The disease-modifying effects of intra-articular corticosteroid injection at the freezing phase of frozen shoulder in an animal model

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Directed by Professor Sang Chul Lee

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This certifies that the Master's Thesis of
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ABSTRACT

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Freezing phase of frozen shoulder is characterized by elevated inflammatory response. It is not known if the effects of intra-articular corticosteroid injection during the freezing phase are limited to transient suppression of inflammation, or if it exerts disease-modifying effects to prevent further progresion of the disease towards fibrosis. This study aimed to assess the disease-modifying effects of intra-articular corticosteroid administration at the freezing phase of frozen shoulder at preventing disease progression.

24 Sprague-Dawley rats were randomly divided into four groups (n=6 in each). Unilateral shoulders were immobillized for the first 3 days in all groups, followed by intra-articular corticosteroid injection in Group A, injection and cessation of immobilization in Group B, no further intervention in Group C, and cessation of immobilization in Group D. All rats were sacrificed in Week 3 of study, at which passive shoulder abduction angles were measured, and axillary recess tissues were retreived for histological, immunohistochemical and Western blot analyses.

Passive shoulder abduction angles at sacrifice were 138.0° ± 7.8° (Group A), 145.7° ± 5.2° (Group B), 94.8° ± 11.2° (Group C), 132.2° ± 8.1° (Group D), and 157.8° ± 2.3° (Control). Group B did not show significnat difference from Control (P=0.069), showing that intra-articular corticosteroid injection at the freezing phase combined with remobilization restored shoulder range of motion to normal range.

Histological assessment showed greater degree of fibrosis and inflammation in groups that did not receive corticosteroid injection (Groups C and D) compared to corticosteroid-injected groups (Groups A and B). Corticosteroid-injected groups showed no significant differences in semi-quantitative histological scores compared to healthy control, Western blot analyses showed similar expression levels of IL-1α and IL-1β in Group B and healthy control. Group A also showed significantly lower expression of IL-1α, IL-1β, TNF-α, TNF-β, and RAGE compared to Group C. These findings demonstrated the long-term anti-inflammatory and disease-modifying effects of corticosteroid injection at the freezing phase of frozen shoulder.

Key words : frozen shoulder; corticosteroid; intra-articular injection; experimental animal model; range of motion

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I. INTRODUCTION

Frozen shoulder is a disease characterized by pain and stiffness of shoulder joint, and is either idiopathic or secondary to other medical conditions such as diabetes, hypothyroidism, cancer and prolonged immobilization[1]. It is a common but potentially burdensome disease as it interferes with patients' activities of daily living by inflicting pain during joint mobilization and causing limitation in passive and active range of motion (ROM)[2].

The disease has been described in the literature as progressing through three clinical phases: freezing, frozen and thawing[3, 4], or four phases including pre-adhesive phase[5]. During the freezing or pre-adhesive phase, inflammatory cell infiltration and inflammatory responses mainly constitute the pathophysiologic mechanism, consistent with the clinical manifestation of diffuse severe shoulder pain without severe limitation in range of motion[6], while full-blown fibrosis is usually not observed[7, 8]. In contrast, as the disease progresses towards the frozen phase, inflammation subsides while fibrotic changes become more salient[7, 8], corresponding to progressive limitation in shoulder movement[6]. These changes gradually reverse during the thawing stage which may take as long as 2 years[9], marking the end to the usually self-limiting disease. Ideally, it would be most desirable to detect and treat the disease at the freezing phase so as to alter the natural course of the disease by preventing progression towards the frozen phase.

However, there are limited evidences that any of the currently available treatment options show such definitive disease-modifying effects that could intervene with the natural course of the disease.

Intra-articular (IA) corticosteroid is frequently administered for frozen shoulder. Considering its anti-inflammatory properties[10], IA corticosteroid is usually prescribed during the freezing phase of frozen shoulder in which active inflammation is present[11]. The clinical effect is usually demonstrated by transient pain reduction after injection[12, 13], which has been shown to persist for up to 12 weeks[14]. As of now, it is not known if IA corticosteroid has a disease-modifying property that prevents fibrosis and further progression of the disease when administered at the freezing phase.

For the assessment of histological and molecular aspects of frozen shoulder in a controlled environment, the use of a variety of animal models have been documented[15-21]. Prolonged immobilization of unilateral shoulder joint by either surgical or non-surgical means (e.g. plaster fixation) has shown to induce secondary frozen shoulder in these models. In case of a Sprague-Dawley rat model, 3 days of immobilization resulted in inflammatory cell infiltration upon histological examination, akin to the freezing phase in human primary frozen shoulder, while 3 weeks of immobilization resulted in histological findings analogous to the frozen phase[22]. There are only limited number of studies that performed IA injection to the rat model[23-25], and in all of these studies, injections were performed at or after 3 weeks of shoulder immobilization. Therefore, the consequences of IA corticosteroid injection at the freezing phase when inflammation is predominant and fibrosis has yet to take place have not been studied for rat models. It is essential to assess if IA corticosteroid administered at the freezing phase exerts a disease-modifying effect to prevent further progression into fibrosis, or if it only exerts temporary anti-inflammatory effect.

This study aimed to assess the disease-modifying effects of IA corticosteroid injection during the freezing phase at preventing disease progression to fulminant fibrosis. We hypothesized that IA administration of corticosteroid combined with release of immobilization at the freezing phase would result in normalization of shoulder ROM and the disease pathology including fibrosis.

II. MATERIALS AND METHODS

The institutional review board and ethics committee for animal experimentation granted approval for this study (DCIAFCR-221129-33-YRA).

1. Study protocol

In order to perform intervention at the freezing phase, we first determined the duration of shoulder immobilization that best simulated the freezing phase in a rat model. A previous report showed inflammatory cell infiltration and capillary proliferation upon histological examination at 3 days after immobilization of rat shoulders, which are findings that correlate with the definition of "freezing phase"[22]. These findings were not as obvious at 1 week after immobilization. However, due to the lack of other related references and the need to precisely simulate the freezing phase in our study, we examined rat shoulders that were immobilized for 3,4,5 days respectively to determine the most optimal simulation of the freezing phase. After this step, we proceeded to perform IA corticosteroid injection during the freezing phase (Figure 1). Healthy male 7-week old Sprague-Dawley rats were used, and the rats were allocated to study groups in a random manner by assigning a random number to each rat. All authors were blinded to the group assignment except for the laboratory technician.

To determine the optimal duration of shoulder immobilization to simulate the freezing phase, 18 rats were allocated into three groups (n=6 in each), with 3,4,5 days of shoulder immobilization, respectively. Unilateral shoulders were fixated in adduction and internal rotation using molding plaster, a well established method for frozen shoulder induction in animal models[22, 24, 26, 27]. Following the designated periods of immobilization, the plasters were removed and the rats were sacrificed by cervical dislocation. The shoulder tissues were then retrieved for histological and Western blot analyses. Intraperitoneal anesthesia with 40 mg/kg of tiletamine hydrochloride and zolazepam hydrochloride (Zoletil; Virbac, Carros, France) and 1.0–5.0 mg/kg of xylazine (Rompun; Bayer AG,

Leverkusen, Germany) was performed during both plaster fixation and removal[24]. As arthroscopic findings during the earliest stage of frozen shoulder show fibrinous synovial inflammation without capsular contracture or adhesion[5], we selected the group which showed highest expression of inflammatory markers and lowest expression of fibrosis markers as most adequately simulating the freezing phase.

Next, to determine the effects of IA corticosteroid injection during the freezing phase, 24 rats were allocated into four groups (n=6 in each), all of which underwent unilateral shoulder immobilization for the duration that was found to best simulate the freezing phase in the first stage experiment. After this period of immobilization and hence the induction of the freezing phase, IA corticosteroid injection with continuation of immobilization was performed in Group A, IA corticosteroid injection and cessation of immobilization in Group B, continuation of immobilization without further intervention in Group C, and cessation of immobilization in Group D. All rats were sacrificed at the third week of study, and the tissues were prepared in the same manner as the first stage experiment. IA injection dosage was determined as 38 μL of 20 mg/ml triamcinolone acetonide in accordance with the animal-equivalent dose calculation formula[28] which was implemented in a previous study on rat frozen shoulder model[24].

