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Metformin increases the expression of proinflammatory cytokines and inhibits supraspinatus fatty infiltration

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Abstract

Background After a rotator cuff (RC) tendon tear, the supraspinatus (SS) inflammatory response induces fatty infiltration (FI). Metformin has the effect of regulating the initial inflammatory response of atrophic muscles. Therefore, this study aimed to investigate the effect of metformin use on modulating the expression of proinflammatory cytokines and SS FI in an acute RC tear rat model.

Methods This study used 26 male Sprague–Dawley rats. Animals were randomly divided into two groups: The metformin group received metformin for 5 days after cutting the RC tendon, and the control group was administered only with saline after cutting the tendon. Metformin 50 mg/kg was intraperitoneally injected for 5 days. Three rats in each group were sacrificed 5 days after SS tendon rupture surgery, and 10 rats in each group were sacrificed 14 days after surgery. The SS was sampled 5 days after SS tendon tear surgery, and the expression of proinflammatory cytokines was measured by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). On day 14 after sampling, histological analysis of the SS was performed using hematoxylin and eosin, Masson's trichrome, and picrosirius red staining.

Results On day 5 of surgery, the expression values of interferon gamma (increased 7.2-fold, $P < .01$), tumor necrosis factor alpha (increased 13-fold, $P < .05$), interleukin-1β (increased 4.7-fold, $P < .001$), and interleukin-6 (increased 4.6-fold, $P < .01$) increased significantly in the metformin group compared with those in the control group. As a result of Oil Red O staining, SS FI was significantly suppressed in the metformin group compared with that in the control group (metformin group, $305 \pm 50.3\ \mu m^2$, $P < .001$; control group, $3136 \pm 662.8\ \mu m^2$, $P < .001$). In addition, the SS volume of the metformin group was not reduced compared with those of the control group, and the morphology and structure of the SS were better preserved.

Conclusions The results of this study revealed that metformin can increase the expression of proinflammatory cytokines and suppress SS fat infiltration in delayed sutures.

Keywords Metformin, Inflammatory response, Fatty infiltration, Supraspinatus atrophy, Histology, Rotator cuff tear

Background

RC tear is one of the most common shoulder disorders in humans and incurs enormous medical costs to patients, severe pain in the shoulder joint, muscle weakness, and limited mobility [1]. Despite successful surgical intervention, RC tears do not heal spontaneously, and poor surgical prognosis and high re-tear rates remain a problem [2–5]. Factors that adversely

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affect improved healing following RC tendon suture include RC muscle fiber atrophy and fat accumulation in and around the muscle fibers. In these RC tears, FI is an irreversible, muscle degenerative change that results in muscle weakness, shoulder dysfunction, limited range of motion, and impaired tendon–bone healing [6, 7].

Massive RC tears induce an inflammatory response in muscles and can have detrimental effects on the development of chronic diseases, leading to muscle atrophy and FI of the skeletal muscles [8–11]. Previous studies have suggested that the RC muscle becomes inflamed in the presence of an RC tendon tear [12] and that this acute inflammation plays a detrimental role in the development of chronic muscle diseases [7, 13]. In an actual animal experiment, the number of inflammatory cells such as neutrophils and monocytes/macrophages peaked at the first 5–7 days in the SS muscles with ruptured full-thickness RC tendons, and significant changes were noted in proinflammatory and anti-inflammatory cytokines [5, 12, 14, 15]. Clinical studies have also reported muscle changes and overall changes in cytokine levels, metalloprotease levels, and growth factors after RC tendon rupture [16]. This muscle inflammation after an RC tendon rupture affects the degree of FI and subcellular changes in the skeletal muscles [5, 17]. Therefore, the modulation of muscle inflammation in RC tears may be an effective target to improve RC tendon–bone healing [16].

Metformin is an FDA-approved drug for hypoglycemic diabetes that has been used to treat type 2 diabetes mellitus since 1957 [18–20]. It does not induce hypoglycemia, has an excellent antihyperglycemic effect, reduces cost, and has excellent pharmacological properties as a first-line drug, such as having fewer side effects [19–23]. In addition to its antihyperglycemic effects, metformin has the potential to influence proinflammatory cytokine levels by activating macrophages within the inflammatory response [24]. Elevated proinflammatory cytokine levels can trigger the apoptosis of fibro-adipogenic progenitors (FAPs) and the activation of satellite cells, consequently mitigating the fatty degeneration of FAPs [24, 25]. The effects of metformin on the inflammatory response of the SS muscle resulting from an RC tear have not been established. Our hypothesis was that the metformin-induced activation of immune cells during the initial stages of RC rupture might have the potential to suppress SS FI.

