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The Effect of Isolated Hyperglycemia on Native Mechanical and Biologic Shoulder Joint Properties in a Rat Model Hyperglycemia and Shoulder Properties

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Abstract

Recently, diabetes has been linked to rotator cuff disease and adhesive capsulitis, conditions with increased stiffness and inflammation. Unfortunately, limited research exists examining how hyperglycemia affects the native shoulder (tendon and capsule) properties. Therefore, the objectives of this study were to compare shoulder joint mechanics, tendon properties (mechanics and immunohistochemistry), and capsule of healthy control and hyperglycemic rats 8 weeks following induction of hyperglycemia with a submaximal dose of streptozotocin (STZ). Eighteen rats were injected with STZ to induce hyperglycemia or citrate buffer (control) and underwent normal cage activity for 8 weeks. Passive joint mechanics demonstrated significantly less external rotation in the hyperglycemic group compared to controls, with no other group differences. Tendon mechanical properties (stiffness and modulus) were not significantly different between groups at both the insertion site and mid-substance. Immunohistochemistry staining of the tendon and capsule demonstrated significantly increased, IL1-β and AGE staining localized to the insertion and mid-substance of the tendon but not the capsule. In addition, TNF-α staining was significantly increased in the superior capsule but not the supraspinatus tendon. This study demonstrates that isolated hypergylcemia does not diminish shoulder mechanical properties but does induce a chronic inflammatory response.

Keywords

hyperglycemia; rotator cuff; tendon; capsule; rat model

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Introduction

Rotator cuff tendon tears are some of the most common soft tissue injuries that occur in the shoulder, especially in the aging population.[1] It has been shown that 25–50% of asymptomatic patients over the age of 50 have a rotator cuff tear [2; 3] and 60% of symptomatic patients over the age of 60 have a partial or full thickness rotator cuff tears.[4] Another age related condition, type II diabetes mellitus, has been rapidly rising in the general population and is now listed as an epidemic. The CDC estimates that 17.9 million Americans have been diagnosed with diabetes and an additional 5.7 million remain undiagnosed. This disease more commonly occurs in obese individuals due to excessive caloric intake, but the likelihood of developing diabetes increases with age even in non-obese individuals. It is estimated that 23% of individuals over the age of 60 have diabetes; this proportion is expected to rise in the next several years.[5] The associated complications of diabetes are of major concern, and include cardiovascular disease, stroke, nervous system disease, blindness, kidney disease, and decreased tissue healing.[5]

All of the aforementioned complications exhibit alterations of collagen, which have been shown to alter the normal organ function.[6; 7] When hyperglycemia is present, glucose binds to collagen in what is referred to as a Maillard reaction,[7; 8] resulting in the production of advanced glycated end-products (AGE). AGEs are deposited in collagen and are associated with activation of an inflammatory response.[9; 10] In uninjured tissue, hyperglycemia produces a fibrotic response, increasing collagen production and creating stiffened tissue due to an increase in collagen cross-linking.[8; 11]

Recently, diabetes has been linked to rotator cuff disease and adhesive capsulitis [12], conditions with both increased stiffness and inflammation. Unfortunately, limited research exists examining how hyperglycemia affects native shoulder (tendon and capsule) properties. A recent type I diabetic rat model (severe hyperglycemia and complete insulin deficiency) study determined that the native mechanical properties of the patellar tendon were decreased after just 19 days of induced hyperglycemia.[13] Although this study highlighted the mechanical changes that may occur in the presence of uncontrolled type I diabetes, it was not designed to address the altered tissue properties and biologic mechanisms in the shoulder joint caused by isolated hypergylcemia.

Therefore, the objectives of this study were to compare shoulder joint mechanics, tendon properties (using mechanical and immunohistochemical analyses), and capsule properties of healthy control and hyperglycemic rats 8 weeks following induction of hyperglycemia with a submaximal dose of streptozotocin (STZ). We hypothesized that there will be an increase in passive shoulder stiffness and a decrease in shoulder range of motion in the hyperglycemic group. Additionally, we hypothesized there will be an increase in tendon properties (mechanics and immunohistochemistry) and capsule properties for AGE, and inflammatory cytokines interleukin 1-beta (IL-1β) and tumor necrosis factor alpha (TNF-α).

Methods

Study Design

Eighteen adult male Sprague-Dawley rats were divided into a hyperglycemia group (N = 8) and a control group (N = 10). The hyperglycemia group was injected with a maximum of three 30mg/kg doses of STZ dissolved in a citrate buffer every three days until hyperglycemia was present. Hyperglycemia was defined as a fasting blood glucose level of ≥ 200mg/dL and was determined with a glucometer (One Touch Ultra, Lifescan, Milpitas, California). All rats required the three doses to induce hyperglycemia except two rats, which were induced following the second dose. Once hyperglycemia was confirmed, the animals remained in normal cage activity and had free access to water and chow over an 8 week period. During the 8 weeks fasting blood glucose levels were recorded on a weekly basis to confirm the maintenance of hyperglycemia. The control animals were injected with just a citrate buffer and had the same dosing schedule as the hyperglycemia group. All animals were then sacrificed 8 weeks following hyperglycemia induction. For histology (n = 5), right whole shoulders were immediately fixed in formalin. The left shoulder of all animals (n = 18) were designated for mechanical testing and stored intact at −20° C. Fasting serum insulin levels were measured by enzyme-linked immunosorbent assay (ELISA), and animals were excluded if the values were less than 70% from control.

Passive Joint Mechanics

Passive shoulder joint range of motion (ROM) and stiffness were measured at 8 weeks following induction of hyperglycemia using a custom instrument and methodology.[14] Briefly, under anesthesia, the forearm was placed through a fixture and secured into a rotating clamp at 90° of elbow flexion and 90° of glenohumeral forward flexion. The scapula was manually stabilized in order to isolate glenohumeral motion. The arm was then rotated through the full range of internal rotation (IR) and external rotation (ER) three times. The range of IR and ER was determined using data from all three cycles. A bilinear fit utilizing least-squares optimization was applied to calculate joint stiffness in the linear region for both IR and ER.

Tendon Mechanical Testing

The animals were thawed, and the scapula and humerus were dissected with the supraspinatus tendon intact. Tendon testing was performed as previously described.[15] Briefly, stain lines for local optical strain measurement (at insertion and midsubstance) were placed on the supraspinatus tendon. Cross-sectional area was measured using a custom laser device. The humerus was embedded in a holding fixture using polymethylmethacrylate, gripped with cyanoacrylate annealed sand paper, and immersed in phosphate-buffered saline (PBS) at 37° C. Tensile testing was performed as follows: preload to 0.08 N, preconditioning (10 cycles of 0.1–0.5 N at a rate of 1% strain/second), stress relaxation to 5% strain at a rate of 5% strain/second for 600 seconds, and ramp to failure at 0.3% strain/second. Stress was calculated as force divided by initial area, and two-dimensional Lagrangian optical strain was determined from stain line displacements using texture tracking software.

Histology

For histology, whole shoulders were left completely intact. All samples were processed, longitudinal sections (7 μm) were collected, and sections were stained with hematoxylin and eosin (H&E) (Fig. 1). H&E stained sections were imaged at the insertion site and midsubstance of each supraspinatus tendon at x200 magnification. In addition, the mid-substance of the superior capsule was imaged. Cell density (number of cells/mm^2) and cell shape (aspect ratio; 0–1, with 1 being a circle) were quantified using a bioquantification software system (Bioquant Osteo II; BIOQUANT Image Analysis Corp, Nashville, TN, USA).

