BASIC SCIENCE RESEARCH

Free Radical Damage in Facsimile Synovium: Correlation with Adhesion Formation in Osteoarthritic TMJs

Donald W. Sheets, Jr., DMD;[1] Toru Okamoto, DDS, PhD;[2] Leonore C. Dijkgraaf, DDS, PhD;[3] Stephen B. Milam, DDS, PhD;[4] John P. Schmitz, DDS, PhD;[5] and Gustavo Zardeneta, PhD[6]

Purpose: **The purpose of this study was to use the rat air pouch model of facsimile synovium to evaluate oxidative stress as a primary mechanism in the pathogenesis of degenerative temporomandibular joint (TMJ) disease.**

Materials and Methods: **Forty-nine Sprague-Dawley adult female rats were used to generate the standard rat air pouch model of facsimile synovium. This was accomplished by daily air injections (20 cc) subdermally through the dorsal skin. Hydrogen peroxide and ferrous iron (components of the Fenton reaction which generate free radicals) were introduced into the pouches of the 4-, 7-, and 14-day groups to generate oxidative stress. Control rats were injected with phosphate-buffered solution (PBS), pH 7.4. Either *N*-acetylcysteine (NAC), a powerful free radical scavenger, or ibuprofen were simultaneously injected with the Fenton reagents into the pouches of the 14-day treatment groups to modulate free radical-mediated protein damage to the synovium. Animals were euthanized at appropriate experimental intervals and biopsies obtained from specimens to analyze: (1) proteins' amino acid modification (carbonyl group formation), (2) protein hydrophobicity, (3) detection of low molecular weight protein degradation products, and (4) histological and gross anatomical observations.**

Results: **Free radicals introduced into the rat air pouch interacted with synovial tissues causing oxidation and breakdown of proteins. Clinical evidence of adhesion formation consistent with features found in osteoarthritis of the TMJ developed. The groups subjected to oxidative stress experienced statistically significant ($p < 0.05$) increases in carbonyl formation, carbonyls/protein, and low molecular weight protein fragments. These groups also showed significant ($p < 0.05$) hydrophobicity changes consistent with free radical attack. Control synovial tissues were statistically undamaged. The 14-day NAC and ibuprofen treatment groups experienced statistically significant ($p < 0.05$) decreases in total carbonyl formation, carbonyls/protein, and hydrophobicity. Histological and gross observations in free radical damaged synovium exhibited features consistent with known arthoscopic and arthrocentesis findings in diseased TMJs.**

Abstract continued on next page

[1]*Captain USAF, Resident, Department of Prosthodontics, Wilford Hall Medical Center, Lackland AFB, San Antonio, TX.*

[2]*Section of Orthodontics, Department of Oral Functional Science, Division of Oral Medical Science, Graduate School of Dentistry, Hokkaido University, Sapporo, Japan.*

[3]*Department of Oral and Maxillofacial Surgery, Groningen University Hospital, The Netherlands.*

[4]*Professor and Chairman, Department of Oral and Maxillofacial Surgery, University of Texas Health Science Center San Antonio, TX.*

[5]*Associate Professor, Department of Oral and Maxillofacial Surgery, University of Texas Health Science Center San Antonio, TX.*

[6]*Director of Research and Assistant Professor, Department of Oral and Maxillofacial Surgery, University of Texas Health Science Center San Antonio, TX.*

Accepted December 2, 2004.

Correspondence to: Gustavo Zardeneta, PhD, Department of Oral/Maxillofacial Surgery, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229. E-mail: zardeneta@uthscsa.edu

1059-941X/06

doi: 10.1111/j.1532-849X.2006.00063.x

Conclusions: **This study suggests that the rat air pouch model of facsimile synovium develops clinical evidence of adhesions and biochemical signs of protein modification when subjected to free radical attack. NAC and ibuprofen prevented carbonyl formation as well as hydrophobicity changes indicative of oxidative stress damage in facsimile synovium. These findings are consistent with features of degenerative human TMJ disease. Future direction may be taken from this study to postulate new analysis techniques and treatment modalities for patients with degenerative TMJ disease.**

J Prosthodont 2006;15:9-19. Copyright © 2006 by The American College of Prosthodontists.

