protocols. While increases in free radical production and lipid hydroperoxides may occur because of rising concentrations of iron, these differences may be too small for the method chosen to differentiate. These levels may also be below the detectable limit. These issues could be resolved by using a different method of detection such as mass spectometry analysis. Collaboration with analytical chemists could also help streamline the procedure. Also, using a greater range of concentrations could improved resolution of results.

Aim #3: Subsequently, each erythrocyte treatment group will have membrane cross-linking evaluated by gel electrophoresis. Cell cross-linking will be evaluated using SDS-PAGE and dual staining of lipids and membrane proteins. Red blood cell membranes are well characterized and they are an ideal membrane model for this purpose due to the simplicity of their membrane components. Detection of cross-linking in membrane proteins only requires the analysis of a limited number of well-characterized protein banding patterns in gel electrophoresis. Therefore they offer the opportunity to evaluate the effects of free radicals on membrane lipids and proteins (Gothoskar, 2004).

Evaluating Lipid and Protein Cross-linking

Cross-linking of lipids and proteins in cell membranes alter the molecular weight. Cross-linking would result in higher molecular weight protein products and lipids. Gel electrophoresis separates proteins and lipids based on molecular weight. Therefore, this technique can be used to initially assess the number of cross-linked products by observing changes of electrophoretic mobility in a SDS-PAGE gel in comparison with a

molecular weight standard as well as the negative controls described previously.

SDS-PAGE will be performed as adapted from Dzandu et al. (1984) and Hixson et al. (1981). After the 24 hour treatment, a portion of each treatment sample will be centrifuged with supernatant removed and washed five times in 5 mM Tris/HCl / 140mM NaCl / 1mM EDTA/ 0.1 mM phenylmethylsulfonyl fluoride (pH 7.4) at 4 C. Cells will be diluted to numbers of equal cells, centrifuged, and then solubilized with 1% sodium dodecyl sulfate (SDS). Electrophoresis will be carried out on 16 x 18 x 0.15 cm acrylamide slab gels. A current of 50 mA for each slab until bromophenol blue tracking dye is 1.5 cm from the bottom of gel. Molecular weight standards will be purchased from Sigma; erythrocyte membrane polypetide band standards will also be used as internal molecular weight standards.

A silver-staining kit to stain lipids from Bio-Rad will be used according to manufacturer's instructions. Each slab will be counterstained with 200 ml of 0.1% (wt/vol) coomassie blue/ 25% (vol/vol) methanol/ 7.5% (vol/vol) acetic acid for proteins (Dzandu et al., 1984). Gels will be loaded with 1.25 μL of packed erythrocytes from treatment sample replicates including controls. Lipids will be stained a yellow color while polypeptides will stained blue (Dzandu et al., 1984). Densitometry will be used to assess changes in protein and lipid densities per band (Tobiason, personal communication).

I would expect that as the iron treatment levels rose, so would the iron catalysis of free radicals. If iron production of free radicals plays a role in cross-linking as stated by Schweitzer et al. (2007), I would expect to see increases in the cross-linking of cell membrane proteins and lipids in the ghost erythrocytes. Increases in cross-linking will correspond to larger protein and lipid sizes because two or more proteins or lipids are polymerized together. Larger sized proteins and lipids will travel slower through the gel matrix, and migration distances will be shorter. Therefore I would expect that ghosts treated with the highest concentration of iron, 100 μM, would show the lowest migration distances for its membrane proteins and lipids when compared to the untreated ghost cell standard. Also, densitometric analysis of bands from treated ghosts and untreated ghost standard would be expected to reveal bands that are more dense (meaning more protein or lipid due to staining intensity) closer to the wells. I anticipate bands will become

denser in coloration because there are more proteins and lipids per band due to polymerization of one or more proteins and lipids.

However, it would be idealistic to expect all proteins and lipids to cross-linked as some would remain unaltered. Perhaps as the iron concentration reaches the upper limits of 100 μM this may occur. Also, the iron concentrations chosen may not be sufficient to display detectable differences in band distance and density. A new, broader range of iron concentrations could then be used like 0-200 μM.

Discussion

In 2005, the discovery of pliable soft-tissues from a 65+ million year old *Tyrannosaurus rex* specimen by Schweitzer et al. prompted discussion of mechanisms that allowed for the preservation of collagen and cellular material such as osteocytes. One of the mechanisms proposed was the triggering of cross-linking of cell membrane consituents by iron, which is oxidized during hemoglobin breakdown and subsequently catalyzes the production of free radicals (Schweitzer et al. 2007). Cross-linking of membrane proteins and lipids makes them resistant to decay and degradative reactions (Schweitzer et al., 2007). I want to investigate iron's role as a preservational mechanism, so I propose to expose cellular material (erythrocyte ghosts) to varying concentrations of iron. Free radical production will be quantified and lipid peroxidation will be detected by HPLC analysis. Cross-linking in proteins and lipids will be assessed using SDS-PAGE with densitometry to evaluate changes in bands.

Increases in lipid and protein band densities and lower migration distances are expected if polymerization of the membrane constituents occurs. Demonstrating polymerization of lipids and proteins in ghost erthrocytes would support Schweitzer's hypothesis, and it would help explain the conservation of endogenous organic material. If iron production of free radicals does not induce detectable cross-linking in this study, it would not support Schweitzer's hypothesis concerning iron's contribution to preservation of soft tissues like osteocytes and endothelial cells (Schweitzer et al., 2007). Iron concentrations were chosen based on previous studies; however, these concentrations of iron may not be relevant because the iron concentrations of decaying remains in a

realistic setting has not been investigated. Future studies could include the examination of iron content in decaying organisms over time, this would elucidate relevant iron concentrations to be used in a similar study such as this.

As stated by Schweitzer et al. (2007), this mechanism alone, if supported by the results of my study, is not adequate to explain preservation of soft-tissue structures across millions of years; the stabilization of organic material through phosphatization and mineral precipitation is the second step. However, investigation of this mechanism is important to authenticate patterns of preservation because the conservation of soft-tissues and cellular material has defied previous expectations (Schweitzer et al., 2007). Iron initiation of cross-linking in cellular membranes could be a central mechanism in the stabilization of ancient organic components.

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