Immunohistochemistry

Immunohistochemistry was performed on the whole shoulder sections following established protocols.[16] Proteins of interest were selected based on previous diabetes research in other organ systems. Briefly, following deparaffinization and rehydration, sections were blocked for endogenous peroxidase which included incubation in 3% H_2O_2 in methanol (48°C) for 30 min. Sections were then washed, incubated in 2% dried milk in PBS (Blotto) for 20 min, and incubated overnight at room temperature with an IL1-β antibody (#AB1832; Chemicon, Temecula, CA; 1: 250 dilution with PBS). After washing, sections were incubated for 2 h at room temperature with goat anti-rabbit peroxidase-conjugated (HRP) secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 with PBS. HRP was visualized as a brown immunoreactive stain using diaminobenzidene (DAB) (Sigma-Aldrich, St. Louis, MO). For TNF-α, sections were incubated in 4% dried milk in PBS (Blotto) for 20 min, and incubated overnight at room temperature with a TNF-α antibody (#NBP1-19532; Novus Biologics, Littleton, CO; 1: 750 dilution with PBS). After washing, sections were incubated for 2 h at room temperature with goat anti-rabbit peroxidase-conjugated (HRP) secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 with PBS. For AGE sections were incubated in 10% goat serum dilution in PBS (Jackson ImmunoResearch, West Grove, PA) for 20 min, and incubated overnight at room temperature with a AGE antibody (#ab23722; Abcam, Cambridge, England; 1: 1000 dilution with PBS). After washing, sections were incubated for 2 h at room temperature with goat anti-rabbit peroxidase-conjugated (HRP) secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 with PBS. Slides were not counterstained. Sections were dehydrated and coverslipped with DPX mounting medium. Negative control slides included omission of the primary antibody. Imaging of immunostained sections followed the same protocol as sections stained with H&E. Stain density (number of stained pixels/total pixels) was quantified using the bioquantification software system.

Statistics

Joint and tendon mechanics and histology (tendon and capsule) were assessed using a one-tailed t-test. Significance was set at $p<0.05$. The statistical program SPSS for Windows (Version 20.0; IBM Corp, Armonk, NY, USA) was used for data analysis.

Results

The hyperglycemic group had a significantly higher fasting blood glucose ($p = 0.0001$) level compared to controls ($p = 0.0001$; Fig. 2A); the fasting serum insulin levels were not significantly different ($p = 0.13$; Fig 2B), which confirms our animal model. Passive joint mechanics (Fig. 3) demonstrated significantly less ER ROM in the hyperglycemic group ($p = 0.03$) compared to controls, with no other group differences. Tendon mechanics (stiffness and modulus) and cross sectional area were not significantly different between groups at both the insertion site and mid-substance (Fig. 4). For histology, cell shape was not significantly different between groups at the insertion site and mid-substance of the tendon or the superior capsule (Fig. 5). Cell density also was not significantly different between groups at the insertion site or superior capsule; however the hyperglycemic group had a greater cell density at the mid-substance of the tendon ($p = 0.003$) compared to the control group (Table 1). Immunohistochemistry staining of the tendon and capsule (Table 2) demonstrated significantly increased IL1-β and AGE staining localized to the insertion (IL1-β, $p = 0.03$; AGE, $p = 0.02$) and mid-substance (IL1-β, $p = 0.005$; AGE, $p = 0.01$) of the hyperglycemic tendon but not the capsule (Fig 5). In addition, TNF-α staining was significantly increased in the superior capsule ($p = 0.02$) of the hyperglycemic group but not in the supraspinatus tendon.

Discussion

While the relation of diabetes to cardiovascular disease has been well studied [17; 18], there is limited information with respect to rotator cuff disease [19] and adhesive capsulitis.[20] Our results demonstrate that 8 weeks of isolated hyperglycemia, defined as hyperglycemia and partial insulin deficiency, leads to a decrease in ER ROM but no other mechanical changes in the native uninjured joint or supraspinatus tendon. However, there was a significant biologic response with elevated levels of inflammatory markers (IL1-β and TNF-α) and AGE. Joint stiffness and ROM results demonstrated a decrease in ER ROM with no other group differences. Although the currently observed decrease in ER ROM is relatively small, adhesive capsulitis patients also present with a loss of ER ROM, which has recently been suggested to be associated with type II diabetes.[20; 21] In fact, patients with adhesive capsulitis demonstrate an increased expression of inflammatory markers within the capsule,[22] which is similar to our immunohistochemistry result that found an increase in TNF-α in the superior region of the capsule. These results provide evidence that hyperglycemia causes both mechanical and biologic responses in the native joint capsule.

We found no group differences for the mechanical properties (stiffness and modulus) of the supraspinatus tendon. These results were surprising and do not support our original hypothesis. We hypothesized that hyperglycemic tendons would have an increased stiffness and modulus, potentially due to collagen crosslinking and increased fibrosis caused by chronic inflammation. However, previous studies that demonstrated increased tissue stiffness were *in vitro* studies of tendon cultured in a hyperglycemic medium.[11; 23] The *in vivo* environment is much more complex with many mechanical and biologic interactions, which may account for the maintenance of the mechanical properties. In addition, longer time points may be required to observe alterations in tendon mechanical properties.

Therefore, the lack of mechanical group differences is surprising and may have important implications, which should be further investigated.

Tendon histology results demonstrated an increased cell density at the mid-substance of the supraspinatus tendon in the hyperglycemic group, however there were no other group differences for cell density or cell shape. The increase in cell density at the mid-substance of the supraspinatus tendon indicates an increase in metabolic activity of the tendon. This finding is similar to previous studies examining tendon injury, such as tendinopathy.[24; 25] Although the tendon was not mechanically injured in this study, the animals were induced with hyperglycemia, which is thought to cause a biologic cascade of tissue damage due to an increase in reactive oxygen species and inflammatory cytokine production.[26; 27] Our results are consistent with this hypothesis since the mid-substance of the tendon is mainly composed of type I collagen, which would be expected to elicit a strong biologic response to hyperglycemia.

We performed immunohistochemistry to identify the specific cellular response of the supraspinatus tendon and joint capsule. We found that the hyperglycemic group displayed increased staining of IL1-β and AGE within both the insertion site and mid-substance of the supraspinatus tendon. This further supports our original hypothesis that hyperglycemia would cause increased expression of AGE and inflammatory factors. Cardiovascular research has also demonstrated similar findings with increased expression of both AGE and several inflammatory markers within the arterial endothelium in a hyperglycemic state.[17; 28] It has been shown that AGE is a byproduct of the Maillard reaction between glucose and collagen.[7] Once AGE accumulates within the tissue, it is thought to initiate a cascade of inflammatory signals, thereby causing chronic inflammation localized within collagen based tissues.[27; 29] Although there was a significant increase in IL1-β and AGE within the tendon, these biologic changes did not affect the mechanical properties at 8 weeks following induction of hyperglycemia. Longer exposures to inflammatory cytokines and other inflammatory mediators may be needed to have an effect on the tendon mechanical properties.

Immunohistochemistry results identified an increased staining within the superior aspect of the joint capsule. Recently there has been more scientific evidence supporting the association between diabetes and adhesive capsulitis.[30; 31] Adhesive capsulitis is a condition that has similar characteristics as those commonly described in cardiac vessel endothelium in diabetics such as an increased inflammation and tissue stiffness. In fact, adhesive capsulitis has three stages, including an initial frozen stage that is defined as severe joint stiffness and limited range of motion.[32] Others have also identified an increased expression of several inflammatory markers within the capsule of adhesive capsulitis patients undergoing arthroscopic capsule release.[22] Therefore, our results are similar to those found in adhesive capsulitis patients and identify that hyperglycemia may be involved in the development of disease.