INDEX WORDS: degenerative temporomandibular joint disease, free radicals, oxidative stress, adhesions, air pouch model, facsimile synovium

TEMPOROMANDIBULAR JOINT (TMJ) disease, its etiology, pathogenesis, and the degenerative changes affecting the articular structures are not yet fully understood. Arthroscopic studies suggest that chronic degenerative TMJ disease, in addition to cartilage degeneration, may initially result in synovial tissue hypervascularity, creeping synovitis, and redundancy of the synovial lining. Further progression of the TMJ disease is characterized by adhesion formation and a reduction of the posterior and anterior recess leading to functional impairment and pain.[1] In recent years, TMJ arthrocentesis and lavage techniques have been refined in order to detect specific molecular metabolites and proteins in lavage fluid of affected TMJs.[2] Specific characteristics and biochemical mechanisms that may cause damage to the synovial tissues have been postulated. This can ultimately result in progression of the TMJ disease and characteristic adhesion formation in the TMJ compartments leading to functional impairment and pain. Our hypothesis is that degraded proteins, generated by oxidative stress, aggregate within the TMJ compartment to form adhesions.[3]

Biochemical and histological analyses of the TMJ fluid and tissues have led to an increased understanding of diseased TMJ pathophysiology, and studies of other joints have led to the advancement of three pathogenic mechanisms of injury associated with degenerative TMJ disease[3]—direct mechanical injury, hypoxia/reperfusion injury, and neurogenic inflammation. Each model of disease hypothesizes that the underlying cause of degenerative TMJ disease is direct or indirect mechanical stress to the TMJ leading to oxidative stress. The term oxidative stress designates any condition that results in the accumulation of free radicals in a tissue.[4,5] Free radicals are molecules capable of independent existence that have one or more unpaired electrons in their outer orbits.[6] These molecules are extremely reactive and may function as second messengers by activating molecules involved in vital cellular functions.[7,8] Excessive free radical-mediated reactions can be extremely damaging to TMJ synovial tissues by causing direct damage to both cellular and extracellular molecules and by excessive activation of normal cellular processes via their second messenger role.[9,10] Oxidative stress in the TMJ can lead to the conversion of extracellular matrix components into protein radicals (Fig 1). Protein radicals can form intramolecular and intermolecular crosslinks making them more resistant to degradation.[11] Our hypothesis is that oxidized proteins aggregate within the TMJ joint space to form adhesions.[12] As these protein aggregates accumulate in TMJ compartments, they can ultimately form nucleation sites for adhesions. An adhesion is defined as the development of abnormal attachments between tissues[13] and is a characteristic degenerative modification of the TMJ extracellular matrix.

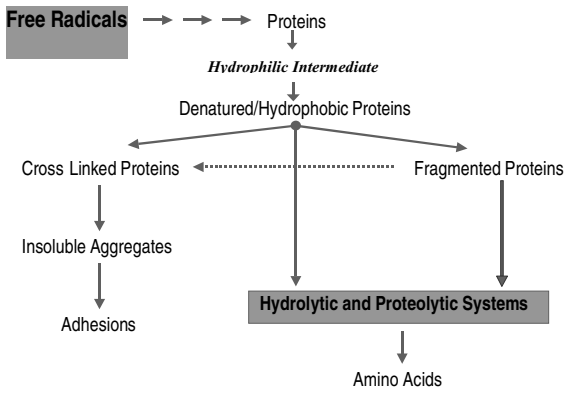
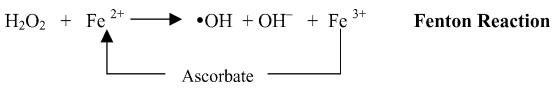


Figure 1. Schematic representation of free radical-mediated protein damage, fragmentation, and aggregation.

The hydroxyl radical is capable of attacking molecules in the TMJ, causing fragmentation and degradation of the extracellular matrix and synovial fluid components.[9,14] Mechanical stress or the TMJ trauma can result in focal bleeding into the TMJ spaces with subsequent extravasation of erythrocytes.[15] As these erythrocytes are destroyed, hemoglobin is released and iron is deposited in the synovial tissues. Zardeneta et al[16] reported a significant amount of redox-active hemoglobin and hydrogen peroxide recovered by arthrocentesis in diseased TMJs. In inflammatory TMJ disease, phagocytes accumulate and produce the superoxide radical and hydrogen peroxide.[17] The superoxide radical dismutes to form hydrogen peroxide and also reduces Fe^{3+} to Fe^{2+}.[19] Hemoglobin provides highly reactive iron to catalyze the conversion of hydrogen peroxide to hydroxyl radicals in what is known as the Fenton reaction.[9,18,19] The hydroxyl radical is also generated when ascorbic acid (present in the synovial fluid of inflamed joints) interacts with hydrogen peroxide and iron in a reaction independent of superoxide.[20]



For the Fenton reaction to be sustained, Fe^{3+} generated by the oxidation of iron Fe^{2+} must be recycled or reduced back to iron Fe^{2+}.