In case of any adverse events, we planned to document the incidence and exclude the animal from the study. However, no complications or deaths occured. Aside from the unilateral shoulder immobilization, the rats were allowed free ambulation while food and water was provided as needed. Each rat was kept in a separate cage in the same room in 24°C and 45% relative humidity.

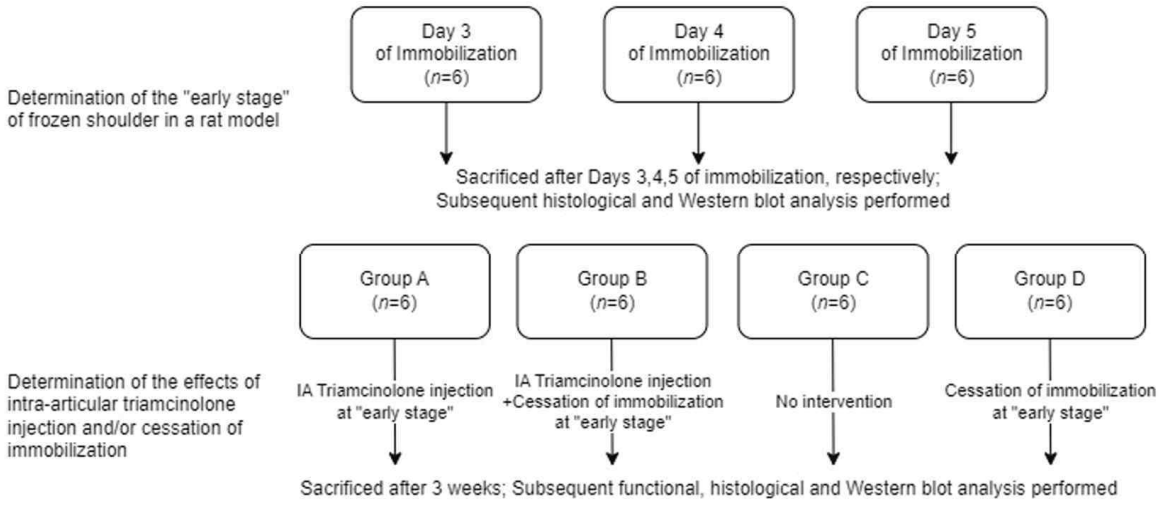


Figure 1. Illustration of the study protocol.

2. Measurement of passive shoulder ROM

Passive shoulder abduction angle (SAA) was assessed as the primary outcome measure. The SAA was measured at baseline, at the “freezing phase”, and at the third week of study or immediately prior to sacrifice. The measurement was performed under intraperitoneal anesthesia, identical to the procedure during plaster fixation and removal. 10 grams of weight was applied to the distal end of humeral shaft, equivalent to approximately 3.92×10^{-3} Nm torque[22, 27]. The angle between the scapular spine and humeral shaft was assessed using a goniometer. The average of three separate measurements by the laboratory technician was used for analysis[27].

3. Histological evaluation

The shoulder tissues from all 6 rats from each group were retrieved for histological assessment. The tissues were formalin-fixed, decalcified with 10% formic acid, and paraffin-embedded. The paraffin-embedded specimens were sectioned in 5-μm-thick slices with a microtome. The prepared sections were stained in Hematoxylin and eosin (H&E) and Masson's trichrome stain (MTS), and examined under a light microscope.

Immunohistochemical staining was performed for detection of inflammatory (i.e. Cluster of Differentiation 68 (CD68), Interleukin-6 (IL-6), and Tumor Necrosis Factor-α (TNF-α) and fibrosis (i.e. Type III Collagen and Vimentin) markers. The paraffin-embedded and microtome-sectioned specimens were treated with phosphate-buffered saline (PBS) and incubated in citrate buffer for 30 minutes at 95°C for antigen retrieval. Endogenous peroxidases were blocked with 0.3% hydrogen peroxide in PBS, and non-specific antibody binding was blocked with PBS with 10% horse, goat, or rabbit serum (Vector Laboratories, Newark, CA, USA) for 30 minutes. Sections were incubated at room temperature with the following primary antibodies at 1:100 to 1:200 dilution: Rabbit anti-CD68 polyclonal antibody (ab125212; Abcam, Cambridge, UK), mouse anti-IL-6 monoclonal antibody (ab9324; Abcam), mouse anti-TNF-α monoclonal antibody (sc-52746; Santa Cruz Biotechnology, Dallas, Texas, USA), rabbit anti-Collagen III polyclonal antibody (COL III) (ab7778; Abcam), and mouse anti-Vimentin monoclonal antibody (sc-6260; Santa Cruz Biotechnology). After incubation with primary antibodies, the sections were incubated with biotinylated anti-mouse or rabbit IgG secondary antibodies at 1:100 dilution. The sections were then washed with PBS and treated with avidin-biotin-peroxidase complex (Vector Laboratories) and underwent peroxidase reaction with 0.05M Tris-HCl (pH 7.6). The sections were counterstained with hematoxylin.

For examination of the stained slides, Axiophot Photomicroscope (Carl Zeiss, Jena, Germany) was used. The images were stored with AxioCam MRc5 (Carl Zeiss) and digitally examined using SlideViewer (3DHISTECH, Budapest, Hungary), including the measurement of capsular thickness at axillary recess (Figure 2). For each section, four fields were selected randomly and photographed for semi-quantitative analysis. Semi-quantitative scoring was done twice in separate occasions, each by two independent physicians blinded to the group allocation, one with more than 10 years of experience as a pathologist (Rater 1) and a trained physician (Rater 2). The intensity and extent of staining in MTS and immunohistochemistry were scored as 0 (negative staining), 1 (weak but detectable), 2 (mildly positive), 3 (moderately positive), or 4 (strongly positive)[24, 29, 30].

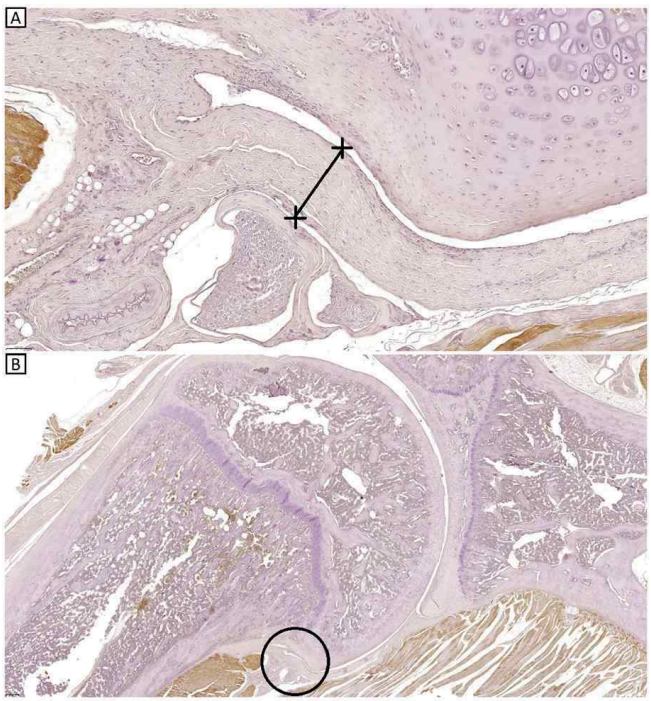


Figure 2. Measurement of capsular thickness at the axillary recess.

(A) at ×40 magnification, the capsular thickness measurement is displayed as a straight line, (B) at ×5 magnification, the axillary recess at which the measurement was made is marked in circle.