One limitation that must be acknowledged is the use of rat rotator cuff model. A well-established rat rotator cuff model was used in this study, and although the rat shoulder has similar anatomy, the use of a quadruped animal does not precisely replicate the human

shoulder.[33] However, due to the large number of potentially confounding variables associated with clinical research, it is often difficult to determine direct cause and effect relationships supporting the use of an animal model.

In conclusion, 8 weeks of hyperglycemia in an animal model led to decreased ER ROM, no changes in tendon mechanical properties, and an increase in cell density at the mid-substance of the supraspinatus tendon. In addition, immunohistochemistry identified increased staining of IL1-β and AGE at the insertion and mid-substance of the supraspinatus tendon, and TNF-α in the superior joint capsule. Interestingly, these results indicate that hyperglycemia initiates a biological response in native uninjured tendon and capsule without mechanical consequences. In addition, the elevated presence of inflammation in native tissue may have detrimental effects to tissue healing. This provides clinicians with basic science evidence that hyperglycemia is pro-inflammatory and should be considered when treating patients. Future studies will examine additional inflammatory markers, microstructural analysis of the collagen (collagen fiber diameter, organization, and cross linking), and begin to investigate the effect of hyperglycemia on tendon healing.

Acknowledgements

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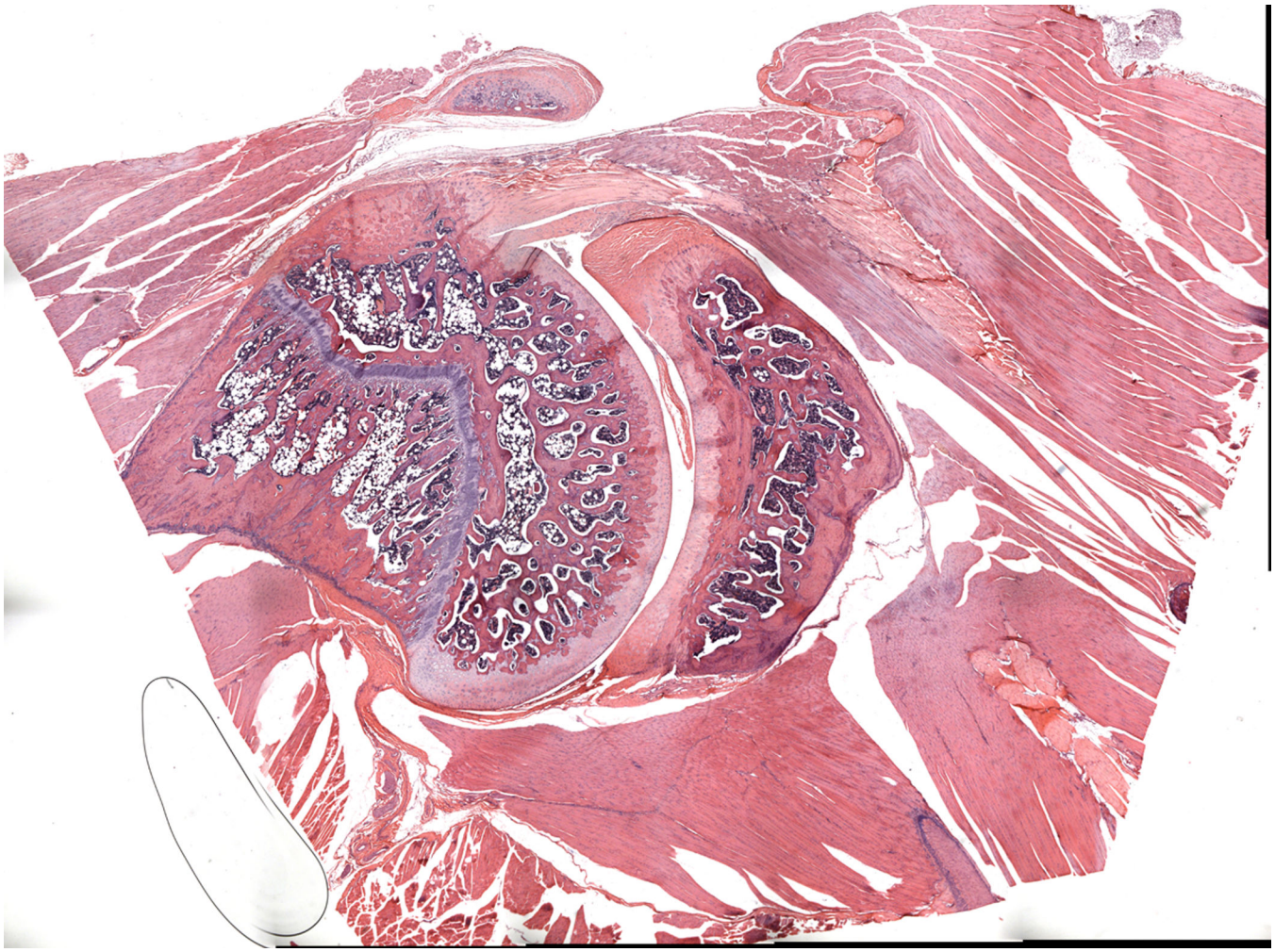
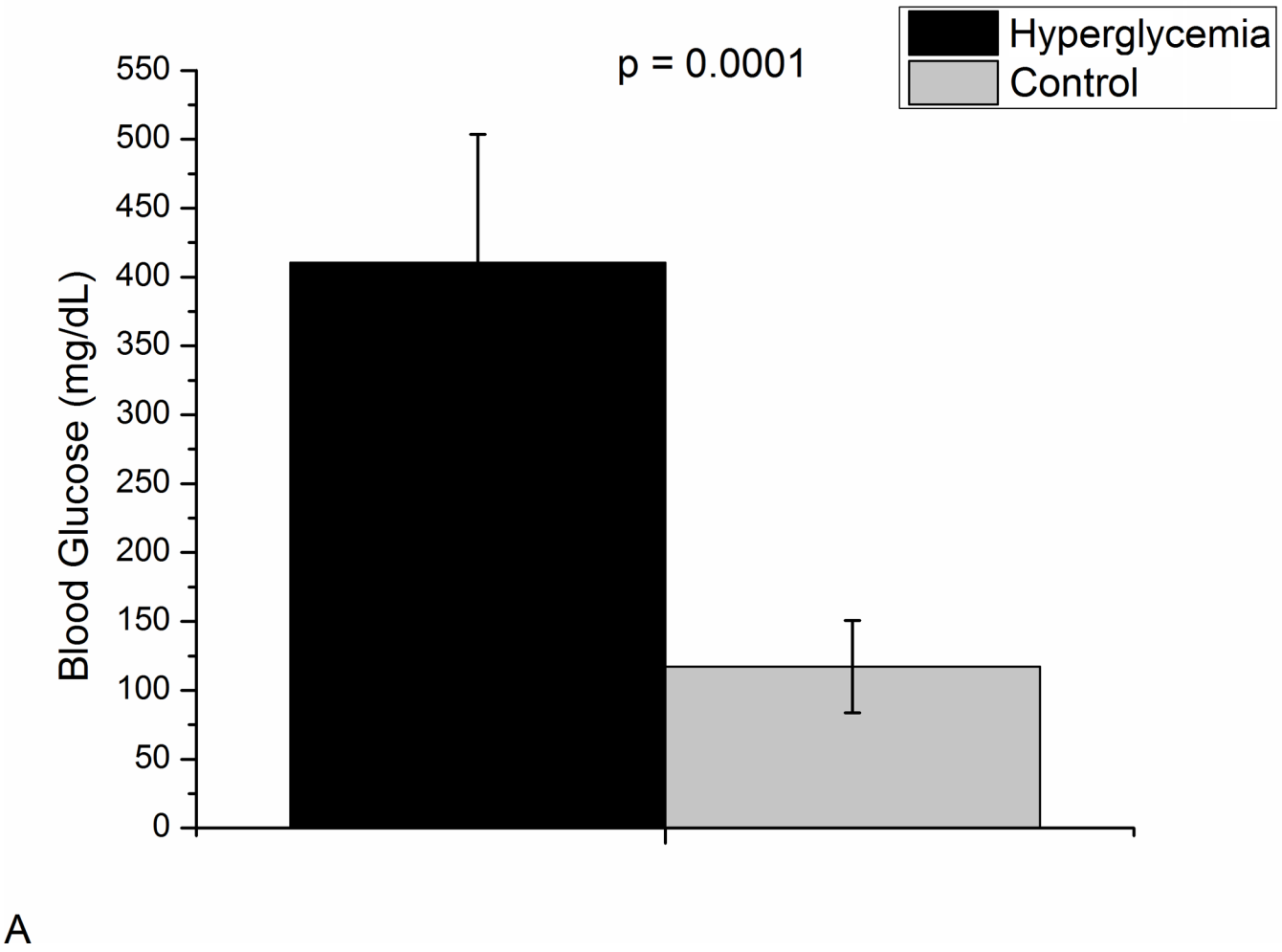