The absence of an appropriate animal model for investigation of the synovial pathophysiology and biochemical mechanisms of degenerative TMJ disease[21] has limited correlation of in vitro data from arthrocentesis specimens with gross histopathological findings. The mechanisms that perpetuate these processes will not be completely understood without a congruous animal model for investigation. Histological and biochemical studies are essential for the study of the pathogenesis of these disorders and development of therapies that are scientifically based on an understanding of the specific disease mechanisms at a molecular level. The purpose of this study was to use the subcutaneous rat air pouch model of facsimile synovium to evaluate oxidative stress as a primary mechanism in the pathogenesis of extracellular matrix protein degradation and adhesion formation in the TMJ disease.

Figure 2. Subcutaneous rat air pouch model. Mechanical disruption of subcutaneous connective tissue on the dorsum of rats by 20 cc air injections over a six-day period develops a synovial membrane-lined cavity by accretion of macrophages and fibroblasts via mechanical cavitation.

Materials and Methods

Rat Air Pouch Model

Forty-nine Sprague-Dawley adult female albino rats (10 rats for pilot study and 39 for final study) weighing 200 to 250 g and housed two per cage were used to generate the standard rat subcutaneous air pouch model of facsimile synovium.[22] This study was approved by the UTHSCSA Instutional Animal Care and Use Committee, protocol #99155-35-01-A. The model was generated by daily 20 cc subcutaneous air injections under the dorsal skin surface of all rats over a six to seven day period (Fig 2).

Materials

$FeSO_4$ and *N*-acetylcysteine (NAC) were purchased from Aldrich Chemical Co., Milwaukee, WI. Ascorbic acid, ibuprofen sodium salt, and phenylmethyl sulfonyl fluoride (PMSF) were acquired from Sigma Chemical Co., St. Louis, MO. The fluorescent probes bis-ANS (1-anilinonaphthalene-8-sulfonic acid) and Bodipy Fluorescein were obtained from Molecular Probes, Eugene, OR. Bio Rad, Hercules, CA, manufactured the 4% to 15% Tris-HCl gradient gels used for SDS-PAGE analysis. Solubilization solution (PBS-TDS) consisted of 1% Triton® X-100, 100 mM deoxycholate, and 1% SDS in PBS, pH 7.5. All other reagents used were reagent grade.

Experimental Design

A pilot study using 10 rats (5 test rats and 5 controls) was accomplished to determine sample sizes for the final study. A power analysis was performed using a power

Table 1. Experimental Design in All Groups

Group	*# Rats*	*Treatment*	*Days*
A	6	F, A, P	4
A control	3	P	4
B	6	F, A, P	7
B control	3	P	7
C	6	F, A, P	14
D	6	F, A, P, NAC	14
E	6	F, A, P, ibuprofen	14
CDE control	3	P	14

Air pouches were developed for six days prior to treatment. Rats were sacrificed in the day following the last treatment day.
F = Fenton reagents; A = Ascorbate; P = PBS; NAC = N-acetylcysteine.

value of at least 85% and $\alpha = 0.05$ to determine final sample sizes.

Thirty-nine rats were used for the final study. Following development of a stable air pouch, Groups A, B, and C (6 rats each) received daily injections of Fenton reagents (4.2 mM $FeSO_4$, 50 mM H_2O_2), 25 mM ascorbate, and pH 7.4 phosphate buffered solution (PBS) in a total volume of 5 ml for 4, 7, and 14 days, respectively. Control Groups A, B, and CDE (3 rats each) received daily 5 ml PBS injections for 4, 7, and 14 days, respectively (Table 1). Groups A, B, and C were used to examine the time-dependent effects of oxidative stress reactions in the rat air pouch.

Following development of a stable air pouch, Group D (6 rats) received daily injections of Fenton reagents, ascorbic acid, PBS, and 21 mM NAC (i.e., 5× molar amount relative to the limiting Fenton reagent iron sulfate), a free radical scavenger for 14 days. Group E (6 rats) received daily injections of Fenton reagents, ascorbic acid, PBS, and 21 mM of ibuprofen (i.e., 5× molar amount relative to the limiting Fenton reagent iron sulfate) for 14 days. Control Group CDE (3 rats) received daily 5 ml PBS injections for 14 days. Groups D and E were used to examine the effect of a free radical scavenger and an anti-inflammatory drug on oxidative stress reactions in the rat air pouch. All experimental groups were run in concurrence and euthanized at appropriate experimental intervals (see experimental design, Table 1).