4. Western blot analysis

Western blot analysis was performed for detection of inflammatory proteins (i.e. CD68, IL-1α, IL-1β, IL-6, TNF-α, TNF-β), fibrosis markers (i.e. type III Collagen), and Alarmin molecules (high mobility group box 1 (HMGB1), and receptor for advanced glycation end products (RAGE)). Approximately 2-3 mm^3 of shoulder tissues were retrieved from 3 out of 6 rats in each group in both the first and second stage experiments for Western blot analysis. The tissue samples were homogenized and denatured using Laemmli buffer. Proteins were separated in order of molecular weight by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis using NUPAGE MOPS SDS running buffer (NP0001; Thermo Fisher Scientific, Waltham, MA, USA). The proteins were transferred to PVDF membrane (10600023; Amersham Cytiva, Amersham, UK) and blocked with casein blocking buffer in PBS (37528; Thermo Fisher Scientific). The PVDF membrane was then incubated with primary antibodies: Rabbit anti-CD68 polyclonal antibody (1:500, ab125212; Abcam, Cambridge, UK), mouse anti-IL-1α monoclonal antibody (1:500, ab239517; Abcam), rabbit anti-IL-1β polyclonal antibody (1:500, ab1832P; Merck Millipore), mouse anti-IL-6 monoclonal antibody (1:500, ab9324; Abcam), mouse anti-TNF-α monoclonal antibody (1:500, sc-52746; Santa Cruz Biotechnology), rabbit anti- TNF-β polyclonal antibody (1:500, PA5-116055; Thermo Fisher Scientific), rabbit anti-Collagen III polyclonal antibody (COL III) (1:500, ab7778; Abcam). Rabbit anti-HMGB1 monoclonal antibody (1:500, ab79823; Abcam), and anti-RAGE polyclonal antibody (1:500, ab37647; Abcam). After incubation with primary antibodies, the PVDF membrane was incubated with anti-rabbit (1:500, LF SA8002; AbFrontier, Seoul, South Korea) and anti-mouse (1:500, 7076S; Cell Signaling, Danvers, MA, USA) secondary antibodies conjugated with horseradish peroxidase. Chemiluminescence reagents were used to visualize the secondary antibodies. For quantification of relative protein band densities, TINA software (version 2.10e) was used.

5. Statistical analysis

Statistical analysis was performed using R version 4.3.1 software (R Foundation, Vienna, Austria). Shapiro-Wilk test was used to demonstrate normal distribution of continuous outcome variables (i.e. passive SAA). Paired *t*-test was used for within-group comparison of passive SAAs at day 3 and week 3 of the second experiment. One-way analysis of variance (ANOVA) was used for among-group comparison of quantitative variables, after which Tukey's post-hoc test or Duncan multiple range test was used for between-group comparisons in case one-way ANOVA showed statistical significance. Statistical significance was determined as $P<0.05$. Weighted Kappa was used to assess the intra- and inter-observer reliability of semi-quantitative scoring: Intra-rater reliability was calculated as 0.881 showing strong agreement, while inter-rater reliability was 0.793 showing substantial agreement.

III. RESULTS

1. Optimal duration of immobilization for simulation of the freezing phase shoulder

A. Histological findings

One-way ANOVA of capsular thickness showed no significant differences among the three groups (0.15 ± 0.06 mm for Day 3 of immobilization, 0.17 ± 0.04 mm for Day 4 of immobilization, and 0.25 ± 0.05 mm for Day 5 of immobilization, P=0.163). However, the capsular thickness tended to increase in proportion to the duration of immobilization. This pattern was also observed in immunohistochemical staining for type III Collagen, as the three groups did not show statistically significant difference in semi-quantitative scores upon one-way ANOVA (P=0.862), although the score tended to rise with increasing duration of immobilization (Figure 3 and 4).

Conversely, with regards to the inflammatory markers (i.e. CD68, IL-6, and TNF-α), the expression patterns were reversed: In general, inflammatory marker expression levels were highest in Day 3 of immobilization and tended to decrease afterwards. One-way ANOVA revealed significant differences in semi-quantitative scores of IL-6 and TNF-α among the three groups (P<0.001 for both). Tukey's post-hoc test showed significant differences in IL-6 expression between Day 3 and 4 (P<0.001), and between Day 3 and 5 of immobilization (P=0.002), while TNF-α expression was significantly different between Day 3 and 4 (P<0.001), and between Day 4 and 5 of immobilization (P=0.016). Although CD68 expression levels were not significantly different among the groups (P=0.064), the semi-quantitative scores were highest in Day 3 and lowest in Day 5 of immobilization. From the above analyses, we determined that 3 days of immobilization most adequately induced the freezing phase in which inflammation was most active while fibrosis was yet to be observed.

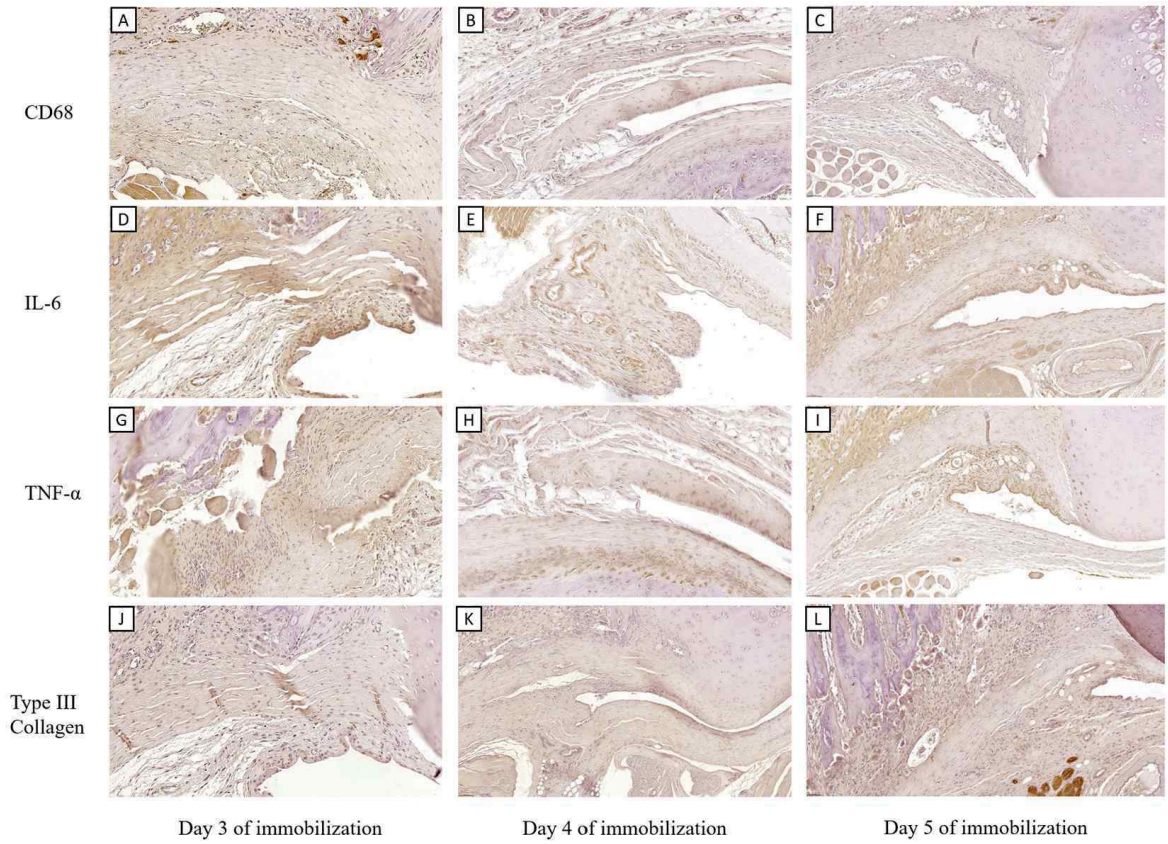
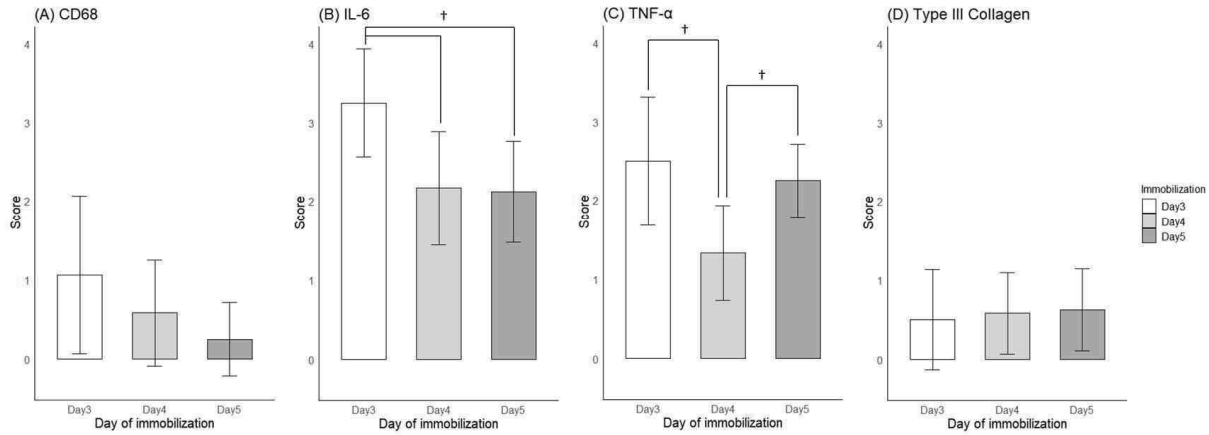