Figure 1.
A representative histology image of the whole shoulder stained with H&E.



A

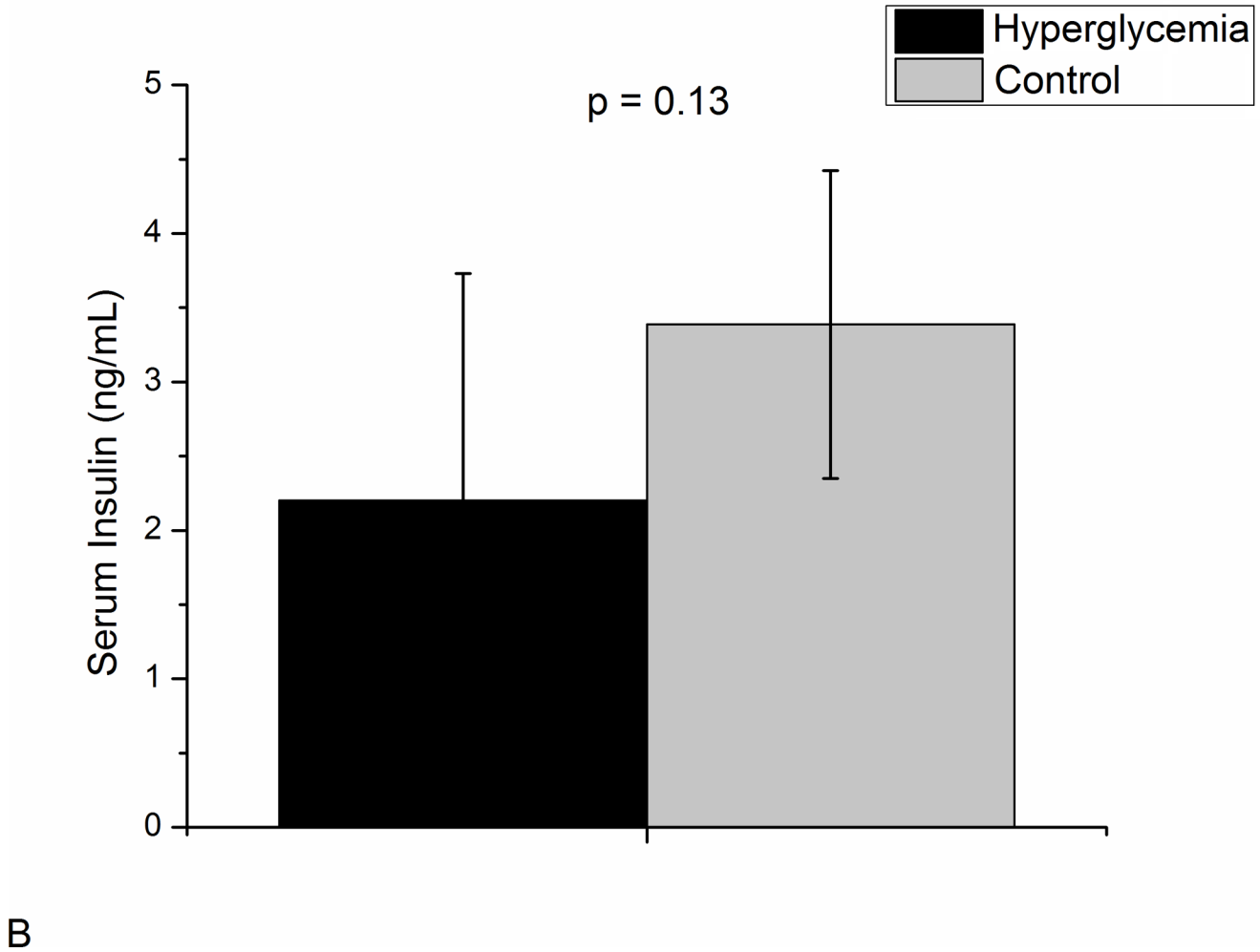
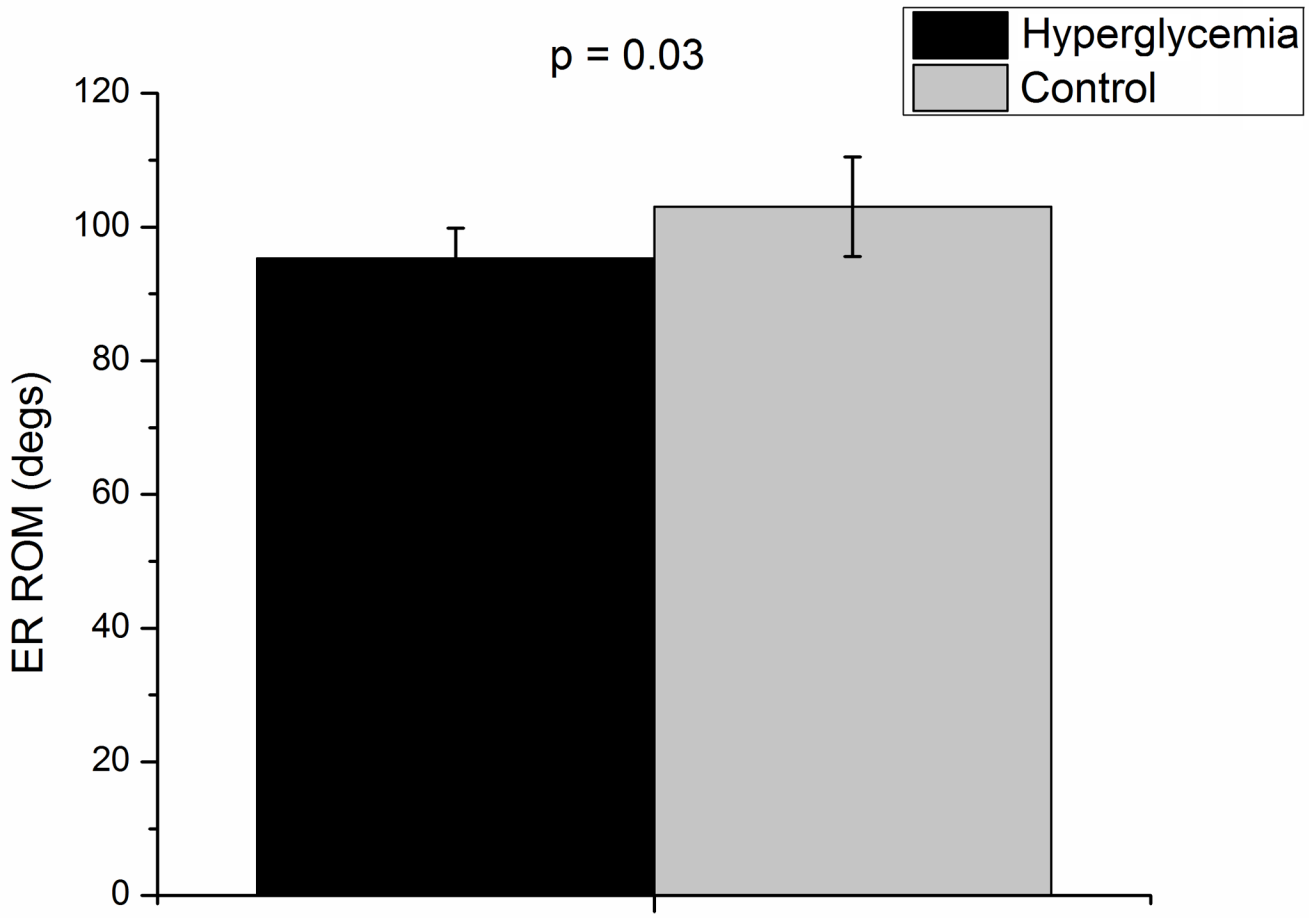


Figure 2.

