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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Purpose. The prevention of early-onset osteoarthritis after traumatic joint injury remains a clinical challenge and may be associated with the poor lubricant quality of the synovial fluid in the injured joint. Scope. The aims of the studies here were to test whether the pathological accumulation of a specific substance found in joint fluid following an injury mediated altered synovial fluid lubrication of articular cartilage and whether modulation of lubricating molecules could restore lubrication function and prevent cartilage wear. Major Findings. Through studies in year 2, we have delineated the time-dependent changes in lubricant molecule gene expression and biochemical content of joint fluid post-injury in a rabbit model. Progress and Significance. Collection and analysis of human synovial fluid is ongoing (Aim 1a), and we have completed most of the analysis of rabbit synovial fluid (Aim 1b). From this data, the time-dependent relationship between joint fluid molecules (both secretion and gene expression) and function could be determined.

The number of pages shall include all pages that have printed data (including the front cover, SF 298, table of contents, and all appendices).

15. SUBJECT TERMS

Cartilage, Wear, Lubrication, Friction, Post-traumatic osteoarthritis, Injury, Blood, Synovial Fluid, Joint

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INTRODUCTION: Deficient lubrication of articular cartilage by synovial fluid after synovial joint injury may advance the deterioration of cartilage, especially in the superficial zone, and hasten the development of osteoarthritis (OA). We recently delineated two mechanisms by which acute injury disrupts the normal lubrication of articular cartilage by synovial fluid: (1) diminished concentration of high-MW HA in SF, and (2) detrimental interaction of blood with SF. The scope of these studies was to test whether the impaired lubrication of articular cartilage by synovial fluid after joint injury contributes to the development of post-traumatic OA and is due, in part, to the pathological accumulation of a specific substance which can be countered by an enhanced lubricant therapy.

2. **KEYWORDS:** cartilage wear, lubrication, friction, synovial fluid, joint, osteoarthritis, post-traumatic osteoarthritis, injury, blood.

3. **ACCOMPLISHMENTS:**

3.a. What were the major goals of the project?

Specific Aim 1 (per proposal)	Timeline	Site 1
1.1. From 60 patients, collect hSF & analyze for lubricant content, quality, & function.	Months	Completion [%]
(a) Obtain IRB approval (VA Palo Alto).	1-3	100
(b) Collect hSF, & transport to UCSD.	3-24	50
(c) Analyze hSF (as fluid is collected) for biochemical properties.	3-30	50
(d) Analyze hSF for lubricant function.	3-30	50
Milestone(s) Achieved:		
A. VA Palo Alto IRB Approval	3	100
B. Characterization of hSF with time after injury and severity/type of injury	15	75
1.2. After rabbit ACL Transection (ACLT), collect & analyze (1) rSF for lubricant content, quality, & function and (2) rAC for content and structure.	Months	Completion [%]
(a) Obtain IACUC approval (UCSD)	1-3	100
(b) Perform rabbit ACLT surgeries, collect fluids & articular cartilage.	3-4.5	100
(c) Analyze rSF for biochemical properties.	3-6	75
(d) Analyze rSF for lubricant function.	3-6	35
(e) Analyze rAC for biochemical and molecular properties.	3-9	100
Milestone(s) Achieved:		
(A) UCSD IACUC Approval	3	100
(B) Characterization of rSF and rAC with time after injury (rabbit ACLT)	9	75

Specific Aim 2 (per proposal)	Timeline	Site 1
2.2. 1.2. After rabbit ACL Transection (ACLT) and time- and dose-variable chelation therapy, collect & analyze (1) rSF and (2) rAC for iron and iron-binding protein content.	Months	Completion [%]
(a) Update UCSD IACUC Approval.	20-22	100
(b) Perform pilot studies (highest chelator dose).	22-24	50
(c) Analyze rSF for iron and iron-binding proteins.	22-30	5
(d) Analyze rAC for iron and iron-binding proteins.	22-30	5
Milestone(s) Achieved:		
A. UCSD IACUC Approval	20	100
B. Characterization of tissues (rSF, rAC) collected in pilot study.	22-30	5

3.b. What was accomplished under these goals?

3.b.i. Aim 1a.

Nothing to report.

3.b.ii. Aim 1b.

Major Activities. In Year 2, studies continued for the analysis of rabbit SF for lubricant content and function (Aim 1b). Analysis of rabbit tissues (cartilage and synovium) for time-dependent changes in gene expression was completed. Studies continued for the analysis of rSF and rAC for iron and iron-binding protein content.