This study aimed to verify the inhibitory effect of metformin on FI in an acute RC tear rat model. In this study, an RC tendon tear model was created, and the expression of cytokines associated with the early postoperative acute inflammatory response was evaluated. FI of the SS muscle was evaluated 14 days after RC tendon cutting, and muscle atrophy was confirmed by histological staining.

Methods

Animal model

All animal testing procedures were approved by the Institutional Animal Committee designated by the author's institution. This study used 9-week-old male Sprague–Dawley rats, which were raised in a specific pathogen-free facility. The temperature of the breeding facility was 22 ± 2 °C, and the light–dark cycle was adjusted to 12 h/12 h, and food and water were given freely. Based on previous study (comparison between 2 groups: mean difference 436 μm^2, standard deviation 96 μm^2, α error = 0.05, b error = 0.2, 2-tailed comparison), power analysis determined that 5 specimens were needed per group to detect a significant difference in a quantitative analysis of FI [26]. A total of 26 rats were randomly divided into the metformin group and the control group. Three rats each of the control and metformin groups were sacrificed for qRT-PCR on day 5. After 14 days, the left shoulders of 5 rats were used for the Oil Red O staining, and the right shoulders of 10 rats were used for the histological analysis.

Surgical procedures

Anesthesia was induced with 5% isoflurane delivered by oxygen at 4 L/min via inhalation in an induction chamber, and anesthesia was maintained with 2% isoflurane via a nose cone. The fur on the shoulder was moderately shaved to expose the skin and sterilized to maintain sterility. An RC tear procedure was then performed [1]. Briefly, the scapula was palpated, and a 3-cm long incision was made. As a result, the deltoid muscle was split, and the SS tendon was exposed (Fig. 1A). The SS tendon was separated using the Metzenbaum, and the tendon was cut at the tendon–bone site using a knife blade (Fig. 1B). The SS where the tendon was cut was marked by tying a knot using Ethibond (Ethicon, Somerville, New Jersey, USA) (Fig. 1C). The metformin group was intraperitoneally injected with metformin after surgery. Metformin (Hanmi Pharm, Suwon, Korea) was prepared at a dose of 50 mg/kg/day and was injected intraperitoneally each time for 5 days. Drug doses and methods were referenced in a previous study [18]. Moreover, 5 and 14 days after surgery, all mice were sacrificed, and the SS was harvested (Fig. 2).

qRT-PCR

According to the manufacturer's protocol, total RNA was isolated from rat SS tissue using NucleoSpin RNA Plus kit (Macherey–Nagel, Duren, Germany) and then reversed into cDNA using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). qRT-PCR was performed on CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) using advanced

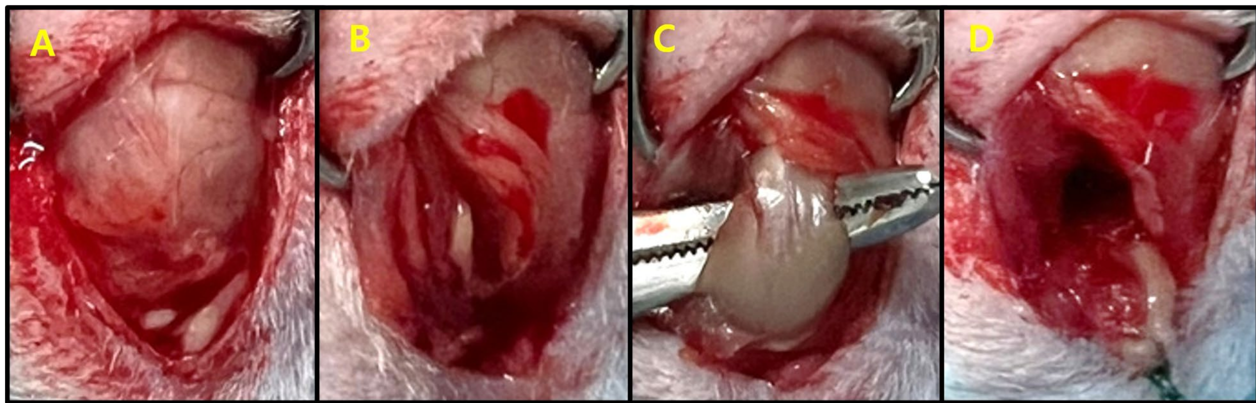

Fig. 1 Animal model and surgical procedure. **A** A longitudinal skin incision of 3–4 cm was made along the scapula, and the deltoid muscle was exposed. **B** A longitudinal 2-cm incision was made in the exposed deltoid muscle. **C** The SS tendon was identified on the inside of the deltoid muscle and marked with a suture. **D** The SS tendon was incised at the greater tuberosity using a knife

