Quantitative and qualitative change of collagen of Achilles tendons in rats with systemic administration of glucocorticoids

ABSTRACT

Background

It is unclear whether glucocorticoid (GC) therapy is directly related to Achilles tendon rupture (ATR), since many of the reported patients were receiving long-term GC therapy for underlying diseases. This study aimed to elucidate the mechanism by which systemic GC administration causes weakening of the Achilles tendon by biochemically, mechanically and morphologically evaluating quantitative and qualitative changes in collagen.

Methods

Male 8-week-old mice were subcutaneously treated with either prednisolone (10 mg/ml/kg; GC group) or saline (1 ml/kg; control group) for 8 weeks and then subjected to the following experiments: 1) a tensile strength test; 2) quantification of the gene expressions of type 1 collagen and lysyl oxidase; 3) quantification of collagen content, enzymatic crosslinks (immature + mature) and senescent crosslinks; and 4) measurement of collagen fiber diameter by electron microscopy.

Results

The maximum tensile load and gene expressions of type 1 collagen and lysyl oxidase were decreased in the GC group. Collagen content was significantly decreased in the GC group compared with the control group. The content of enzymatic crosslinks was significantly lower in the GC group than in the control group. The corresponding amount of senescent crosslinks was not significantly different. The mean collagen fiber diameter was significantly smaller in the GC group than in the control group. Histogram analysis showed a decreased number of thick fibers and an increased number of thin fibers in the GC group.

Conclusion

These observations suggest that systemic GC administration causes decreased strength of the Achilles tendon by decreasing its collagen content, hindering the formation of enzymatic crosslinks and thereby keeping collagen fibers in an immature state with smaller diameters.

Clinical relevance

Systemic GC administration directly prevents maturation of tendon collagen fibers and decreases tendon strength, regardless of the presence or absence of underlying disease.

LEVEL OF EVIDENCE

Level III

KEYWORDS

Glucocorticoid; General Administration; Rat; Collagen; Crosslink; Tensile Strength Test; Electron Microscope

INTRODUCTION

It is generally understood that GC causes weakening of tendons as there have been several case reports of subcutaneous Achilles tendon rupture (ATR) in patients receiving long-term treatment with glucocorticoid (GC) for underlying diseases, such as chronic obstructive pulmonary disease, rheumatoid arthritis, systemic lupus erythematosus and chronic renal failure.[4,7,8,12,14,18] However, it is also possible that these underlying diseases have negative effects on tendons. The mechanism by which GC administration causes weakening of the Achilles tendon remains to be fully elucidated.

The strength of a tendon is critically determined by its main component, collagen. The tendon is composed of collagen, elastin, proteoglycan, water and other components. Collagen accounts for approximately 65% to 80% of the dry weight of a tendon and its amount partially determines the strength of a tendon. We have previously demonstrated that the structure of intermolecular crosslinking of neighboring collagen molecules is associated with the quality of collagen fibers and also serves as a determinant of tendon strength.[21] The purpose of this study was to elucidate the mechanism by which systemic GC administration causes weakening of the Achilles tendon by biochemically, mechanically and morphologically evaluating the quantitative and qualitative changes of collagen.

MATERIALS AND METHODS

All experiments were approved by the animal ethics committee of our institution and carried out in accordance with institutional guidelines for laboratory animal handling. A total of 26 male, 8-week-old, SPF Wistar-Imamichi rats obtained from the Institute for Animal Reproduction were stratified by body weight and then randomized into two groups (13 animals per group). The animals randomized to receive steroid (GC group) were treated with a subcutaneous injection of prednisolone (Kawasaki Pharmaceuticals, Kanagawa, Japan) to the back at a dose of 10 mg/ml/kg

once daily for 8 weeks. The animals in the control group were given saline (Otsuka Pharmaceuticals, Tokyo, Japan) at a dose of 1 ml/kg. After completion of the course of treatment, all animals were euthanized under inhalation anesthesia with isoflurane. The right and left Achilles tendons were collected from ten animals in each group and used for tensile strength testing and collagen analysis, respectively. Achilles tendons collected from three animals in each group were used for electron microscopy.

Tensile strength test

Each specimen of the Achilles tendon was collected *en bloc* including the muscle-tendon junction and the calcaneus. A tensile testing machine (SV-52N, Yasui Kikai Corporation, Osaka, Japan) was used to fix the specimen by placing the calcaneus in a vise and lifting the tendon upward, and a tensile force at a cross-head speed of 0.5 mm/min was applied until the tendon was ruptured to determine the maximum tensile load.