Specific Objectives. The objectives for Year 2 were to a) analyze rSF for biochemical properties, b) analyze rSF for lubricant function, c) analyze rAC for biochemical properties, d) determine if the concentrations of iron and major iron-binding proteins are elevated at early times after injury in rSF, and e) determine the time-dependent concentrations in cartilage of iron and major iron-binding proteins following joint injury.

Significant Results, Key Outcomes, Major Findings, Developments, or Conclusions. Lubricant molecule concentrations and quality were modulated by injury relative to contralateral control (CTRL) samples. Relative to CTRL samples, ACLT rSF protein concentrations were generally higher at days 1, 4, and 7 (+38% to +72%) and similar at days 14, 28, and 42. HA concentration in CTRL samples ranged from 1.2 to 2.3 mg/mL. Relative to CTRL, rSF HA concentrations were lower (36% to 67%) during the acute (1-7 days post-ACLT) and chronic stages (14-42 days) of joint degeneration. ACLT HA

concentrations were significantly decreased compared to CTRL at 1, 4, 28, and 42 days post-ACLT, suggesting that the initial decrease in HA within the knee persisted over the full time course. CTRL knees had a larger fraction of high MW HA as compared to injured knees. The concentration of high MW (2.5-7.0 MDa) HA was higher CTRL knees at 4 (+194%), 28 (+167%), and 42 (+80%) days post-ACLT. Mid-range (1.0-2.5 MDa) HA was also higher in CTRL knees at 4 days post-injury (+201%). Low MW forms of HA increased for ACLT conditions, especially at longer time points post-ACLT. ACLT knees had higher concentrations of low MW (0.03-0.5 MDa) HA at 28 days post-ACLT (+226%).

The concentrations and quality of SF lubricant constituents were altered following injury involving the ACL, in a manner consistent with our previous studies. In upcoming studies, SF samples will be analyzed for the suspected pathological substance as well as lubrication function.

3.b.ii. Aim 2b.

Major Activities. Pilot studies for the rabbit experiments of Aim 2b were initiated in the final quarter of Year 2, to examine the effect of chelator on tissue properties and joint lubrication.

Specific Objectives. The objective for Year 2 were to a) identify (*in vivo*) the dose-dependence of the effects of chelation therapy after ACL transection (ACLT).

Significant Results, Key Outcomes, Major Findings, Developments, or Conclusions. The UCSD IACUC animal protocol (#S10018) was updated to include 3 doses of chelation therapy based on preliminary *in vitro* cytotoxicity studies: 4 mg/200 µl/joint, 1.33 mg/200 µl/joint, and 0.4 mg/200 µl/joint. As a pilot study, n=6 rabbits underwent ACLT and the highest chelator dose (4 mg/200 µl/joint) was injected at 2 (n=2), 4 (n=1), 7 (=2) days, and n=2 rabbits served as uninjected controls. All animals were sacrificed at 10 days post-ACLT, and blood, rSF, and rAC was collected. Analysis of these samples is on-going.

3.c. What opportunities for training and professional development has the project provided?

Undergraduate and graduate students were trained in animal procedures, biochemical techniques, project management skills, and research presentation.

3.d. How were the results disseminated to communities of interest?

An abstract was submitted and accepted to the Tissue Engineering & Regenerative Medicine International Society, North America Conference (December 2016): Raleigh AR, Sun Y, Qian T, Temple-Wong M, Kato K, Murata K, Firestein GS, Sah RL, Masuda K. Synovial Tissue Gene Expression Following Acute Injury: Implications for Lubricant Secretion. *Trans Tiss Eng Regen Med Intnl Soc-North America*, 2016.

An abstract was submitted and accepted to Orthopaedic Research Society Conference (March 2017): Raleigh AR, Sun Y, Qian T, Temple-Wong M, Kato K, Murata K, Firestein GS, Masuda K, Sah RL. Synovial Fluid Hyaluronan Fluctuation in Post-Traumatic Osteoarthritis: Dependence on the Dynamic Balance between Biosynthesis, Loss, and Fluid Flux. *Trans Orthop Res Soc*, 2017, 2017.

3.e. What do you plan to do during the next reporting period to accomplish the goals?