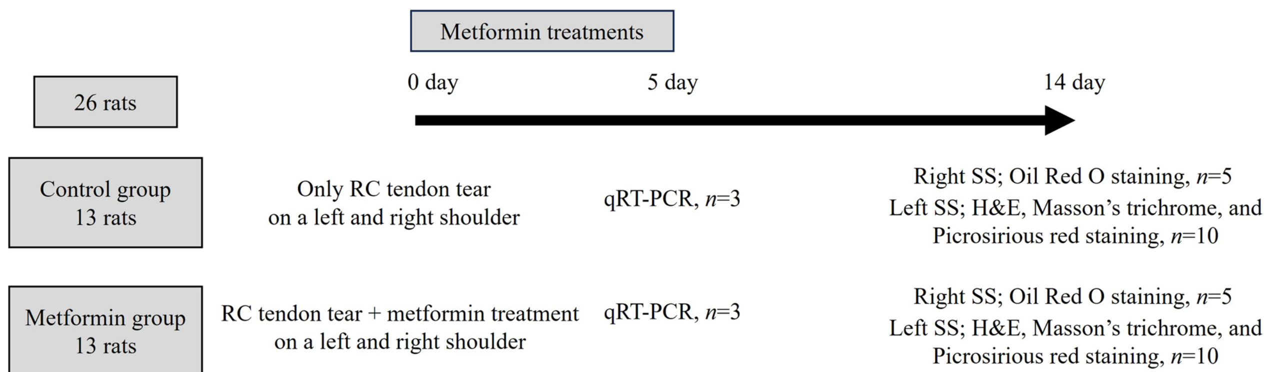

Fig. 2 Experimental timeline schematic for animal experiments. A total of 26 rats were randomly allocated into two groups, each consisting of 13 rats. RC tendon tear surgery was performed on both shoulders of all rats. The metformin group received drug administration for a duration of 5 days. Additionally, all rats were sacrificed 14 days post-surgery. Oil Red O staining was conducted on the five SS extracted from the left shoulder, while histological evaluation was carried out on the ten SS from the right shoulder

universal SYBR Green Supermix (Bio-Rad, Hercules, California, USA) at the following conditions: 95℃ for 30 s, 40 cycles of 95℃ for 15 s, and 60℃ for 30 s. PCR primers used are summarized in Table 1. Fold changes in the mRNA expression levels of interferon (IFN)-γ, interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α were determined relative to the control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression using the $2^{-\Delta\Delta}$CT method.

Oil Red O staining

The SS was harvested from five specimens of each group, after which the muscular portion was meticulously dissected. Each sample was frozen at − 80 °C and cut to frozen tissue sections at a thickness of 8 mm for Oil Red O staining (MAK194, Sigma-Aldrich, St. Louis, Missouri, USA) and counterstaining with hematoxylin [1, 26, 27].

Table 1 Primer sequences for quantitative reverse-transcription polymerase chain reaction analysis

Target gene	Primer sequence (5′–3′)	Product size (bp)
IFN-γ	Forward: 5′- GGCAAAAGGACGGTAACACG-3′ Reverse:5′- TTCACCTCGAACTTGGCGAT-3′	196
IL-1β	Forward: 5′- GGCTGACAGACCCCAAAAGA-3′ Reverse:5′- TGTCGAGATGCTGCTGTGAG-3′	101
IL-6	Forward: 5′- AGAGACTTCCAGCCAGTTGC-3′ Reverse:5′- AGTCTCCTCTCCGGACTTGT-3′	85
TNF-α	Forward: 5′- CTGTGCCTCAGCCTCTTCTC-3′ Reverse:5′- ACTGATGAGAGGGAGCCCAT-3′	126
GAPDH	Forward: 5′- GCGAGATCCCGCTAACATCA-3′ Reverse:5′- CTCGTGGTTCACACCCATCA-3′	178

Oil Red O staining is used for the histologic visualization of fat cells and neutral fat. The FI of specimens was evaluated under a light microscope and with ImageJ.