(A) Fasting blood glucose and (B) fasting plasma insulin levels for the hyperglycemic and control groups at 8 weeks post-induction. Results demonstrated significant difference in blood glucose but not plasma insulin.



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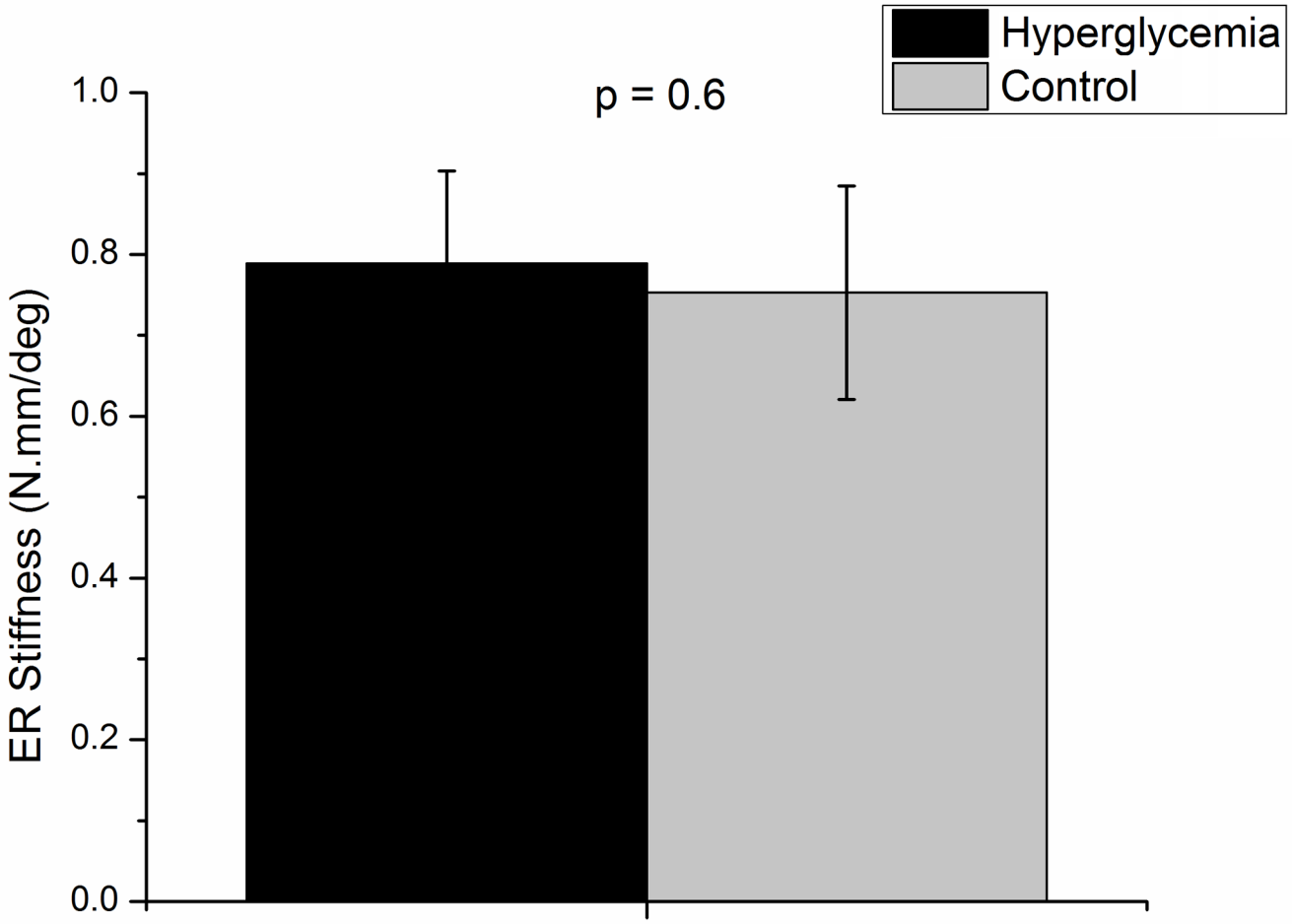

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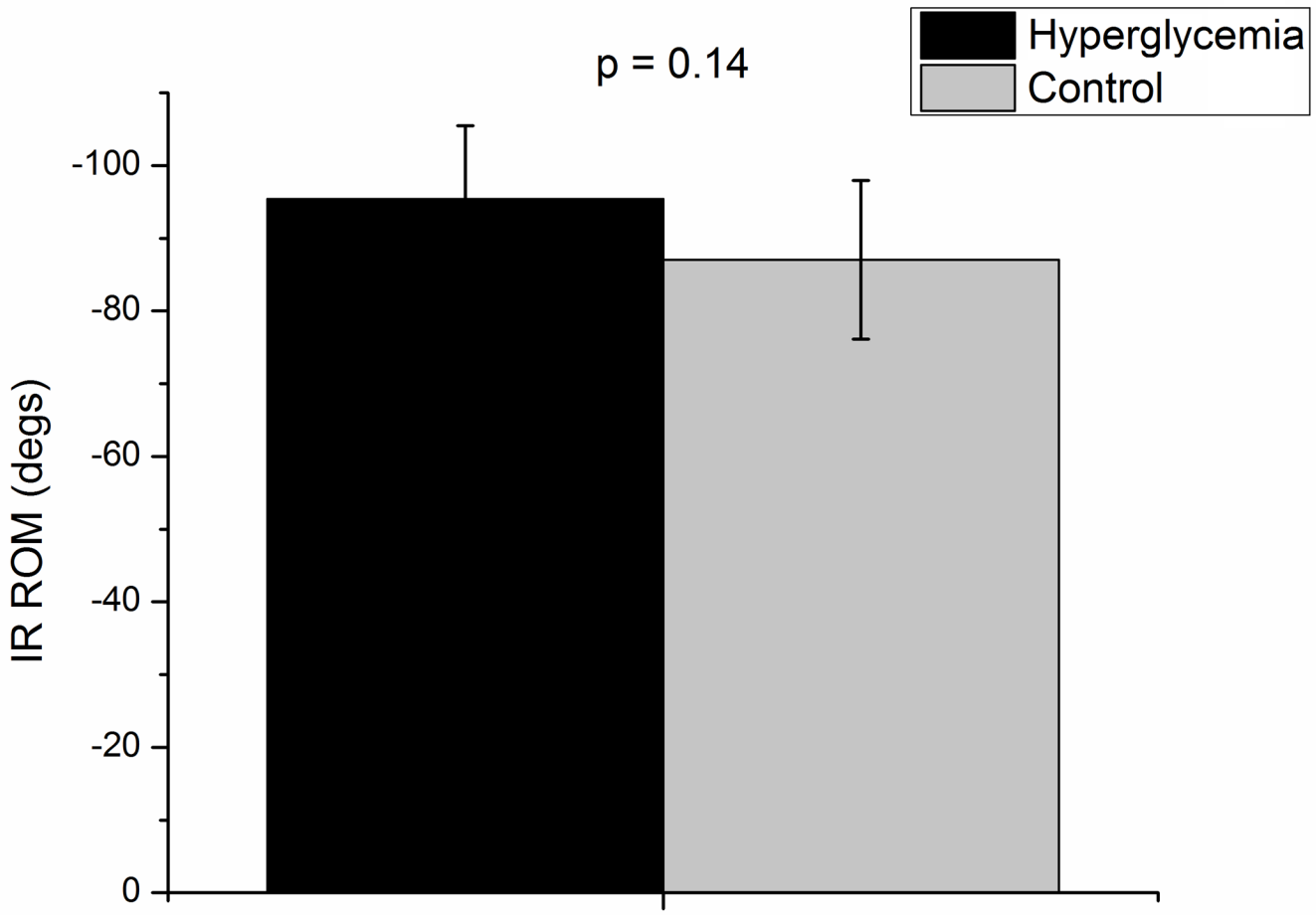