In the next reporting period, it is planned to complete on-going studies for both Aim 1a and Aim 1b. For Aim 1a, we plan to continue analyzing hSF as the samples arrive to UCSD. In addition, we plan to analyze the hSF for iron and iron-binding proteins. We are aiming for a publication by Fall 2017 of hSF biochemical and lubricating properties following injury and the relationship between iron and lubrication function of hSF.

For Aim 1b, we are targeting several publications relating to the time-dependent, post-injury biochemical and lubrication properties of rSF for March-April 2017. For Aim 1b, we plan to measure iron and iron-binding proteins in the rSF and rAC. We anticipate to complete the lubricant analyses in rSF, and we plan to assess the rSF for lubrication function. The iron and iron-binding protein analysis is anticipated to be completed for Aim 1b by May 2017.

For Aim 2b, the first pilot study in rabbits to assess the ability of a chelator to restore lubrication function in the joint has been performed. We anticipate completing the rabbit surgeries for Aim 2b by September 2017.

4. IMPACT:

4. a. What was the impact on the development of the principal discipline(s) of the project?

Nothing to report. However, several publications are pending.

4.a.What was the impact on other disciplines?

Nothing to report. However, several publications are pending.

4. c. What was the impact on technology transfer?

Nothing to report.

4. d. What was the impact on society beyond science and technology?

Nothing to report. However, several publications are pending.

5. CHANGES/PROBLEMS:

5.a. Changes in approach and reasons for change.

Nothing to report.

5.a.Actual or anticipated problems or delays and actions or plans to resolve them.

Analyses of rabbit synovial fluids required some scaling of the various assays. That work has been completed and analyses are proceeding.

5.c. Changes that had a significant impact on expenditures.

Nothing to report.

5.d. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Nothing to report.

5.a.i. Significant changes in use or care of human subjects
5.a.ii. Significant changes in use or care of vertebrate animals.
5.a.iii. Significant changes in use of biohazards and/or select agents

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

6.a. Lay Press:
6.b. Peer-Reviewed Scientific Journals:
6.b.i. Raleigh AR, Sun Y, Qian T, Temple-Wong M, Kato K, Murata K, Firestein GS, Masuda K, Sah RL. Synovial fluid hyaluronan fluctuation in post-traumatic osteoarthritis: dependence on the dynamic balance between biosynthesis, loss, and dilution. *Arthritis Rheum*, 2017, *in preparation*.
6.c. Invited Articles:
6.d. Abstracts:
6.d.i. Raleigh AR, Sun Y, Qian T, Temple-Wong M, Kato K, Murata K, Firestein GS, Sah RL, Masuda K. Synovial Tissue Gene Expression Following Acute Injury: Implications for

Lubricant Secretion. *Trans Tiss Eng Regen Med Intnl Soc-North America*, 2016.

6.d.ii. Raleigh AR, Sun Y, Qian T, Temple-Wong M, Kato K, Murata K, Firestein GS, Masuda K, Sah RL. Synovial Fluid Hyaluronan Fluctuation in Post-Traumatic Osteoarthritis: Dependence on the Dynamic Balance between Biosynthesis, Loss, and Fluid Flux. *Trans Orthop Res Soc*, 2017.

6.e. Presentations:

6.e.i. Raleigh AR, Sun Y, Temple-Wong MM, Firestein GS, Sah RL, Masuda K: Cartilage and synovium gene expression in a chronic model of PTOA. *UC San Diego Jacobs Research Expo*, 2016. (Poster)

6.e.ii. Raleigh AR, Sun Y, Qian D, Temple-Wong MM, Kato K, Murata K, Firestein GS, Masuda K, Sah RL: Synovial tissue gene expression following acute injury: Implications for lubricant secretion. *Trans Tiss Eng Regen Med Intnl Soc-North America*, 2016. (Oral Presentation)

6.e.iii. Raleigh AR, *et al.*: Synovial fluid hyaluronan fluctuation in post-traumatic osteoarthritis: Dependence on the dynamic balance between biosynthesis, loss, and fluid flux. *Trans Orthop Res Soc* 42:1506, 2017. (Poster)

7. INVENTIONS, PATENTS AND LICENSES:

Nothing to report.

8. REPORTABLE OUTCOMES:

Nothing to report.

9. OTHER ACHIEVEMENTS:

Nothing to report.