Real-time RT-PCR

RNA samples were extracted and subjected to RT-PCR for analyzing intracellular gene expressions. After the cytoplasmic layer was collected, total RNA was extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen Inc., Hilden, Germany) and first-strand cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Life Technologies Corp., Carlsbad, CA). Real-time PCR was performed using probe-primer sets specific to rat Col1A1 and LOX, respectively, and data were analyzed with StepOne^{TM} Real-Time PCR System (Applied Biosystems). The expressions of each gene were quantified using housekeeping gene GAPDH as an internal standard and corrected for sample volume.

Collagen analysis

Measurement of collagen crosslinks in tendons was carried out as previously

described.[13,19] Briefly, tendon specimens were frozen and pulverized in liquid nitrogen. Specimens were suspended in potassium phosphate buffer (pH 7.6) and reduced at 37 °C with sodium borohydride ($NaBH_4$; Sigma-Aldrich, St Louis, MO). The specimens were hydrolyzed in 6 N HCl at 110 °C for 24 hours. Hydrolysates were analyzed for crosslinks and hydroxyproline levels on a Shimadzu LC9 HPLC fitted with a cation exchange column (0.9 × 10 cm, Aa pack-Na; JASCO, Ltd., Tokyo, Japan). It was assumed that collagen weighed 7.5 times the measured weight of hydroxyproline, with a molecular weight of 300,000 Da.[19] The resulting data were used to calculate crosslink values as mol/mol of collagen. We determined the levels of enzymatic immature reducible and mature non-reducible pyridinium crosslinks such as pyridinoline (Pyr) and nonenzymatic senescent crosslinking pentosidine, which is a well characterized advanced glycation end product (AGE). The immature crosslinks (dihydroxylysinonorleucine [deH-DHLNL] and hydroxylysinonorleucine [deH-HLNL]) were identified and quantified according to their reduced forms (DHLNL and HLNL, respectively). Immature crosslinks and hydroxyproline were detected with O-phthalaldehyde derivatization, whereas Pyr and pentosidine were detected by natural fluorescence. Our established HPLC system enabled us to determine enzymatic and non-enzymatic crosslink concentrations within a linear range from 0.2 to 600 pmol in specimens.

Electron microscopy

The body of the tendon was examined. Tissue was fixed in 2% glutaraldehyde solution (pH 7.4, diluted in 0.1 M phosphate buffer) and then in 1% osmium tetroxide solution (pH 7.4, diluted in 0.1 M phosphate buffer). The fixed tissue was subjected to step-wise dehydration with ethanol, embedded in epoxy resin, cut into very thin slices, and stained with tannic acid, uranyl acetate, and lead citrate. Under a transmission electron microscope (Hitachi H-7500, Hitachi, Tokyo, Japan), 40,000-fold magnified images were taken to determine collagen fiber diameter,

collagen fiber density (number of collagen fibrils per square micrometer), and the percentage of tendon cross-sectional area occupied by collagen fibers.

Statistical analysis

For statistical analysis, significant differences were assessed using Student's t-test, with a significance level of $p < .05$. The distributions of histograms of both groups were compared using a two-sample Kolmogorov-Smirnov test.

RESULTS

Tensile strength test

The site of the Achilles tendon rupture was the body of the tendon. The maximum tensile load was lower in the GC group than in the control group (48.1 ± 6.6 vs. 55.4 ± 7.2 N, $p = .034$; Figure 1).

Real-time RT-PCR

The gene expression of type 1 collagen in the GC group was decreased by 99% compared with the control group (Figure 2). In the GC group, the gene expression of lysyl oxidase, an inducible enzyme for enzymatic crosslinks, was also decreased by 83% compared with the control group (Figure 3).

Collagen analysis

Collagen content was lower in the GC group than the control group (82.8 ± 4.2% vs. 87.3 ± 4.6% of dry tissue weight, $p = .033$; Figure 4). The content of total immature crosslinks (DHLNL+ HLNL) in the corresponding groups was 0.389 ± 0.129 and 0.595 ± 0.215 (mol/mol of collagen), being significantly lower in the GC group ($p = .018$). The content of mature crosslinks (Pyr) was significantly lower in the GC group (.069 ± .021 vs. .102 ± .036 mol/mol of collagen, $p = .021$; Table 1). The content of total enzymatic crosslinks (immature + mature) was also significantly lower in the GC group (0.458 ± 0.137 vs. 0.697 ± 0.225 mol/mol of collagen, $p = .01$; Figure 5). The content of pentosidine, a nonenzymatic crosslink, was 1.481 ± 0.998

and 1.783 ± 0.879 (mol/mol of collagen), showing no significant difference between the groups with the numbers available (p = .481; Figure 6).

Electron microscopy

The mean collagen fiber diameter was significantly smaller in the GC group (147.4 ± 15.1 vs. 169.7 ± 19.6 mm, p = .002). The mean percentage of tendon cross-sectional area occupied by collagen fibers in the GC and control groups was 77.0 ± 3.0% and 80.5 ± 1.8% (p < .001). The mean collagen fiber density was significantly higher in the GC group (43.2 ± 8.4% vs. 33.1 ± 1.8%, p = .002; Figure 7 and Table 2).