Histological evaluation

The RC SS muscles of the experimental and control groups were harvested. Harvested samples were fixed in neutral buffered 10% formalin (pH 7.4), and paraffin blocks were fabricated [27]. The paraffin blocks with a thickness of 4-lm were deparaffinized and rehydrated. Second, sample slides were randomly selected and stained with hematoxylin and eosin, Masson's trichrome, and picrosirius red. Analyses were performed by two investigators who were blinded to the animal groups. Each tissue slide was imaged under a microscope (Leica DM IL LED; Leica Microsystems, Wetzlar, Germany) using LAS V4.8 (Leica Microsystems) imaging system [1]. To quantify changes in muscle fiber area, H&E images were analyzed using ImageJ software (Public domain JAVA image processing program, National Institute of Health, Bethesda, Maryland, USA).

Statistical analysis

Statistical analysis was performed using SPSS Statistics for Windows version 12.0 (SPSS Inc., Chicago, IL, USA). An independent t test was performed to evaluate the comparison of proinflammatory cytokine expression and quantitative comparison of FI of the SS between the two groups. Data are presented as mean and standard deviation (SD). Statistical significance was set at $P<0.05$.

Results

In acute RC tendon rupture, metformin increases the expression of proinflammatory cytokines

To confirm the inflammation-modulating action of metformin in RC rupture, changes in the expression of proinflammatory cytokines were confirmed. The SS muscle sampling point was 5 days, when the inflammatory response peaked after RC rupture, which was confirmed in a previous study. As shown in Fig. 3, proinflammatory cytokines IFN-γ (7.2-fold, $P<.01$), TNF-α (13-fold, $P<.05$), IL1β (4.7-fold, $P<.05$), and IL6 (4.6-fold, $P<.01$) in the metformin group were significantly increased compared with those in the control group (Fig. 3).

In the presence of RC tendon rupture, metformin inhibits SS FI

Oil Red O staining was performed to evaluate metformin's inhibitory effect of SS FI after RC rupture. As shown in Fig. 4, the metformin group had significantly lower FI than the control group (Fig. 4A, B). In the quantitative analysis, results confirmed that the FI of the metformin group (305 ± 50.3 μm^2, $P<.001$) was suppressed by nearly 10 times compared with that of the control group (3135.6 ± 662.1 μm^2, $P<.001$) (Fig. 4C). These results