10. REFERENCES:

11. APPENDICES: Raleigh AR, Sun Y, Qian T, Temple-Wong M, Kato K, Murata K, Firestein GS, Masuda K, Sah RL. Synovial Fluid Hyaluronan Fluctuation in Post-Traumatic Osteoarthritis: Dependence on the Dynamic Balance between Biosynthesis, Loss, and Fluid Flux. *Trans Orthop Res Soc*, 2017.

Synovial Fluid Hyaluronan Fluctuation in Post-Traumatic Osteoarthritis: Dependence on the Dynamic Balance between Biosynthesis, Loss, and Fluid Flux

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INTRODUCTION: Injury to knee joint tissues (cartilage, subchondral bone, ligament, or meniscus) increases the likelihood of post-traumatic osteoarthritis (PTOA) [1]. PTOA may be due, in part, to a decrease in concentration of synovial fluid (SF) lubricants, including hyaluronan (HA) [2]. HA is a large, non-sulfated, anionic glycosaminoglycan, normally present at high concentrations in the SF and produced primarily by HA synthases (HAS) in fibroblast-like synoviocytes (FLS) [3-6]. However, the biophysical mechanisms affecting HA concentration are complex, involving cell, tissue, and organ scale-biology, modulating synthesis, degradation, and (in/ef)flux [7]. Mechanisms of joint degradation may be elucidated in animal models of PTOA, such as with anterior cruciate ligament transection (ACLT). Following ACLT in rabbit knees, the synovial lining undergoes marked proliferation and neovascularization [8,9], while HA residence time is reduced [8]. We sought to combine transport theory and experiment to elucidate how the concentration of HA in SF (c^{SF}_{HA}) after ACLT is affected by synthesis and loss processes, in addition to fluid influx.

METHODS: (1) ***In Vivo Model of PTOA.*** With IACUC approval, 36 adult (~12mo) NZW rabbits were subjected to unilateral ACLT to induce PTOA. Knee joints were harvested at 1, 4, 7, 14, 28, and 42 days post-injury (n=6/group). Synovium (SYN) and SF, both the initial (neat) and lavage aspirates, were harvested from ACLT and contralateral non-operated control (CTRL) joints. SF volume (V^{SF}) was estimated from protein concentration within neat and lavage fluids [10]. **(2)** ***In Vitro Synoviocyte Culture.*** FLS from four additional healthy adult rabbits were isolated and cultured in basal media (DMEM + 0.5% FBS) ± 0.1 or 1.0 ng/mL TGF-β1 and IL-1β. Gene Expression and Biochemical Assays. SYN and cultured FLS were analyzed for expression of *HAS1, HAS2,* and *HAS3* by qPCR with *RPS18* and *GAPDH* as reference genes. HA, in SF and in conditioned media, was assessed using an ELISA-like assay. Statistics. Data were analyzed using 1- or 2-way repeated measures ANOVA with Sidak post-hoc testing. **(3)** ***Modeling and Data Integration*****.** A compartmental mass balance model (**Fig. 1A**) was used to account for the rate of change of SF HA, the product $V^{SF}\cdot c^{SF}_{HA}$, due to HA synthesis, HA loss by efflux or degradation, and net fluid influx [7,11]. Data from *in vivo* CTRL and ACLT knees was used for V^{SF} and c^{SF}_{HA}, while the rate of HA synthesis by FLS ($r_s^{SYN,FLS}{}_{HA}$) was estimated from qPCR data, calibrated to secretion rates *in vitro*. The density of synoviocytes was estimated from previous studies [12]. The loss rate, due to diffusion and/or degradation was thus the only unknown and was fit from the model and data.

RESULTS: (1) *In vivo*, ACLT led to both acute and chronic changes. V^{SF} was greater in ACLT (1.9-4.4X) than that of CTRL knees (0.1-0.2 mL), and peaked at 14 days (**Fig. 1B**). *HAS2* expression was induced in ACLT SYN (1.75-3.6X) as compared to CTRL and peaked at 28 days (**Fig. 2A**). SYN expression of *HAS1* and *HAS3* were below the limits of detection. c^{SF}_{HA} for ACLT knees was lower (to 0.36-0.67X) than that of CTRL knees (1.2-2.3 mg/mL) during the acute and chronic stages (**Fig. 3A**). (2) *In vitro* stimulation of FLS led to correlated increases in gene expression and secreted HA. Treatment with TGF-β1 & IL-1β cytokines stimulated *HAS2* expression and HA secretion (**Fig. 2B**) in a dose-dependent manner (to 3.5-4.2x basal, R^2=0.45, **Fig. 2B**). (3) Modeling to fit c^{SF}_{HA} (**Fig. 3A**) indicated that the ACLT-induced increase in HA synthesis due to proliferation and expression per cell (**Fig. 2A**) was countered both by net fluid influx, causing increased V^{SF} (**Fig. 1B**), and increased rate of HA loss (**Fig. 3B**). The HA loss rate was increased in ACLT (~13X) over CTRL.