Figure 3. Immunohistochemical findings at Days 3-5 of immobilization.

The images show the synovium and subsynovial structures of axillary recess. (A-C) CD68, (D-F) IL-6, (G-I) TNF-α, (J-L) Type III Collagen immunohistochemical staining at Days 3-5 of immobilization (×40). Immunostaining intensities of inflammatory markers (CD68, IL-6, and TNF-α) were generally greater at Day 3 compared to Day 4 or 5 of immobilization, whereas that of fibrosis marker (Type III Collagen) was generally lower at Day 3 of immobilization.



† indicates P <0.05 upon post-hoc testing between groups.

Figure 4. Semi-quantitative scores of immunohistochemical findings.

(A) CD68, (B) IL-6, (C) TNF-α and (D) Type III Collagen at Days 3-5 of immobilization.

B. Western blot results

One-way ANOVA and post-hoc Duncan multiple range test showed significant differences of expression levels of four proteins among and between groups of varying durations of immobilization (Figure 5). For inflammatory markers, Day 3 of immobilization showed significantly greater expression of IL-6 and CD68 compared to Day 4 and 5 of immobilization. TNF-α expression was greater in Day 3 and 4 compared to Day 5 of immobilization, and there was no significant difference between Day 3 and 4 of immobilization. On the contrary, pattern of type III Collagen expression was the opposite from that of inflammatory cytokines, as Day 3 showed significantly lower expression compared to Day 4 and 5 of immobilization, showing fibrosis progressed as immobilization continued. The Western blot results were in accordance with immunohistochemical staining, as Day 3 of immobilization showed greatest expression of inflammatory markers and lowest expression of fibrosis markers.

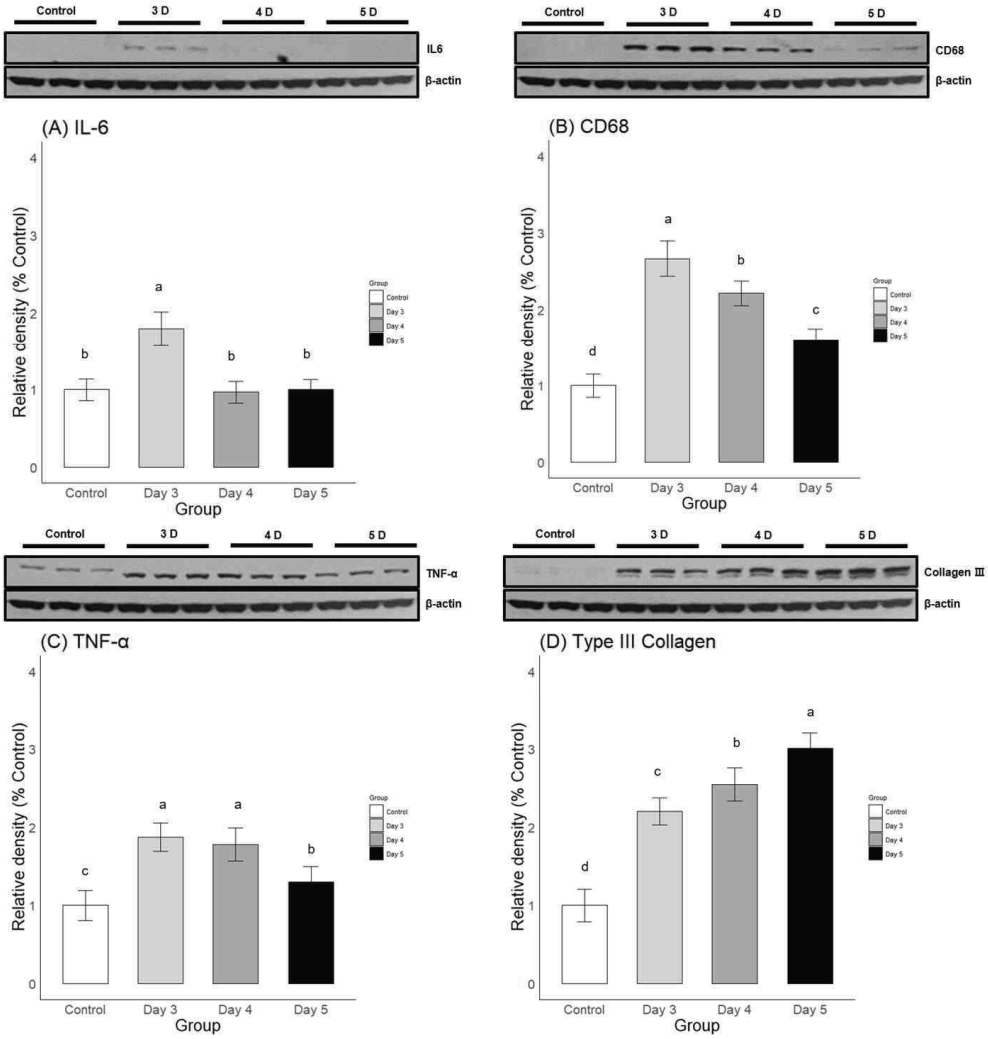


Figure 5. Western blot results of shoulder tissues with varying durations of immobilization.

Relative densities of protein bands were compared with one-way ANOVA and post-hoc Duncan multiple range test. Different letters (a-d) on the bar represent significant difference upon Duncan multiple range test.

2. Efficacy of IA corticosteroid injection at the freezing phase

A. Passive shoulder ROM

The passive SAA values for each group at baseline, day 3, and week 3 of study, and the differences in values before and after intervention (ΔSAA = (SAA at week 3) – (SAA at day 3)) are shown in Table 1. While Group D and Control group did not show significant changes in SAA between day 3 and week 3 (P=0.334, and 0.880, respectively), Group A and B, both of which received IA injection at day 3, showed significant improvement in SAA (P=0.048, and <0.001, respectively). Group C, which continued to be immobilized, showed significant reduction in passive SAA (P<0.001) (Table 1).