Euthanasia and Biopsy Protocol

The animals were euthanized at the appropriate experimental intervals via carbon dioxide inhalation. Dorsal pouch dissection was accomplished via sharp and blunt techniques to gain access to the internal surface of the pouch and synovial lining. First, the pouches were inspected macroscopically for adhesion formation and other pathologiccal changes. Subsequently, four biopsy samples, two 10% neutral formalin-fixed and two frozen samples (−80° C), per test and control rats were used for eventual histological and biochemical analysis. Tissue specimens were taken from adhesions located at the lateral border of the pouch along the anterior line of transition between the normal adherent skin and that detached by the air pouch.

Tissue Sample Preparation

Frozen tissue specimens used for biochemical analysis were solubilized in a solution containing 1.5 mM PMSF and 1 ml of PBS-TDS. Each sample was sonicated two times each for 1 minute with a Microson ultrasonic cell disruptor (Misonix, Inc., Farmingdale, NY) to solubilize tissue, and stored at −80°C. Tissues to be used for histological analysis were fixed in 10% neutral formalin and embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin.

Protein Quantification

The BCA colorimetric assay (Pierce Chemical Co., Rockford, IL) with a bovine serum albumin (BSA) standard was used with a Dynatech MR 5000 spectrophotometer (Dynex Technologies, Chantilly, VA) to determine protein concentration.

Amino Acid Modification (Detection of Carbonyl Groups by Fluorescence)

Assessment of protein modification by free radicals was measured with the fluorophore, Bodipy Fluorescein, which reacts with carbonyl groups yielding stable hydrazones to give a bright fluorescence.[23] The extent of fluorescence correlates with the amounts of carbonyl groups indicative of free radical damage.

Protein samples (100 μg) were incubated with 10 μg Bodipy and PBS in a final volume of 100 μl at 25°C in the dark for 1 hour. Then, protein was precipitated by the addition of 1 ml 10% trichloroacetic acid (TCA) to each sample, and incubated at 4°C for 10 minutes. Samples were centrifuged 5 minutes at 5000*g* at 4°C. The supernatant was discarded and the TCA precipitation repeated. The remaining pellets were washed with 1 ml ethanol:ethylacetate, 1:1 v/v solution three times each to remove the free dye. The pellets were resuspended in 120 μl of Tris-HCl, pH 8.8. Samples (100 μl) were aliquoted into a flat bottom polystyrene 96-well plate. The total relative fluorescence was measured with a Cytofluor 2350 fluorescent plate reader (Millipore, Bedford, MA) using excitation and emission wavelengths of 495 and 520 nm, respectively.

Bodipy-labeled samples were also analyzed by SDS-PAGE (sodium dodecyl sulfate—polyacrylamide gel

electrophoresis). Aliquots (50 μl) from the 96-well plate after fluorometric analysis were run in 4% to 15% Tris-HCl gradient gels, and the protein bands were visualized and photographed using a multi-image light cabinet (Alpha Innotech Corp., San Leandro, CA). The most prominent fluorescent band at ~70 kDa was quantified by densitometric analysis using NIH Image 1.54-computer software. Relative densitometric units were calculated from the integration of peak areas in comparison with standards.[24]

Detection of Low Molecular Weight Protein Degradation Products

Protein samples (50 μg) of solubilized control and test samples were subjected to SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250 (Bio Rad, Hercules, CA). Protein fragments were quantified by densitometric analysis using the relative staining intensities which can reliably detect a 5% loss of a given protein band.[25]

Protein Structure Modification (Detection of Hydrophobicity Changes by Fluorescence)

Bis-ANS binds to hydrophobic sites on proteins and was used to detect protein conformations. Initially, as a protein denatures, bis-ANS fluorescence decreases and then increases as a protein becomes more denatured.[26] Protein (100 μg) solubilized from pouch synovial tissues was incubated in the dark for 5 minutes with 0.05 μM bis-ANS in a total volume of 100 μl in a 96-well plate. Fluorescence was quantitated in a plate reader using excitation and emission wavelengths of 360 and 460 nm, respectively.

Histological Observations

Histology samples were assessed based on parameters described previously.[27-31] The observations were based on the following parameters: synovial lining features (hyperplasia, vascularity, inflammatory cell infiltrate, morphology, fibrinoid extracellular matrix tissue) and subintima features (inflammation, vascularity, type, myxoid degeneration, perivascular fibrosis, erythrocyte extravasation).

Statistical Management of the Data

Pilot study data were analyzed via Student's *t*-test to assess statistically significant differences and a power analysis to determine sample size for the final study. A two-way analysis of variance was used for the time course study (Groups A, B, and C) to compare groups across three time periods. A one-way analysis of variance was used for treatment Groups D and E. A Tukey HSD post hoc test was used to assess the effects of treatment.