DISCUSSION: These results indicate that SF lubricant concentration depends not only on cellular secretion rates, but also on loss rates from, and fluid influx into, the SF compartment. The model-based deduction that HA loss is increased after ACLT is consistent with the decreased residence time of injected HA at particular times after ACLT, especially for high MW HA [8]. The increased loss of HA may be due to increased permeability or degradation.

These results provide a foundation for developing, calibrating, and testing a predictive synovial joint model for the dynamics of lubricants, and potentially other molecules, in SF in health and disease. A variety of *in vitro* analyses, such as to correlate gene expression and lubricant secretion, in a controlled environment allow quantitative estimation of individual model parameters in the absence of the complexities of the joint as a system. The accuracy and assumptions of both measures and models can be further analyzed and improved, including for individual patients and animal models.

SIGNIFICANCE: The inverse relationship between *HAS2* expression and c^{SF}_{HA} suggests that metrics and markers of synovial joint health need to include not only biological indices at the levels of cells (biosynthesis) and molecules (causing degradation), but also physical properties of tissues (permeability) and organs (fluid influx). Because SF lubricant function depends on concentration [2,11], a predictive model for changes during joint injury and arthritis could be used to inform the development of patient-specific therapies.

REFERENCES: [1] Gelber AC+, *Ann Intern Med* 133:321, 2000. [2] Elsaid KA+. *Arthritis Rheum*. 52:1746, 2005. [3] Dahl LB+. *Ann Rheum Dis*. 44:817, 1985. [4] Lee HG+. *Anal Biochem.* 219:278, 1994. [5] Spicer AP+. *J Biol Chem*. 273: 1923, 1998. [6] Oguchi T+. *Connect Tissue Res*. 45:197, 2004. [7] Blewis ME+. *Eur Cell Mat*. 13:26, 2007. [8] McCarty WJ+. *Arthritis Rheum*. 64:3993, 2012. [9] Lukoschek M+. *J Orthop Res*. 6:475, 1988. [10] Coleman PJ+. *J Physiol*. 503:645, 1997. [11] McCarty WJ+. In: Comprehensive Biomaterials, 2011. [12] Boyd RD+. *Arthritis Rheum*. 34:515, 1991.

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FIGURES:

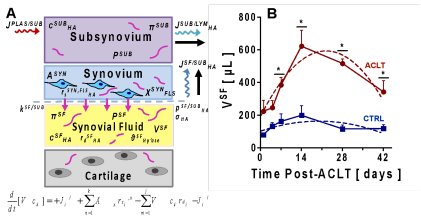

Figure 1. Compartmental model of synovial joint and effect of PTOA on V^{SF}. (A) Model schematic and mass balance equations. **(B)** V^{SF} measured as a function of time after ACLT, with curve fits. Mean + SE, n=4-7, *p<0.05.

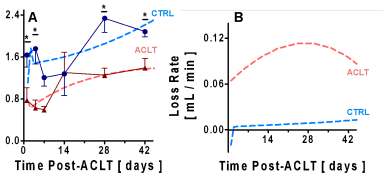

Figure 3. Experimental and theoretical assessment of c^{SF}_{HA} and HA loss rates. (A) *In vivo* data (symbols, mean+SE, n=4-7, *p<0.05), and model predictions (dotted lines). **(B)** HA loss rate.

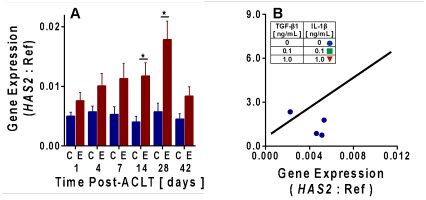

Figure 2. HA synthesis by FLS. (A) *In vivo* SYN expression of *HAS2*. Mean+SE, n=4-7, *p<0.05. **(B)** *In vitro* correlation of *HAS2* expression to HA secretion for FLS cultures, n=12.