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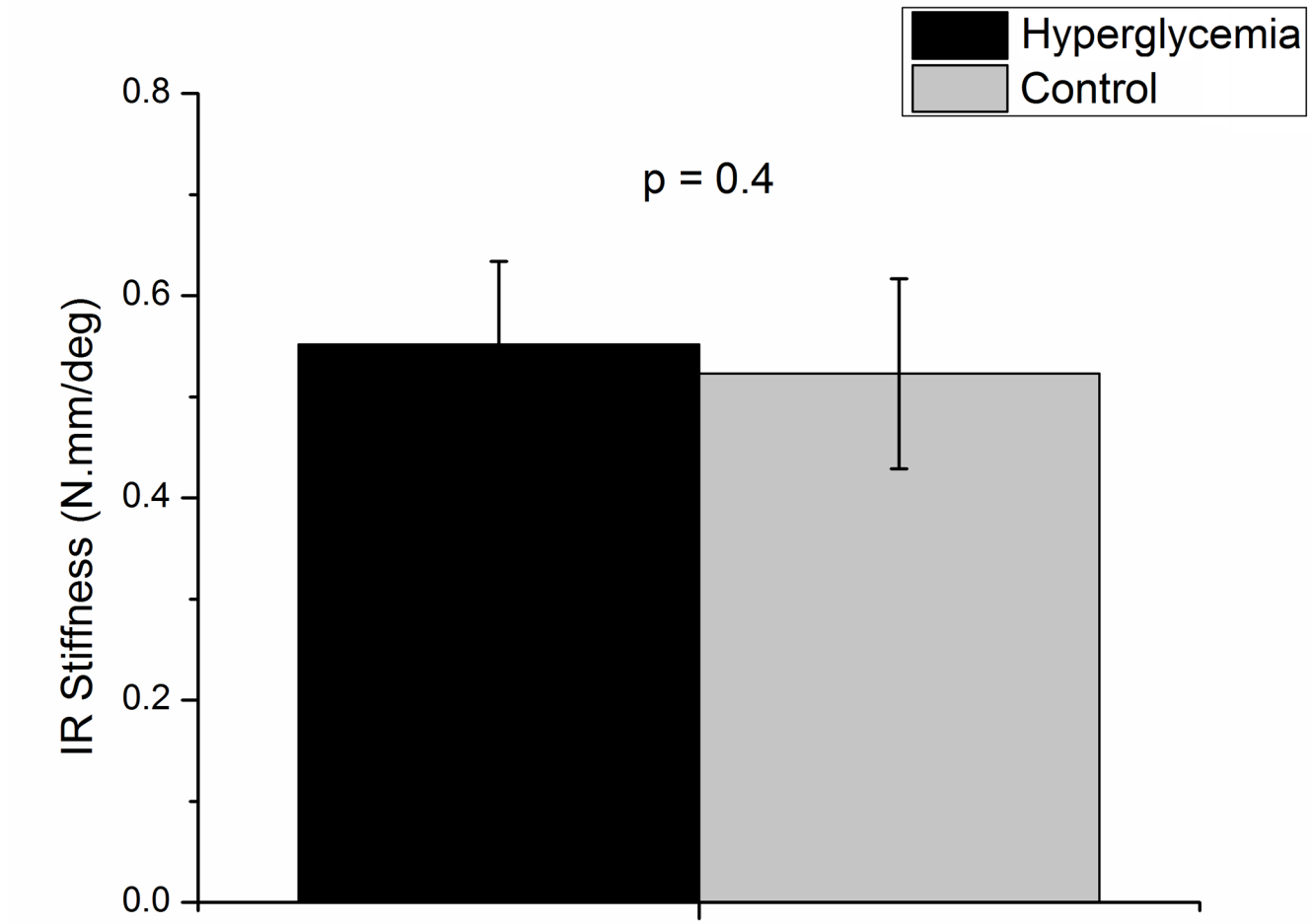
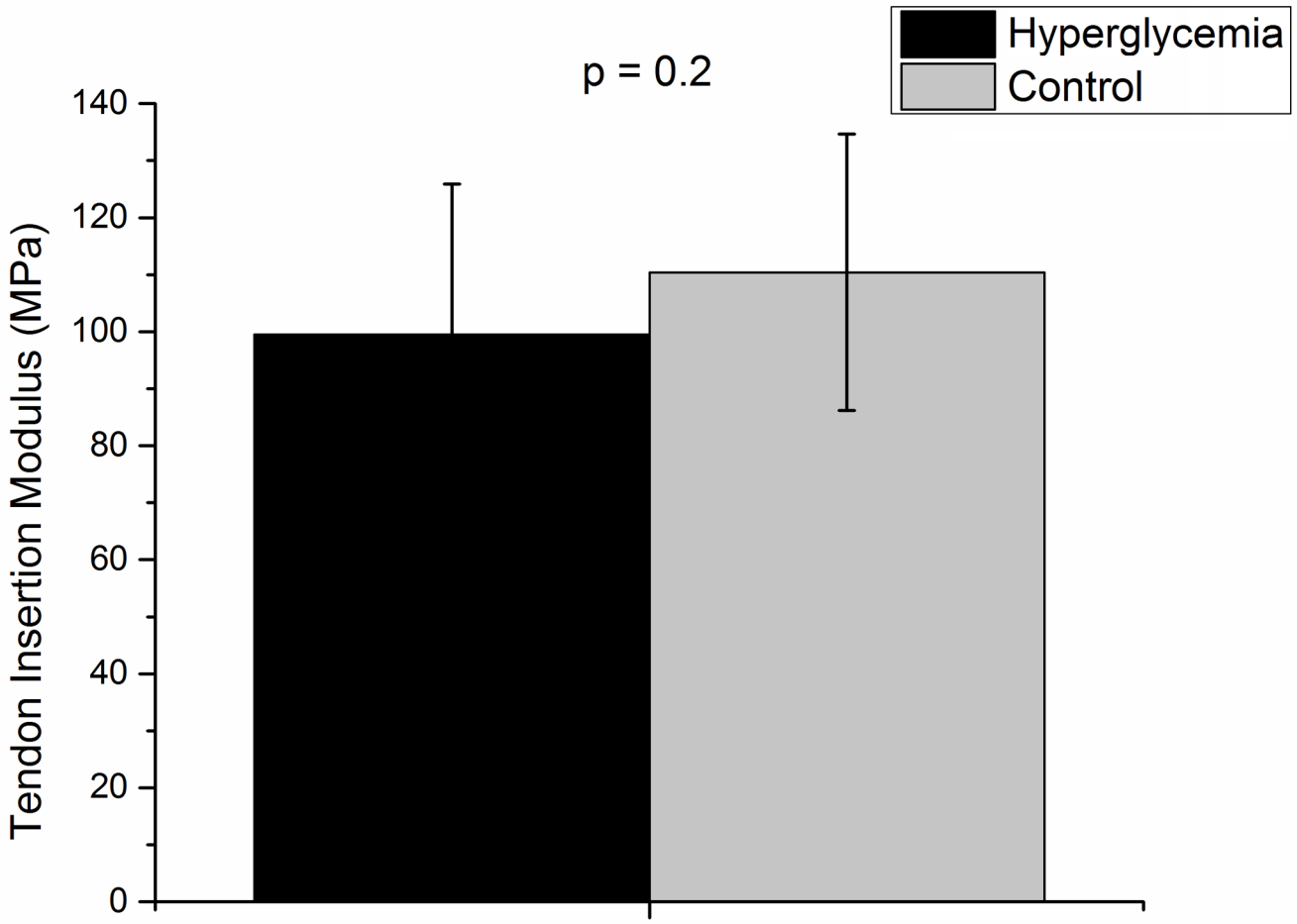