Results

Macroscopic Findings

Clinical evidence of adhesion formation was present in areas anterior and lateral to the air pouch in the test subjects (4-, 7-, and 14-day Fenton-treated Groups A, B, and C) (Fig 3). This was characteristic of the formation of abnormal attachments between the air pouch synovial tissues and consistent with a degenerative modification of the extracellular matrix of the test subject vs.

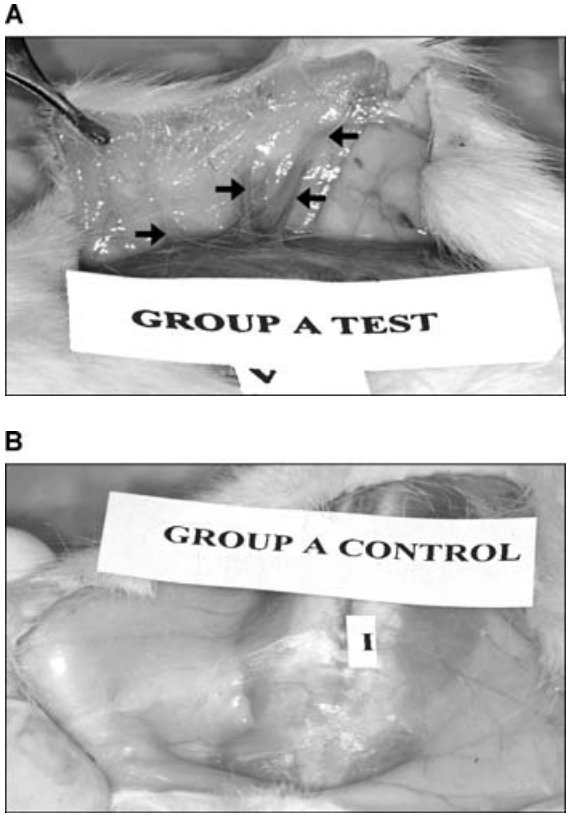


Figure 3. Macroscopic presentation of adhesion formation in rat air pouch. (A) After a 4-day treatment with Fenton reagents (Group A), the lateral borders of the dissected air pouch show adhesions (depicted by arrows). Note the change in synovial tissue character to include hypervascularity, whereas a control specimen (B), shows clinically undamaged synovium. Note pink, noninflamed synovial tissues.

controls. NAC and ibuprofen treatment groups (D and E) also experienced adhesion formation relative to controls.

Histological Observations

Histological observations of all test subjects (including the 4-, 7-, and 14-day Fenton-treated groups and the 14-day NAC and ibuprofen groups) consistently demonstrated synovial lining hyperplasia, fibrous intima matrices, fibrous subintimal tissues, hypervascularity, inflammatory infiltration, perivascular fibrosis, myxoid degeneration, and erythrocyte extravasation relative to respective control specimens (Fig 4).

Fluorophore-Quantitated Presence of Carbonyl Groups (Total Protein)

The presence of carbonyl groups in total proteins derived from air pouch adhesions represented a significant increase over controls (Fig 5). At 7 and 14 days, the carbonyl group formation was significantly increased over controls. This represented a 65% and 75% increase, respectively. Carbonyl group formation was inhibited by both NAC and ibuprofen at the 14-day period. NAC inhibited carbonyl formation 58% compared to 14-day samples, while ibuprofen inhibited carbonyl formation 51% compared to 14-day samples. This demonstrated that certain free radical scavengers (NAC and ibuprofen) could modulate carbonyl formation in a facsimile synovium model of oxidative stress generated by the Fenton reaction.

Detection of Carbonyl Groups in 70 kDa Proteins

Solubilized tissue samples were labeled with Bodipy fluorescein and analyzed by SDS-PAGE. The most prominent fluorescent protein band was seen at ~70 kDa. This was analyzed by densitometric analysis. The 7-, and 14-day Fenton-treated groups showed significant increases in carbonyls/70 kDa protein relative to their controls by 44%, 235%, and 280%, respectively (Fig 6). Carbonyl group formation was inhibited by NAC at the 14-day period. Carbonyl group formation was not inhibited in the 14-day ibuprofen group. In view of the total protein inhibition of carbonyl formation by ibuprofen, this data suggests that the ~70 kDa protein may be more susceptible to free radical attack and resultant carbonyl formation.