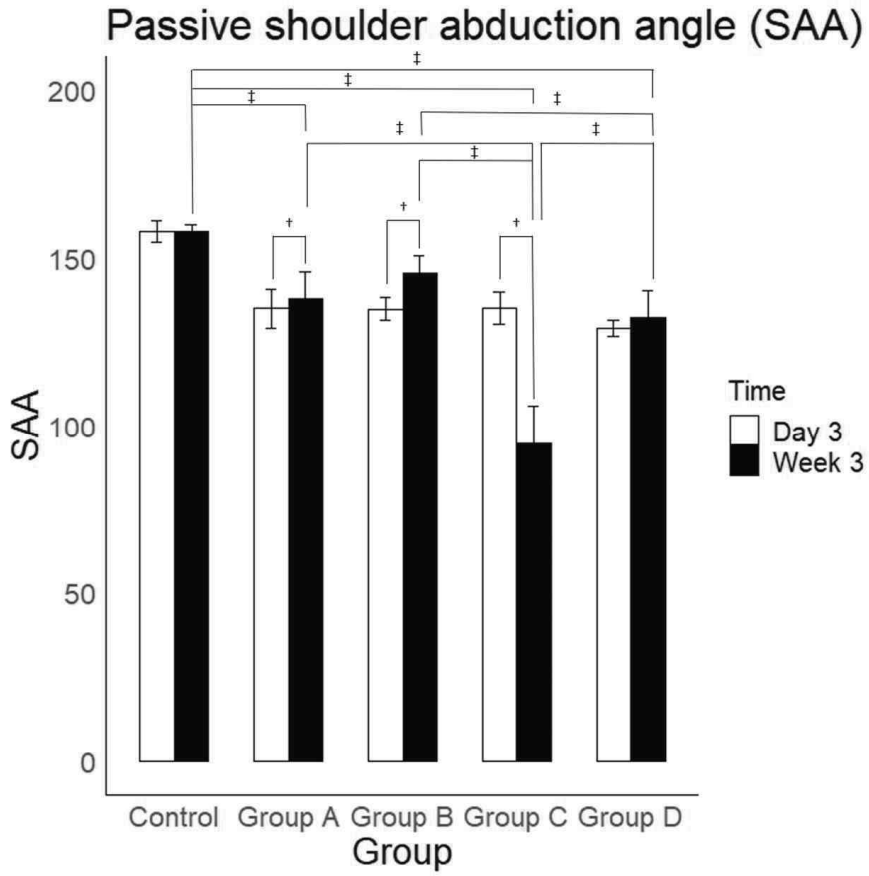
One-way ANOVA of ΔSAA values revealed significant differences among groups (P<0.001). Tukey's post-hoc test showed significant differences between Groups A and C (P<0.001), Groups B and C (P<0.001), and Groups C and D (P<0.001); compared with Control group, Groups B and C showed significant differences in ΔSAA (P=0.009, and <0.001, respectively).

One-way ANOVA of the final SAA values (at week 3) revealed significant differences among groups (P<0.001). Tukey's post-hoc test showed significant differences between Groups A and C (P<0.001), Groups B and C (P<0.001), Groups B and D (P=0.035), and Groups C and D (P<0.001); compared with Control group, Groups A, C, and D showed significant differences in SAA at week 3 (P=0.001, <0.001, and <0.001, respectively).

Table 1. Passive SAA (average ± standard deviation) at the third day of study and the third week of study (or immediately prior to sacrifice) in the groups.

	Passive SAA				
	SAA at baseline	SAA at day 3	SAA at week 3	ΔSAA	*P*
Group A	159.3 ± 3.1	135.0 ± 5.8	138.0 ± 7.8	3.0 ± 2.8	**0.048**
Group B	160.2 ± 3.4	134.8 ± 3.5	145.7 ± 5.2	10.8 ± 3.8	**<0.001**
Group C	159.0 ± 4.0	135.2 ± 4.8	94.8 ± 11.2	-40.3 ± 7.2	**<0.001**
Group D	158.0 ± 4.8	129.0 ± 2.4	132.2 ± 8.1	3.2 ± 7.3	0.334
Control	157.5 ± 3.9	158.0 ± 3.2	157.8 ± 2.3	-0.2 ± 2.6	0.880

The differences between SAA at day 3 and at week 3 are presented as ΔSAA, and P values of the paired t-test performed between the SAAs at day 3 and at week 3 are shown.



† indicates P <0.05 upon within-group comparison of SAA at Day 3 and Week 3 of study using paired *t*-test.

‡ indicates P <0.05 upon between-group comparison of final SAA at Week 3 of study using post-hoc Tukey's test.

Figure 6. Graphical representation of passive SAA at the third day of study and the third week of study in the groups.

B. Histological findings

Upon histological assessment, corticosteroid-injected groups (Groups A and B) showed comparable degree of capsular fibrosis with healthy control, which was markedly increased in groups which did not receive corticosteroid treatment (Groups C and D) (Figure 6 (A-E)). This gross observation was supported by quantitative measurements of capsular thickness. One-way ANOVA of capsular thickness showed significant differences among the five groups ($P<0.001$, 0.28 ± 0.09 mm for Group A; 0.21 ± 0.03 mm for Group B; 0.39 ± 0.08 mm for Group C; 0.34 ± 0.11 mm for Group D; 0.19 ± 0.04 mm for Control). Post-hoc Tukey's test revealed significant differences between Control and Group C ($P=0.002$), Control and Group D ($P=0.031$), and between Groups B and C ($P=0.004$). Capsular thickness of corticosteroid-injected groups were not significantly different from that of Control ($P=0.396$, 0.998, for comparison with Group A and B, respectively).

For examination of fibrosis, MTS (Figure 6 (F-J)) and Vimentin immunostaining (Figure 7 (F-J)) showed greater staining intensity in groups that did not receive corticosteroid injection compared to healthy control or corticosteroid-injected groups. This was supported by semi-quantitative analysis (Figure 8 (A), (C)). For MTS, one-way ANOVA showed significant differences in semi-quantitative scores among groups ($P<0.001$). Post-hoc test showed significant differences between Control and Group C, Group A and Group C, and Group B and Group C ($P<0.001$, 0.011, 0.001, respectively). For Vimentin immunostaining, one-way ANOVA showed significant differences in semi-quantitative scores among groups ($P<0.001$). Post-hoc test showed significant differences between Control and Group C, Group A and Group C, Group B and Group C, Control and Group D, Group A and Group D, Group B and Group D, and Group C and Group D ($P<0.001$, <0.001, <0.001, 0.008, 0.033, 0.012, 0.008, respectively).

For examination of inflammatory marker expression, IL-6 immunostaining (Figure 7 (A-E)) showed similar pattern among groups as fibrosis markers, but differences among groups were less prominent. One-way ANOVA of semi-quantitative scores of IL-6 immunostaining (Figure 8 (B)) showed significant differences among groups (P=0.017). Post-hoc test showed significant difference only between Control and Group C (P=0.019).