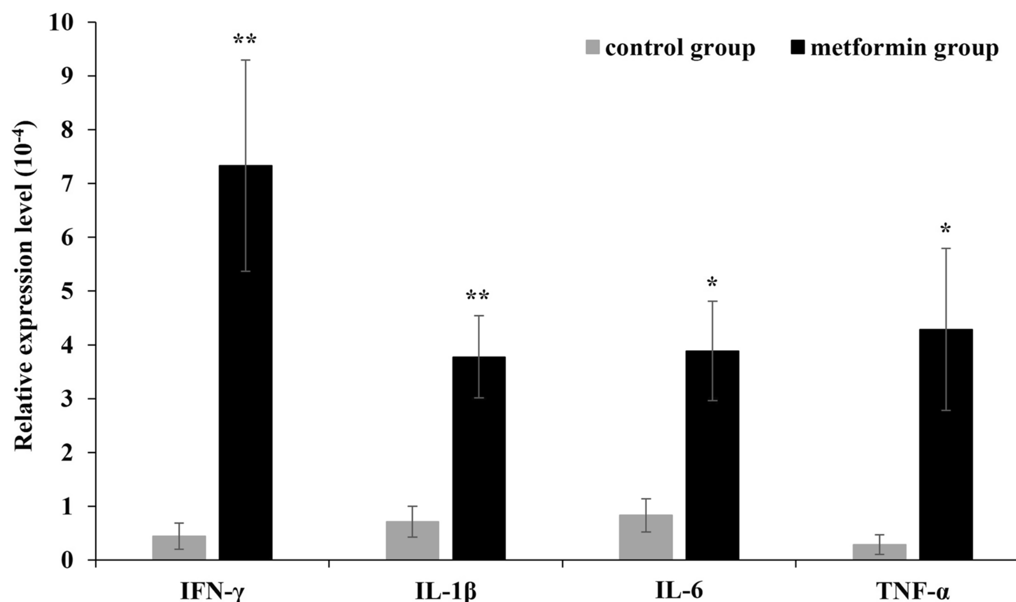

Fig. 3 Expression level of proinflammatory cytokines on the SS treated with metformin. In this graph, proinflammatory cytokines interferon gamma (7.2-fold, $P<.01$), tumor necrosis factor alpha (13-fold, $P<.05$), interleukin 1-beta (4.7-fold, $P<.05$), and interleukin-6 (4.6-fold, $P<.01$) in the metformin group were significantly increased compared with those in the control group. The values are presented as means, with the error bars depicting the standard error of the mean ($n=9$). *$P<.05$; **$P<.01$; ***$P<.001$

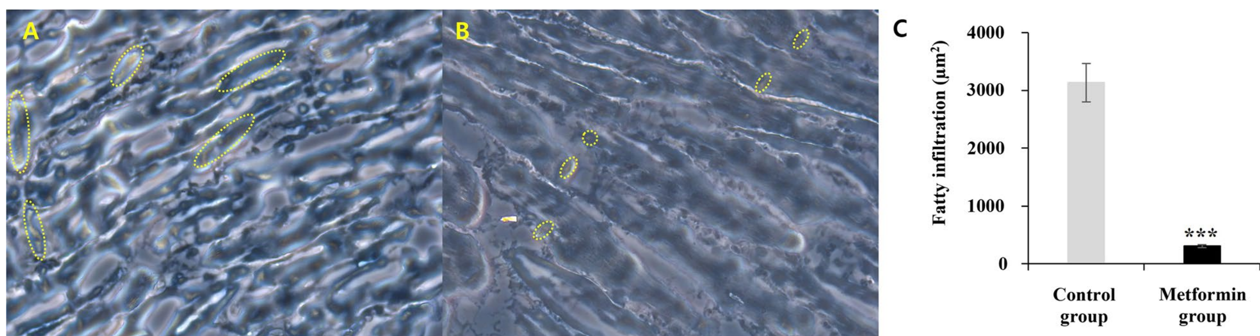

Fig. 4 Oil Red O staining of the metformin group **A** and the control group **B** 14 days after surgery. The spot (yellow arrows) of orange represents FI in the SS after tendon tear (magnification, ×50). The metformin group **B** showed a significantly lower FI than the control group. In graph **C**, the FI of the metformin group (305 ± 50.3 μm^2) was suppressed by nearly 10 times compared with that in the control group (3135.6 ± 662.1 μm^2). The values are presented as means, with the error bars represent the standard deviation of the mean (*n* = 5). ****P* < .001

demonstrate that the use of metformin in the acute inflammatory phase after RC rupture can inhibit SS FI.

Metformin prevents SS atrophy and structural damage to muscle fibers

Through histological macroscopic analysis, structural changes were observed in the SS muscle tissue of the metformin group. As shown in Fig. 5, muscle fibers from rats with delayed suturing after RC rupture showed morphological changes such as atrophy, heterogeneous arrangement, and wide interfibrillar voids. Overall, myofibrillar structure and morphology were disrupted by muscle atrophy (Fig. 5A, C, E). By contrast, the muscle fibers of metformin-treated rats after RC rupture showed a homogeneous arrangement of collagen fibers and narrow interfiber voids (Fig. 5B, D, F). In the quantification of the atrophied muscle, the control group (80.27 ± 6.76%, *P* < .001) showed a 21.6% reduction in muscle fiber area compared to the metformin group (97.60 ± 1.22%, *P* < .001) (Fig. 5G). This indicates that metformin