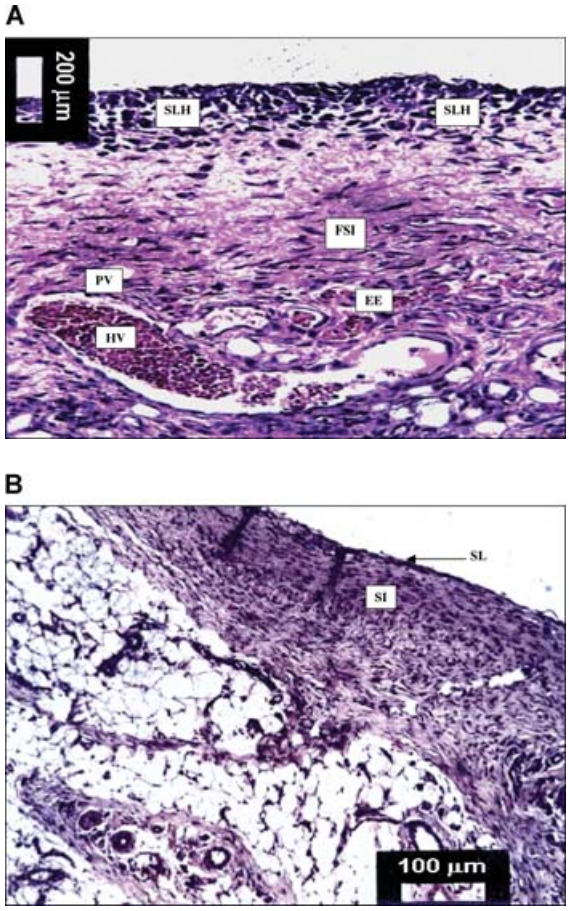


Figure 4. Histologic observations of the rat air pouch model of facsimile synovium. (A) Shows a photomicrograph of a sample from Group A (4-day Fenton-treated group). SLH = synovial lining hyperplasia with dense fibrinoid extracellular matrix, FSI = fibrous subintima with acute inflammatory infiltrate and myxoid degeneration, HV = hypervascularity (ingrowth of multiple vascular structures to support hyperplasic synovial lining) with perivascular fibrosis (PV). EE = erythrocyte extravasation. (B) Shows a photomicrograph of an untreated sample from Group A control. SL = 1 to 3 cell layer thickness synovial lining (nonhyperplasic), SI = mixed subintima of areolar and adipose type with relative lack of inflammatory infiltrate.

Detection of Low Molecular Weight Protein Degradation Products

Low molecular weight protein degradation products (less than 70 kDa) were detected using

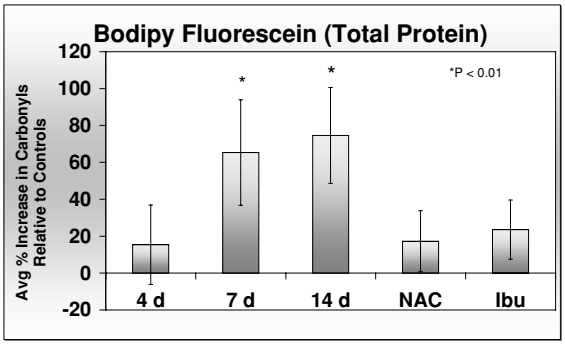


Figure 5. Presence of total protein carbonyl groups quantified by fluorescence. Solubilized protein samples were labeled with Bodipy fluorescein and subjected to fluorometric analysis. Higher levels of fluorescence correlate to higher degrees of carbonyl group formation and hence oxidative modification. Significant statistical differences between the 4-, 7-, and 14-day Fenton-treated groups as well as the 14-day NAC and ibuprofen groups and their respective controls are denoted with asterisks.

densitometric analysis. The 7-, and 14-day Fenton-treated groups showed a 5%, 41%, and 101% average increase, respectively, in protein degradation. This represents significant increases over their controls (Fig 7). Thus, there was a time-dependent increase in protein degradation upon exposure to oxidative stress. The 14-day NAC and ibuprofen groups showed significant average increases in protein degradation of 147% and 142%, respectively, over controls.

Protein Structure Modification (Detection of Hydrophobicity Changes by Fluorescence)

The solubilized protein samples were incubated with bis-ANS and analyzed fluorometrically. The initial hydrophilic intermediate of the oxidatively damaged proteins was detected as indicated by decreases in hydrophobicity of all samples versus their controls (Fig 8). The 4-, 7-, and 14-day Fenton-treated groups showed a significant 31%, 36%, and 38% average decrease in hydrophobicity, respectively, relative to their own controls. The 14-day NAC and ibuprofen groups showed a significant 44% and 31% average decrease in hydrophobicity, respectively, relative to controls.