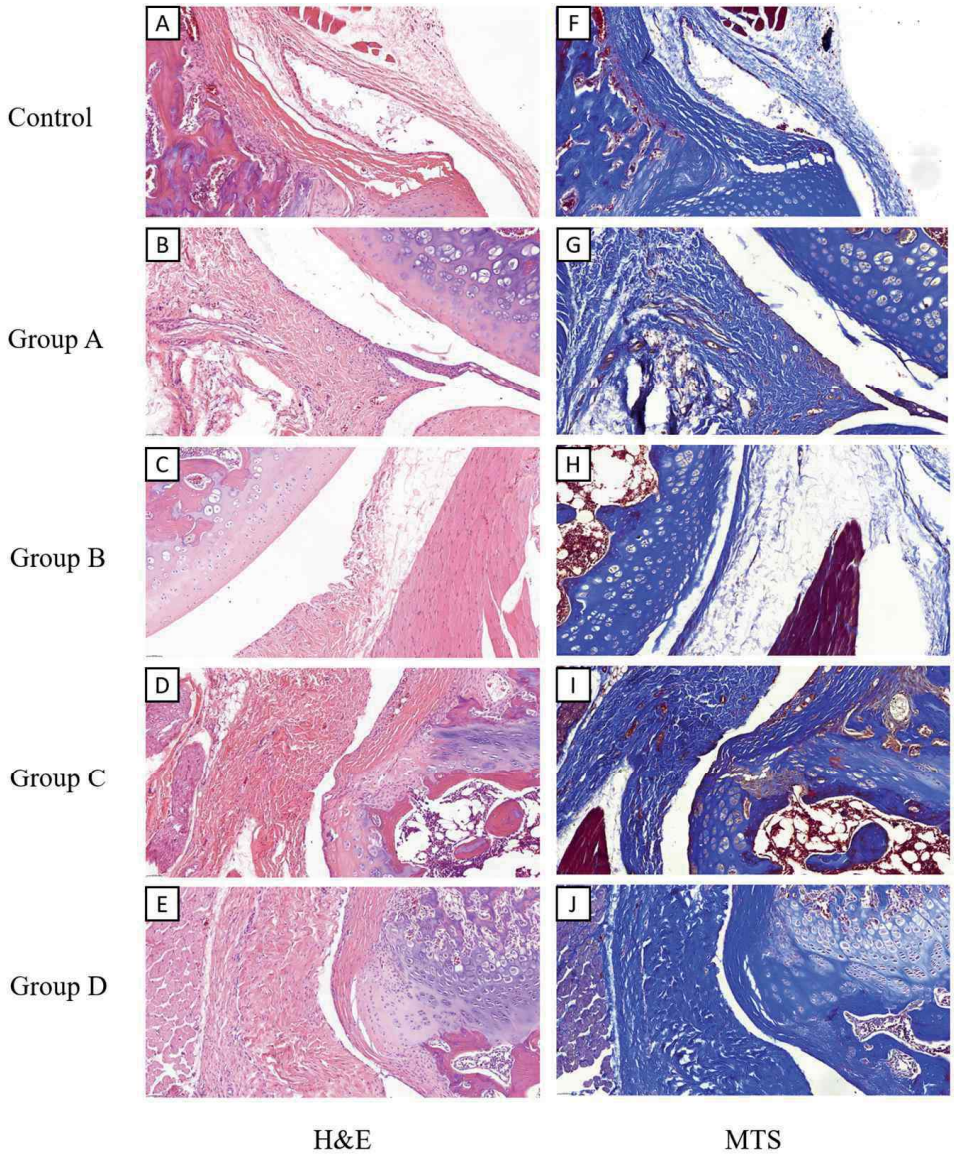


Figure 7. Staining of Control and Groups A-D.

The images show the synovium and subsynovial structures of axillary recess. (A-E) H&E, (F-J) MTS of Control and Groups A-D (×40). In general, corticosteroid-injected groups (Groups A and B) showed lower staining intensities for shoulder capsule in MTS compared to groups that did not receive injection (Groups C and D).

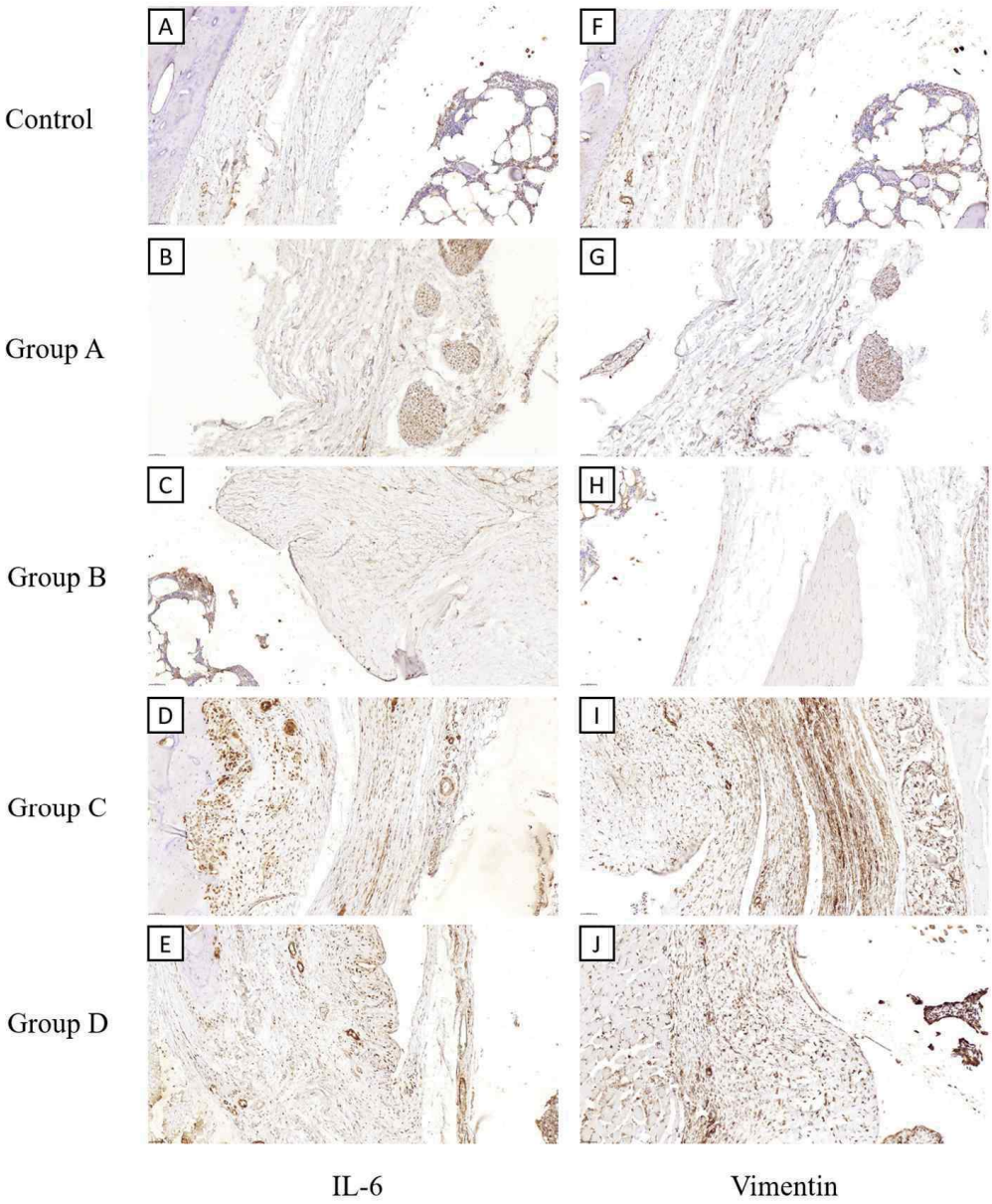
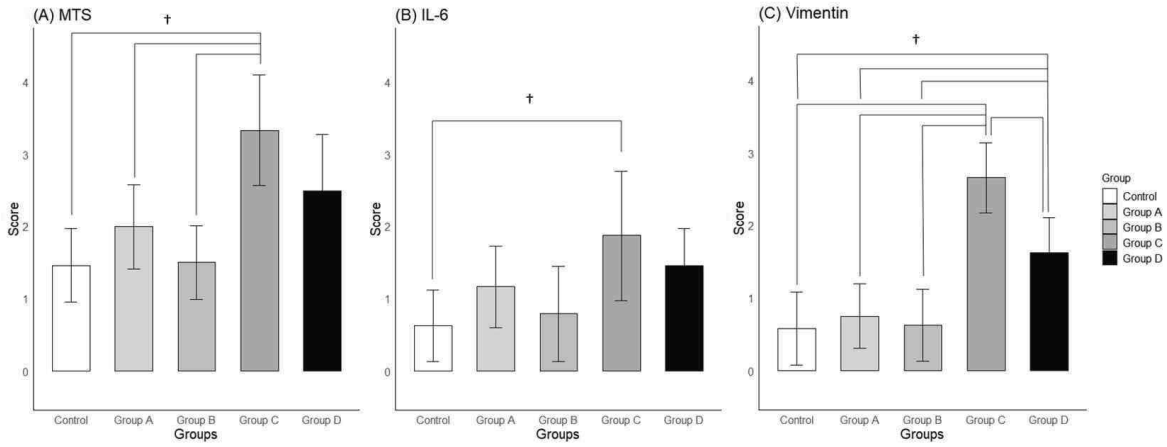


Figure 8. Immunohistochemical findings of Control and Groups A-D.