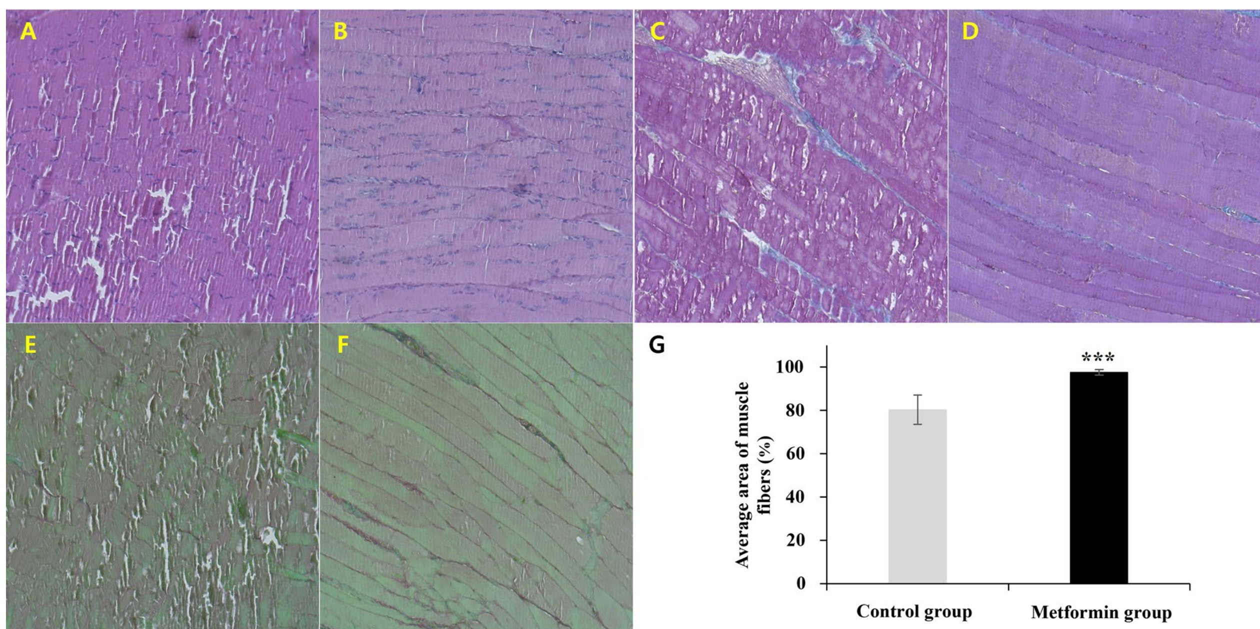

Fig. 5 Effect of metformin on morphological changes in the SS muscle. Metformin group: **B**, **D**, and **E**. Control group: **A**, **C**, and **E**. **A**, **B** Hematoxylin and eosin. **C**, **D** Masson's trichrome. **E–F** Picrosirius red staining. **G** represents the average area of muscle fiber measured by ImageJ software. The values are presented as means, with the error bars depicting the standard deviation of the mean (*n* = 10). ****P* < .001. All images by ×100 magnification

prevented muscle tissue atrophy and myofibrillar structural damage induced in delayed repair after RC tear.

Discussion

In this study, we confirmed metformin-induced increase in proinflammatory cytokines and suppression of SS FI in acute RC tendon rupture. Proinflammatory cytokines, released by immune cells under inflammatory conditions, play a pivotal role in muscle fat degeneration [14, 28, 29]. Metformin has the potential to activate T cells and macrophages and modulate adipocyte function [30]. This study suggests the effectiveness of metformin for inhibition of SS FI induced by acute RC tear.

Metformin is known to exhibit anti-inflammatory effects by inhibiting the release of proinflammatory cytokines, which generally play an important role in the occurrence and development of inflammation [31–33]. This may differ from our results. However, this discrepancy could be explained by metformin-induced activation of inflammatory macrophages in RC tear. Intramuscular adipocytes arise primarily from the FAPs population that resides between muscle fibers. In fact, infiltrating inflammatory macrophages can directly induce apoptosis of FAPs through the secretion of TNF-a [24] and can strongly suppress the adipogenesis of FAPs by satellite cell-derived muscle fibers by secreting IL-6 and G-CSF [25]. In a previous study showed that pretreatment with metformin in concanavalin A hepatitis increased the total number of hepatic infiltrating immune cells (CD_4^+ T, $CD8^+$ T, and $NK_{1.1}^+CD3^-$ NK cells) and correspondingly increased TNF-α, IFN-γ and IL-17 [30]. In addition, it was confirmed that metformin increased proinflammatory cytokines (TNF-α, IL-1α, IL-1β, IL-6, IL-18, IL-8, and IFN-α) in *Mycobacterium tuberculosis* (causative agent of tuberculosis)-infected macrophages [34]. Overall, in the present study, the increased proinflammatory cytokines by metformin treatment in RC tear could be due to activated macrophages, and the suppression of SS FI could be due to inhibition of the differentiation ability of FAP by inflammatory macrophages or induction of apoptosis.