Discussion

The subcutaneous rat air pouch model, originally described by Selye in 1953,[32] has been found to be

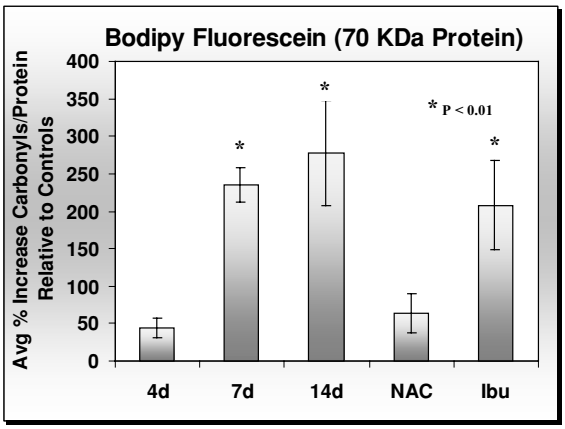


Figure 6. Carbonyl groups in ~70 kDa band. Bodipy-labeled samples from Figure 5 were analyzed by SDS-PAGE. The most prominent fluorescent band at ~70 kDa was quantified by densitometric analysis. Greater relative densitometric units correlate to higher degrees of carbonyl group formation and hence oxidative modification. Significant statistical differences between groups and their respective controls are denoted with asterisks.

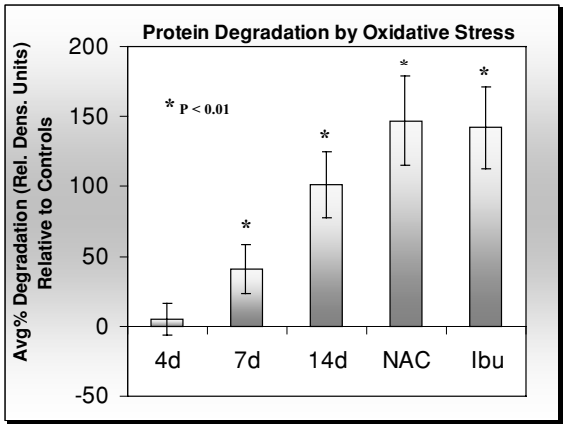


Figure 7. Detection of low molecular weight protein degradation products by SDS-PAGE analysis. Solubilized protein samples were subjected to SDS-PAGE. Protein fragments were quantified by densitometric analysis. Increased relative amounts of low molecular weight protein bands correlate to increased degradation. Significant statistical differences between groups and their respective controls are denoted with asterisks.

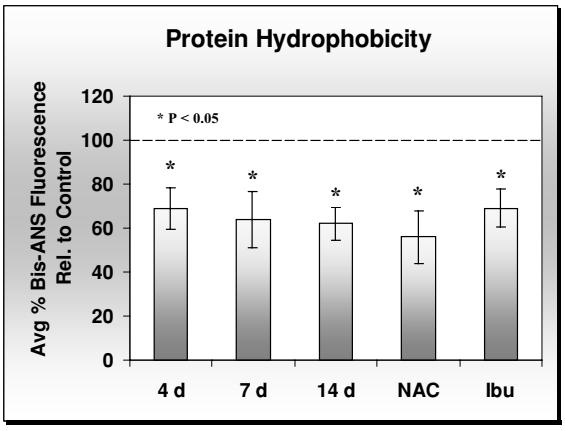


Figure 8. Hydrophobicity of proteins from facsimile synovium. Solubilized protein was incubated with bis-ANS and analyzed fluorometrically. Changes in bis-ANS fluorescence depict protein modification indicative of the initial stages of oxidative synovial damage. Significant statistical differences between groups and their respective controls are denoted with asterisks.

a suitable model to perform time course measurements and biochemical analyses of synovial inflammatory reactions and their ramifications.[33] Advantages of the rat air pouch model include its close approximation to synovial tissue,[22] reproducible ease of induction, and the presence of a blind connective tissue cavity that lacks a mesothelial basement membrane.[34] In as early as six days, the resulting cavity develops a lining having a mixed population of macrophage (synovial-like A) and fibroblast (synovial-like B) cell types arranged in a manner similar to synovial tissue. In the present study, histological analysis demonstrated that rat air pouch synovial linings underwent consistent and pronounced alterations comparable in many aspects to the proliferative synovitis that develops in human TMJ disease.