The images show the synovium and subsynovial structures of axillary recess. (A-E) IL-6, (F-J) Vimentin immunohistochemical staining of Control and Groups A-D (×40). In general, corticosteroid-injected groups (Groups A and B) showed lower immunostaining intensities for both IL-6 and Vimentin compared to groups that did not receive injection (Groups C and D).



† indicates P <0.05 upon post-hoc testing between groups.

Figure 9. Semi-quantitative scores of (A) MTS, (B) IL-6 immunostaining, and (C) Vimentin immunostaining for Control and Groups A-D.

C. Western blot results

One-way ANOVA and post-hoc Duncan multiple range test showed significant differences of expression levels of six proteins among and between groups (Figure 6). For inflammatory markers (Figure 6 (A-D)), Group B showed significantly lower levels of all four markers (i.e. IL-1α, IL-1β, TNF-α, and TNF-β) compared to Groups A, C, and D. In case of IL-1α and IL-1β expression, Group B showed no significant difference from the control group. Group A showed significantly lower levels of all four markers compared to Group C. Also, Group A showed significantly lower levels of TNF-α and TNF-β, but showed higher level of IL-1β compared to Group D. Finally, Group D showed significantly lower levels of IL-1α, IL-1β, and TNF-β levels compared to Group C.

For Alarmin molecules (Figure 6 (E-F)), Group B showed significantly lower levels of the two markers (HMGB1 and RAGE) compared to Groups A, C, and D. However, the expression levels of both HMGB1 and RAGE were greater compared to the control group. Group A showed significantly lower level of RAGE, but not HMGB1 expression compared to Group C and D. Finally, Group D showed significantly lower level of RAGE, but not HMGB1 expression compared to Group C.

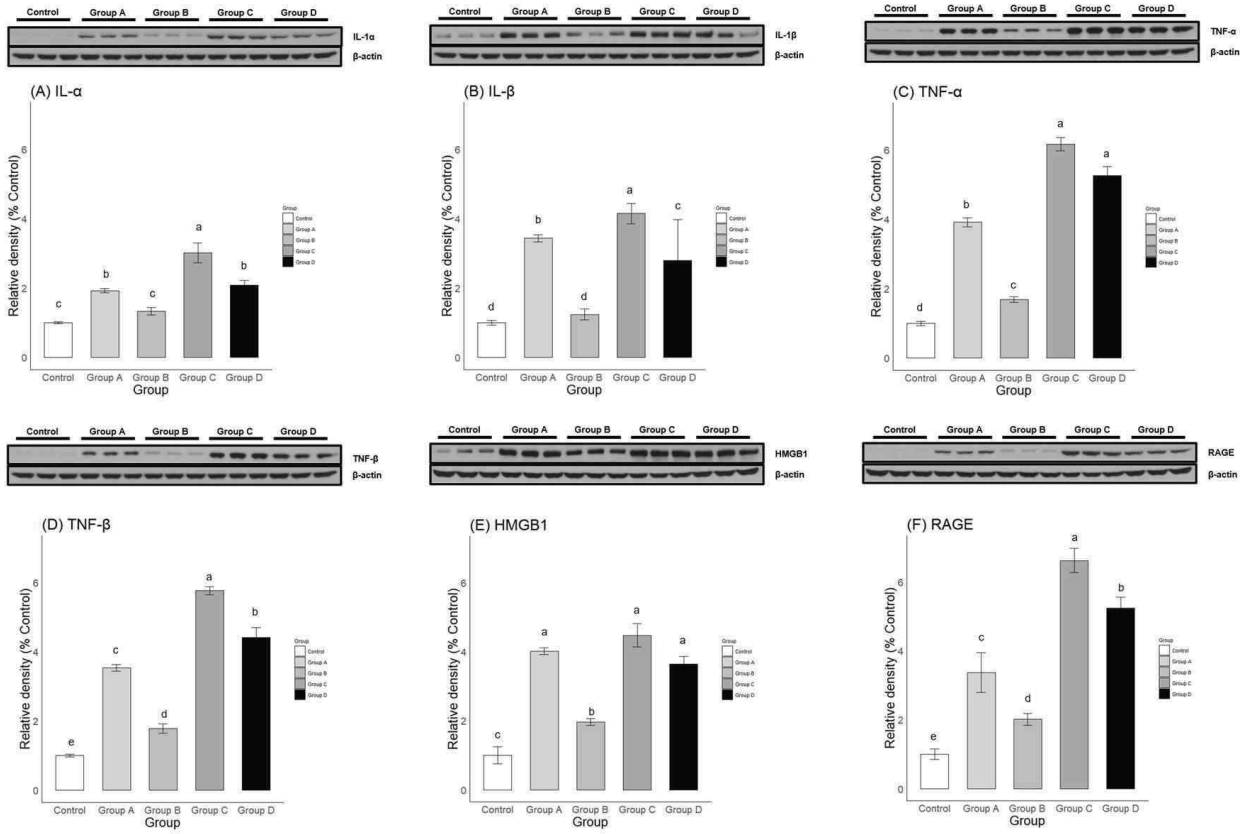


Figure 10. Western blot results of (A) IL-1α, (B) IL-1β, (C) TNF-α, (D) TNF-β, (E) HMGB1, and (F) RAGE post-intervention at the freezing phase.

Relative densities of protein bands were compared with one-way ANOVA and post-hoc Duncan multiple range test. Different letters (a-e) on the bar represent significant difference upon Duncan multiple range test.

IV. DISCUSSION

Passive shoulder abduction angle assessment showed significant improvement in corticosteroid-injected groups (Groups A and B) at Week 3 of study compared to Day 3 of immobilization. Release of shoulder immobilization alone (Group D) did not induce significant improvement in range of motion at Week 3 of study, while continuation of shoulder immobilization (Group C) significantly aggravated the limitation in range of motion, simulating progression to the classical "frozen phase", in accordance with previous reports with rat models[22]. Histological evidences were in accordance with the above-mentioned functional assessment of shoulder, as staining intensity of MTS and Vimentin immunohistochemistry were significantly lower in corticosteroid-injected groups compared to those that did not receive injection, signifying prevention of fibrotic changes and fibroblast proliferation by IA corticosteroid injection, hence the "disease-modifying" effects.

Regarding inflammatory marker expression, IL-6 immunostaining intensities were generally lower across all groups examined at Week 3 of study compared to Days 3 through 5 of immobilization as observed in differences in semi-quantitative scores (Figures 4 and 8), showing that the inflammatory process is greatest during the freezing phase of the disease, which is also corroborated by previous reports on rat models[22]. Comparison within groups at Week 3 of study showed significant difference only between healthy control and fully-immobilized Group C. Western blotting of inflammatory cytokines and alarmin molecules showed no significant difference in IL-1α and IL-1β expression between Group B and Control, while Group A showed significantly lower expression of IL-1α, IL-1β, TNF-α, TNF-β, and RAGE compared to Group C. These show the direct anti-inflammatory effects of corticosteroid injection, which is intensified by re-mobilization of shoulder.

The anti-fibrotic effect of corticosteroid is not clearly elucidated in previous literature, especially in musculoskeletal systems. Systemic corticosteroid therapy is widely used in fibrotic lung diseases (e.g. idiopathic pulmonary fibrosis), and in these circumstances, it is regarded that anti-fibrotic effects are achieved only indirectly by attenuation of inflammatory process such as alveolitis[31, 32]. The modulatory mechanisms on inflammation include suppression of macrophage function which subsequently affects T-lymphocytes[33, 34], and direct inhibitory effects on T-lymphocyte proliferation and effector function[35, 36].