Although the underlying pathogenesis of RC SS FD remains unclear, most agree with the importance of immune cell infiltration [35]. In RC rupture, the SS shows severe signs of acute inflammation, premature degeneration, and FI. The rupture of the RC SS tendon increases the presence of immune cells such as leukocytes, neutrophils, and macrophages and increases the levels of proinflammatory cytokines [1, 17]. This acute inflammation peaks on days 5–7, and the SS fatty infiltrate worsen in the absence of other therapeutic interventions [5]. A large-scale RC tendon tear animal model study also reported similar or more pronounced FI results [28]. The results of this study showed that early administration of metformin in RC rupture could effectively inhibit SS FI. This is because metformin upregulates proinflammatory cytokines, and this is confirmed by our results. In addition, the regulation of cytokines by metformin prevented the decrease in the volume of SS muscle tissue caused by muscle atrophy on day 14 after surgery and prevented the disintegration of the shape and structure.

Muscular FD by RC tear cannot be reversed by suturing and may increase extramuscular fatty accumulation. In addition, since RC FD adversely affects postoperative prognosis, potential therapeutic targets must be developed [36]. Several animal studies have shown that FD and atrophy can be inhibited through the inhibition of specific genes [13, 37], application of adipose-derived stem cells [38], application of perivascular stem cells [39], or improvement of hypercholesterolemia [40]. However, data on the regulation of proinflammatory cytokines in the acute inflammatory response underlying RC muscle FD are limited. Through this study, we suggest that the therapeutic intervention of metformin, which can promote the expression of proinflammatory cytokines in the early stages of RC rupture, can effectively inhibit FI.

The effect of metformin to inhibit SS FI in RC tears was reported in this study for the first time; however, some limitations should be considered in interpreting the results. First, since the expression of time-dependent cytokines in the early stages of RC rupture is a much more dynamic phenomenon, it must be measured at more points. In addition, the expression of cytokines in cells that play important roles in the immune response, such as neutrophils and macrophages, should be directly investigated. Second, the limited evaluation period of 2 weeks does not consider all possible subsequent events and may not be sufficient for the final analysis. Third, we only assessed a singular dose of metformin, which may not adequately account for its efficacy. Therefore, future investigations should encompass the evaluation of SS FI inhibition across a minimum of two doses or even higher doses. Fourth, we did not explore the impact of FI inhibition on enhancing the biomechanical aspects of tendon healing following tendon repair. Subsequent research endeavors should validate the potential biomechanical enhancements in tendon healing attributed to metformin, utilizing an acute RC repair rat model. Fifth, the histological evaluation was semi-quantitative, and a histological system specific to RC tears was not used. Finally, this study successfully confirmed the expression of proinflammatory cytokines. Nonetheless, for a thorough assessment of FI inhibition, it is necessary to conduct molecular marker (PPAR-γ and C/EBP-α) analysis and include immunohistochemical staining experiments.

In conclusion, our study demonstrates that the initial use of metformin promotes the expression of proinflammatory cytokines in an acute RC rupture rat model. SS FI promoted by delayed RC tear suturing could be effectively inhibited by metformin. In addition, metformin can preserve the morphology and structure of the SS muscle following muscle atrophy. Therefore, metformin can be an effective treatment to inhibit muscle atrophy and FI following RC tears.

Conclusions

In acute RC rupture, the use of metformin may increase the levels of proinflammatory cytokines, which are mediators of inflammatory response. The control of this acute inflammatory response could be a new therapeutic strategy to prevent muscle FD. Metformin promotes the inflammatory response in the early stages of RC rupture and can effectively prevent muscle atrophy and FI.

Abbreviations

RC	Rotator cuff
SS	Supraspinatus
FI	Fatty infiltration
FD	Fatty degeneration
IFN-γ	Interferon-gamma
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
TNF-α	Tumor necrosis factor-alpha
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
SD	Standard deviation

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Author contributions
JPY was involved in main and study design. SJP contributed to main and corresponding author. DHK was involved in data analysis and experiment co-worker. SWC contributed to experiment co-worker and data review.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
All animal procedures were approved by the Institutional Animal Care and Use Committee of the author's organization. (Kyungpook National University Institutional Animal Care and Use Committee, IACUC NO: KNU 2023-0110).

Consent for publication
Not applicable.

Competing interests
All authors declare that they have no competing interests.

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