Figure 3.

(A) ER ROM (B) ER stiffness (C) IR ROM (D) IR stiffness for the hyperglycemic and control groups. Results demonstrated a significant decrease in ER ROM for the hyperglycemic group compared to the control group.



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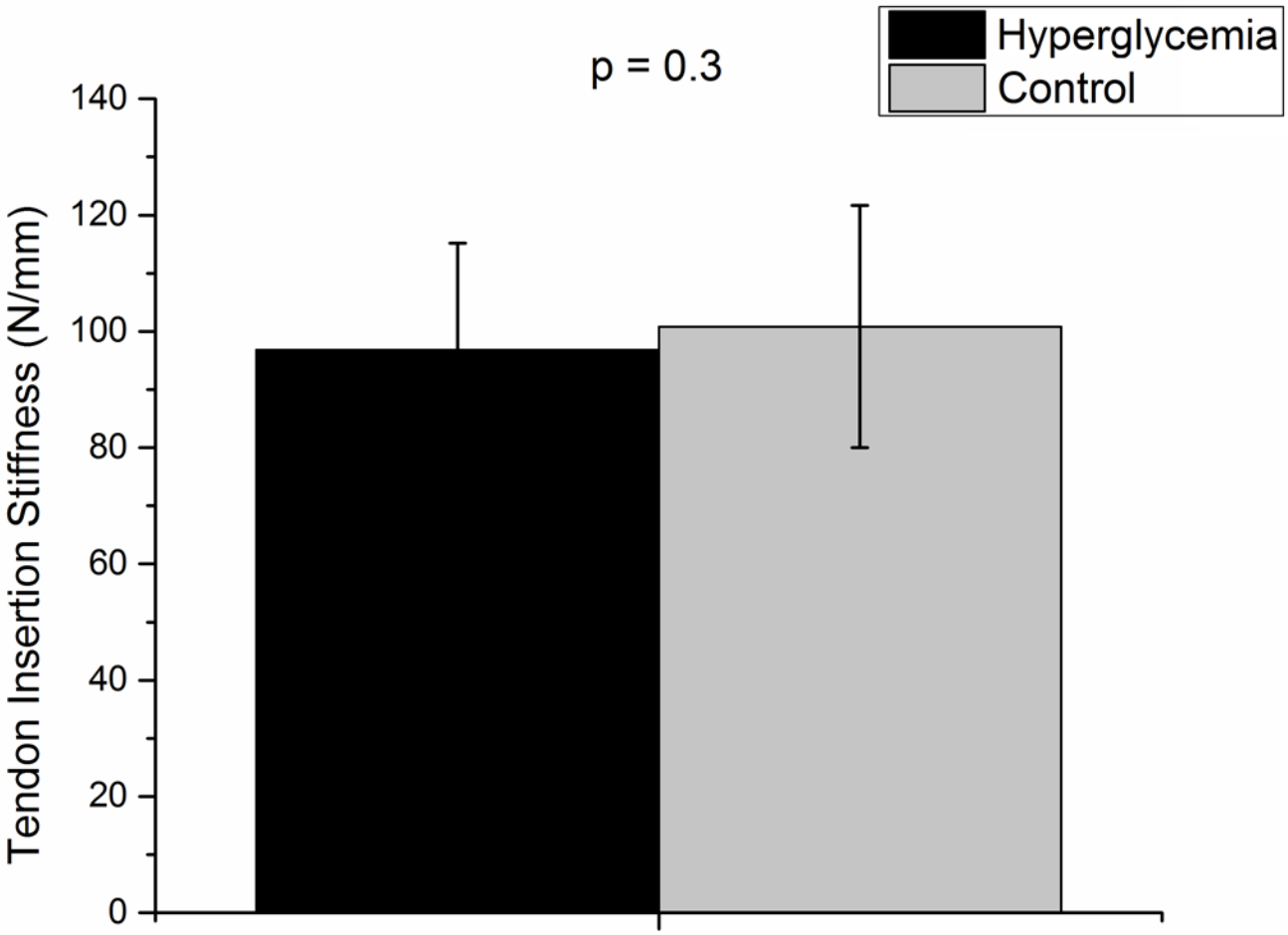

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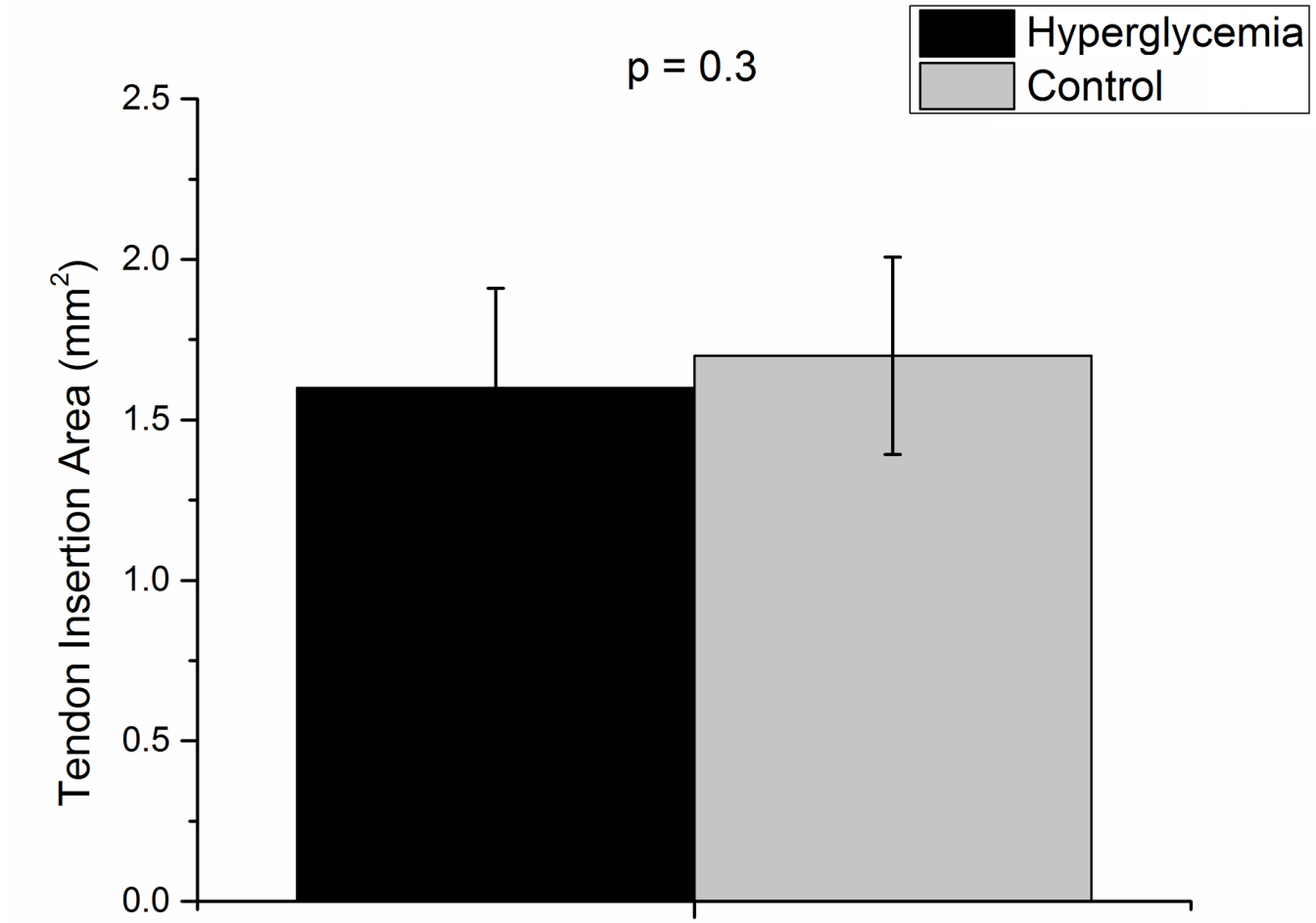