However, there are in vitro studies documenting direct inhibitory effects of corticosteroid on fibroblast proliferation and production of components of connective tissue matrices (e.g. Collagen or mucopolysaccharide)[37, 38]. Also, corticosteroid has shown to regulate secretion of elastase and collagenase by macrophages[39], which also directly affects fibrotic process. In our study, immunohistochemical staining of Vimentin, a fibroblast marker, showed significantly lower expression in corticosteroid-injected groups (Groups A and B) compared to groups that did not receive corticosteroid injection (Groups C and D). This could be due to the direct inhibitory effects of corticosteroid on fibroblast proliferation. However, considering the time difference between corticosteroid administration (Day 3 of study) and acquisition of histological samples (Week 3 of study), it is also possible that such inhibition of fibroblast proliferation was mediated secondarily by other factors, such as inflammatory cells. In context of frozen shoulder, IL-17 has been found to be produced by a subpopulation of T-cells in the diseased shoulders, and this cytokine was shown to affect fibroblast cell viability by increasing anti-apoptotic gene expression (e.g. BCL2)[40]. Therefore, corticosteroid administration may affect inhibit fibroblast proliferation through both direct and indirect processes, the latter involving inhibition of T-lymphocytes and subsequent release of IL-17. Although our study did not include assessment of IL-17 expression, Western blot analysis in our study showed decreased expression of a variety of inflammatory cytokines, which could have

contributed to secondary modulation of fibroblast proliferation and activity. Both Groups A and B in our study demonstrated the potent anti-fibrotic effects, whether direct or indirect, regardless of cessation or continuation of immobilization.

The anti-fibrotic effects of corticosteroid were also quantitatively assessed by capsular thickness measurements. In our study, capsular thickness in corticosteroid-injected groups (Groups A and B) did not show significant difference from that of healthy control, while groups that did not receive injection showed significant difference. This observation aligns with the semi-quantitative scores of MTS and Vimentin immunostaining (Figure 8 (A), (C)). In a previous report which measured capsular thickness in frozen shoulder rat model, no significant difference was observed between group that received shoulder immobilization for 8 weeks and group that received corticosteroid injection after the 8-week immobilization[23], which is contrary to our findings. However, this study did not report the unit of measurement (which was milimeters in our study), preventing direct comparison with our results. Also, the difference in results may have resulted from different periods of immobilization.

Although the rat model used in our study is analogous to the most commonly reported ones in the literature, as it is induced secondarily through immobilization of shoulder joint, it is not an immaculate simulation of idiopathic primary frozen shoulder. In order to validate the model's similarity to primary frozen shoulder and to establish the immobilization protocol that best simulates the natural course of primary frozen shoulder, our study included groups that continued immobilization throughout the study (Groups A and C), and groups that released immobilization after induction of "freezing phase" (Groups B and D). In a classical primary frozen shoulder model, the freezing phase progresses spontaneously to the "frozen phase" with greatly decreased range of motion[5], even without immobilizing the joint as in our rat model. Therefore, it is logical to suspect that in secondary frozen shoulder animal models, induction of freezing phase and hence

inflammation in glenohumeral joint through short period of immobilization may suffice to induce progressive reduction in range of motion even after release of immobilization. However, this was not the case, as demonstrated by a mild increase in range of motion in Group D after release of immobilization at Day 3. In order to induce the classic frozen phase with limited range of motion, prolonged immobilization to Week 3 was needed, as seen in Group C. Similar findings have been reported in a canine secondary frozen shoulder model, in which 12 weeks of immobilization followed by another 12 weeks without immobilization resulted in normalization of shoulder range of motion[19]. Therefore, Group A, which maintained immobilization after corticosteroid administration, may be a more accurate representation of performing injection to human subjects than Group B.

There are several limitations to our study. Firstly, as the sample size calculation was powered on passive shoulder range of motion, our study was not powered to detect differences in secondary outcomes such as differences in semi-quantitative scores of histological findings and Western blot results. Secondly, we included only limited number of proteins for immunohistochemical analysis. For a more thorough observation of fibrosis, proteins such as α-SMA and CD31 could have been included as markers for myofibroblast and myoendothelium, respectively. Also, further molecular studies are needed to elucidate the effects of various cytokines examined in our study on fibroblasts or fibrosis in general. Finally, as extensively discussed above, rat frozen shoulder models which are induced secondary to immobilization cannot be perfect substitutes to study primary frozen shoulder in humans. For a closer simulation of primary frozen shoulder, either genetically engineered or biochemically induced frozen shoulder animal models could be conceived, but these models have not been established so far according to our knowledge.

V. CONCLUSION

This study demonstrated the potentially disease-modifying anti-fibrotic effect of corticosteroid administration during the freezing phase of frozen shoulder. IA corticosteroid injection significantly improved passive range of motion while preventing progression of fibrosis and fibroblast proliferation, and these effects were potentiated by release of immobilization allowing for free shoulder movement. This property should be considered in clinic when administrating IA corticosteroid, which may be not only be symptom-relieving, but also disease-modifying when administered at the freezing phase. Further clinical studies should follow in order to verify the disease-modifying effect of corticosteroid in human subjects.

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ABSTRACT (IN KOREAN)

오십견 동물 모델에서 동결 진행기 관절강내 스테로이드 주사의 질병 완화 효과

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안용진

오십견의 동결 진행기 (freezing phase)에는 염증 반응이 주로 일어나므로 항염증 작용이 있는 코티코스테로이드를 사용하게 된다. 그러나, 관절강내 코티코스테로이드 투여의 효과가 단순히 일시적인 염증 완화에만 그치는지, 또는 섬유화를 억제하여 동결기로의 진행을 억제하는지는 연구된 바가 없다. 본 연구는 랫드의 오십견 모델에서 동결 진행기에 관절강내 코티코스테로이드 주사가 질병의 진행 억제에 미치는 영향에 대해 분석하고자 한다.

총 24마리의 Sprague-Dawley 랫드를 6마리씩 4군으로 나누어 진행하였다 (Group A – 3일간 일측 견관절 고정 후 관절강내 스테로이드 주사 후 견관절 고정 유지; Group B – 3일간 일측 견관절 고정 후 관절강내 스테로이드 주사 후 견관절 고정 제거; Group C – 총 3주간 일측 견관절 고정 유지; Group D – 3일간 일측 견관절 고정 후 나머지 기간 동안 고정 제거). 모든 군에서 실험 3주차에 견관절의 passive abduction angle을 측정하고, axillary recess 부위의 어깨 관절 조직을 채취하여 H&E, Masson trichrome, 면역조직화학염색 및 Western blot 분석을 시행하였다.

실험 종료 시점인 3주차에 측정한 수동적 견관절 외전 각도는 각각 138.0° ± 7.8° (Group A), 145.7° ± 5.2° (Group B), 94.8° ± 11.2° (Group C), 132.2° ± 8.1° (Group D), 157.8° ± 2.3° (Control)로 확인되었다. Group B의 경우 건강한 대조군과 유의미한 각도 차이를 보이지 않았다. 이는 오십견의 동결 진행기에 관절강내 코티코스테로이드 주사와 함께 견관절 고정의 중단 이후 견관절 기능이 정상적 수준까지 회복됨을 보여준다.

Masson trichrome 염색 및 IL-6와 Vimentin 면역조직화학염색의 육안적 분석 및 반정량적 분석 방법 상, 코티코스테로이드를 투여하지 않은 군 (Group C와 D)에서 대조군 및 코티코스테로이드 투여군 (Group A와 B)에 비해 섬유화 및 염증의 정도가 더 강함이 확인되었다. 또한, 코티코스테로이드 투여군의 경우 대조군과 비교했을 때 섬유화 및 염증의 정도가 큰 차이를 보이지 않았다. Western blot 검사 상 IL-1α, IL-1β의 경우 Group B와 건강한 대조군의 발현 정도가 유의미한 차이를 보이지 않았다. Group A 또한 IL-1α, IL-1β, TNF-α, TNF-β, RAGE의 발현이 Group C에 비해 유의미하게 낮았다. 이는 동결 진행기의 관절강내 코티코스테로이드 주사가 장기적으로 염증 반응을 감소를 유발하며, 이를 통해 섬유화로의 진행을 억제하는 질병경과 변형 (disease-modifying)의 효과가 있음을 보여준다.

핵심되는 말 : 오십견; 코티코스테로이드; 관절강내 주사; 실험 동물 모델; 관절 가동 범위