Degenerative changes of the TMJ can occur focally in the articular cartilage due to changes in external compressive and shear stresses resulting in fragmentation of collagen fibrils.[38] These fibrils are released into the TMJ cavity and resorbed by the synovial membrane causing inflammatory synovitis. Cytokines released from the synovial membrane may stimulate chondrocytes to synthesize and release degradative enzymes. This process, coupled with damage generated by hydroxyl free radical production via the Fenton reaction, impacts the progression of synovitis to degenerative joint disease characterized by adhesion formation, limited mandibular mobility, pain, and decreased function. Oxidized proteins can also undergo hydrolytic and proteolytic degradation.[10,11] As a result of attack by free radicals, there is an initial decrease in protein hydrophobic pockets. Eventually, the protein loses its original conformation and an increase in hydrophobicity occurs.[36] Protein oxidation may also cause modifications of amino acid side chains. The presence of carbonyl groups in the amino acid side chains indicates that the protein may have been subjected to free radical damage.[11,37] As the intensity of oxidative stress increases, fibrotic degeneration of connective tissue and inflammation ensues. This can result in irreversible cell damage[38] and degenerative TMJ pathology.

This study shows that the rat air pouch model of facsimile synovium develops clinical evidence of adhesions and degraded proteins when subjected to free radical attack. The effect intensities varied based on time course interactions with Fenton reaction-generated oxidative stress reactions.

The histological observations made in this investigation indicate a close agreement with studies done by Dijkgraaf et al,[28] where synovial membrane biopsies from diseased human TMJs were examined. Their findings suggest that the initial signs of degenerative TMJ pathology include synovial intima hyperplasia and cell hypertrophy, as well as deposition of fibrous material in the intima matrix. Eventually, fibrosis of the subintimal tissue occurred in combination with degeneration of the subintimal layer. The histological findings of this study also agree with other studies[29-31] in that degeneration of the synovial lining of the TMJ may initially result in synovial tissue hypervascularity, redundancy, and subsequent adhesion formation. NAC and ibuprofen were successful in preventing carbonyl group formation—an indication of oxidative stress. NAC successfully prevented carbonyl formation in the ~70 kDa protein; however, ibuprofen acted as a pro-oxidant in this assay. Since it was shown that ibuprofen did elicit an effective treatment effect against carbonyl group formation in total protein experiments, a conclusion can be drawn that it was not effective in preventing carbonyl formation in the 70 kDa protein. It is possible that the

70 kDa protein is more susceptible to oxidative modification than other proteins since proteins vary in their susceptibility to free radical attack. It is also likely that the 70 kDa protein is albumin extravasated from blood vessels. Albumin comprises the major protein in the human TMJ lavage. The transient presence of albumin may explain why it was modified to produce carbonyl groups and not degraded, as were most tissue proteins that were exposed to oxidative stress for longer periods of time, and why NAC was effective in preventing carbonyl formation in this protein. It must be pointed out that the time course of this experiment was not sufficiently long enough to observe the increases in hydrophobicity seen when proteins become fully denatured, e.g., after long-term, sustained free radical attack.

Recently, it has been found that free radical oxidations are not necessarily exclusively initiated by the hydroxyl radical formed from the well-accepted Fenton reaction, but rather, by iron in the form of "Fe-O" complexes.[39] This may explain why NAC and ibuprofen were unsuccessful treatment modalities in the concentrations used against protein degradation by oxidative stress. In fact, they seemed to act as pro-oxidants. Studies by Sprong et al[40] have reported that low-dose NAC protects against oxidative stress, while higher doses may have the opposite effect. Other studies[41,42] suggest an oxidative imbalance seems to play an important role in living systems. These studies found an increased ability of NAC-exposed cells to adhere to other cells and extracellular matrix with high concentrations of NAC. Others have demonstrated that NAC could induce Fe-dependent H_2O_2 generation and postulated a possible mechanism of oxidative damage induced by NAC based on Fe (II) auto-oxidation to generate Fe (I) and a radical of NAC.[43] Pro-oxidant activities of ibuprofen seen in protein degradation have been substantiated by several studies[44-47] that implicate the deleterious effect of high doses of ibuprofen. Ibuprofen has been shown to augment and/or potentiate tumor necrosis factor-α, interleukin-6, elastase, interleukin-1 induced collagenase, and cytokine-induced pro-IL-1β. General pro-oxidative mechanisms of ibuprofen range from inhibition of negative feedback mechanisms to direct activation of these molecules.

Conclusion

The use of the subcutaneous rat air pouch model in this study may become a primary means of understanding more completely the pathophysiological processes contributing to degenerative TMJ disease. This study demonstrated macroscopically, histologically, and biochemically that free radicals introduced into the rat air pouch interact with extracellular matrix molecules causing oxidation and breakdown of proteins and promote adhesion formations consistent with features of human TMJ disease. Future directions may be taken from this study to postulate new analysis techniques and therapeutic modalities for patients afflicted with degenerative TMJ disease.

Acknowledgment

This project was funded in part by NIH grant no. DE 12542.

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