Figure 4.

(A) Tendon insertion site modulus (B) tendon insertion site stiffness (C) tendon insertion site cross sectional area for the hyperglycemic group and control group. Results demonstrated that there were no significant differences between the groups for any mechanical properties.

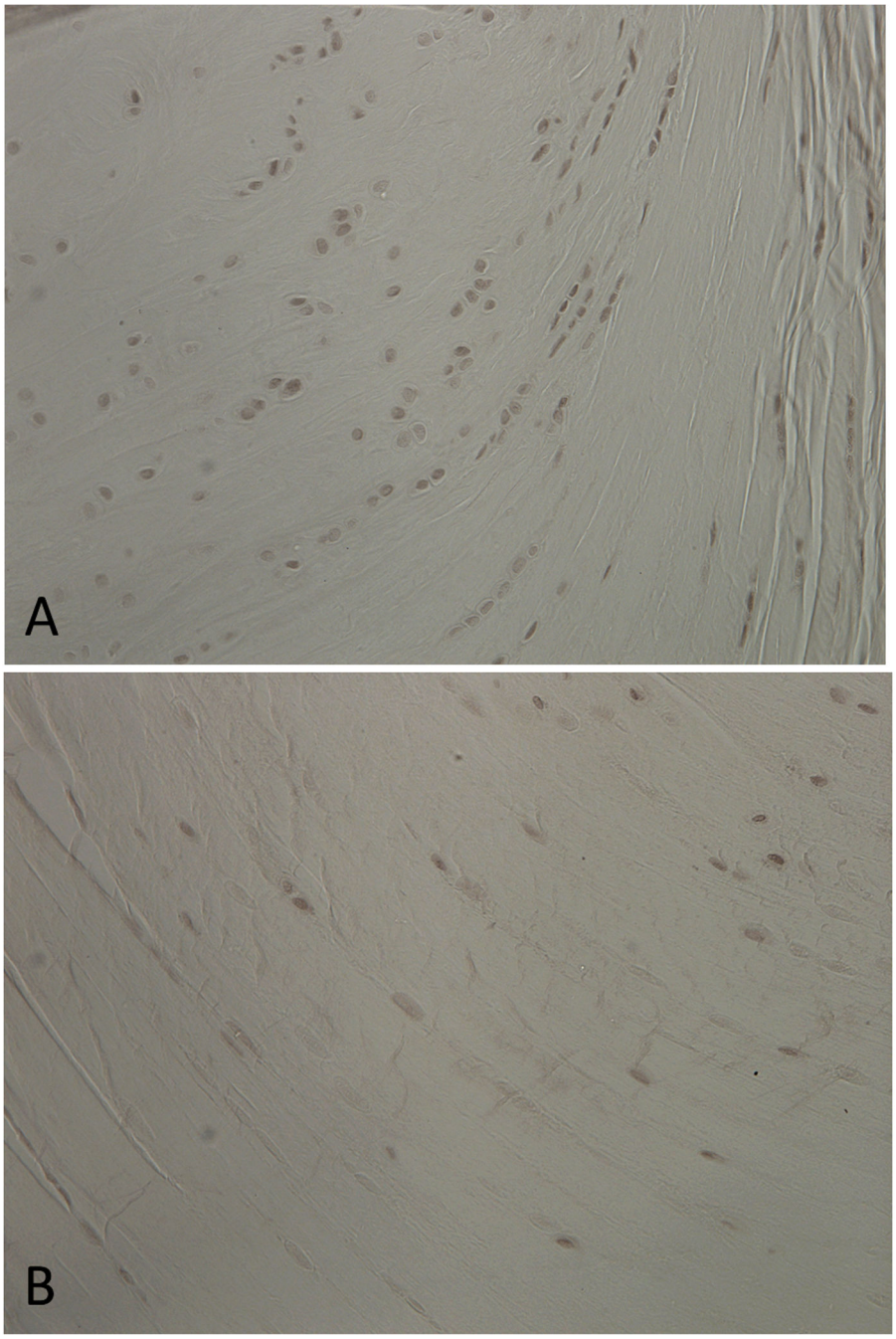

Figure 5.

Immunohistochemistry staining density for IL1-β, AGE, & TNF-α was quantified at the insertion and midsubstance of the supraspinatus and superior capsule. A representative image for IL1-β at the supraspinatus insertion is displayed. IL1-β staining density was significantly increased ($p<0.05$) in the hyperglycemic group (A) compared to the control group (B).

Table 1

Histology data

Tissue	Group	Region	Cell Density (cells/mm^2)	p-value	Cell Shape (aspect ratio)	p-value
Supraspinatus tendon	Hyperglycemia	Insertion	260.96 ± 81.93	0.4	0.72 ± 0.03	0.6
	Control		218.61 ± 63.5		0.72 ± 0.03	
	Hyperglycemia	Mid-substance	425.78 ± 47.57	0.003*	0.4 ± 0.02	0.13
	Control		268.44 ± 37.09		0.49 ± 0.14	
Superior capsule	Hyperglycemia	Mid-substance	1016.0 ± 291.56	0.2	0.61 ± 0.07	0.3
	Control		819.02 ± 66.56		0.58 ± 0.02	

Table 2

Immunohistochemistry data

Tissue	Group	Region	IL1-β Staining Density (%)	p-value	AGE Staining Density (%)	p-value	TNF-α Staining Density (%)	p-value
Supraspinatus tendon	Hyperglycemia	Insertion	1.78 ± 1.29	0.03*	0.57 ± 0.41	0.02*	2.7 ± 1.44	0.5
	Control		0.51 ± 0.4		0.06 ± 0.05		2.74 ± 1.88	
	Hyperglycemia	Mid-substance	3.34 ± 1.54	0.005*	2.37 ± 0.95	0.01*	1.81 ± 1.05	0.3
	Control		0.61 ± 0.34		0.62 ± 0.51		1.31 ± 1.1	
Superior capsule	Hyperglycemia	Mid-substance	4.41 ± 1.6	0.4	1.18 ± 0.83	0.4	6.69 ± 3.65	0.02*
	Control		4.17 ± 1.89		1.01 ± 0.65		2.51 ± 1.0	