The histograms of collagen fiber diameter showed a decreased number of thick fibers (defined as a diameter of less than 260 nm) and an increased number of thin fibers (defined as a diameter of 100 to 240 nm) in the GC group compared with the control group (p < .0001; Figure 8).

DISCUSSION

GC is known to have anti-inflammatory, immunosuppressive and anti-allergic actions and has demonstrated efficacy in the treatment of various autoimmune and intractable diseases.[23] At the same time, it is associated with side effects, such as steroid-induced osteoporosis, associated bone weakening[24] and subcutaneous rupture of weakened tendons.[15]

We previously showed the mechanism of bone weakening associated with GC-induced osteoporosis using GC-treated rats and found that abnormal intermolecular crosslinking of bone collagen leads to deterioration of bone quality and decreased bone strength without a change in bone mineral density.[20] Several reports are available regarding the mechanism of GC-related tendon weakening. Torricelli et al. investigated the effect of systemic GC administration using rat Achilles tendons and reported that the treatment resulted in pronounced decrease in tendon cell proliferation and synthesis of type 1 collagen and proteoglycan.[26] Hossain et al. reported that in vitro treatment of canine-derived Achilles tendon cells with

dexamethasone resulted in inhibited proliferation of tendon cell proliferation and induction of cell death.[9] Zhang et al. treated injured tendons with dexamethasone and evaluated its therapeutic effect.[27] They found that the proliferation and differentiation of human tendon stem cells (hTSCs) have direct impact on the healing of tendons and that treatment with high-concentration dexamethasone causes hTSCs to differentiate into non-tendon cells, which gave rise to fat, and cartilage-like and bone-like tissues when transplanted into animals. This impaired collagen maturation may be induced by impairment of the proliferation of normal tendon cells and cellular synthetic activities and hinders the repair of injured tendons by exerting negative effects at the cellular level.

Meanwhile, no detailed analysis focusing on quantitative and qualitative collagen deterioration has been conducted on the effect of systemic GC administration on tendons, even though collagen is the major component of the Achilles tendon. Collagen biosynthesis occurs in ribosomes of fibroblasts, such as tenocytes, where a single polypeptide chain (proto-collagen) forms a procollagen molecule, which is then excreted from the fibroblast and forms a collagen molecule (tropocollagen).[10,11] Thus, the decreased collagen content in the Achilles tendon following systemic GC administration observed in this study is likely to be due to inhibited proliferation of fibroblastsð the site of collagen biosynthesisð and induced cell death, as reported elsewhere.

Collagen crosslinks are known to affect the biological and mechanical properties of collageneous tissue.[2] In an early stage of collagen fiber formation, weak, non-covalent bonds, such as hydrogen bonds and ionic bonds, are formed between collagen molecules. This is followed by the formation of stronger covalent crosslinks in and between collagen molecules as a result of enzymatic oxidative deamination of lysine and hydroxylysine by lysyl oxidase. These enzymatic crosslinks are considered to directly influence the strength of collagen fibers.[3] Enzymatic crosslinks are divided into divalent immature[25] and trivalent mature[6] types. The former represents

$NaBH_4$-reduced cross-links that are abundant in relatively young tissues while the latter represents non-reduced pyridinoline crosslinks formed by non-enzymatic reaction in maturing tissues. A portion of divalent immature crosslinks undergoes a spontaneous maturation reaction to form trivalent mature crosslinks such as pyridinoline. Enzymatic immature and mature crosslink formation has positive effects on bone mechanical properties.[2,21]

In the course of maturation from infancy to youth and adulthood, immature crosslinks diminish and mature ones increase. In other words, physicochemically unstable reduced crosslinks are replaced by non-reduced, stable crosslinks in the course of the maturation of collagen fibers[19]. Pentosidine[22] is an AGE formed by Maillard reaction between collagen molecules. It is a crosslink that randomly binds to the helical structure of adjacent collagen molecules with aging. Pentosidine crosslinks are also termed as "senescent crosslinks", as they are formed in proteins with low turnover rates, such as collagen, accumulated in tissue with aging, and causes structural and qualitative changes that are disadvantageous to the host organism.[1,5] We found that excessive formation of crosslinking pentosidine leads to the loss of proper elasticity of collagen fibers and decreased bone strength and thus can cause plasticity and weakening of the bone.[21]

In the present study, GC administration reduced the maximum tensile load in the tensile strength test and made the Achilles tendon prone to rupture. The reduced tendon strength related to the impaired enzymatic crosslink formation and thin collagen fiber in GC-treated rats. Meanwhile, the GC treatment did not affect the content of pentosidine, a senescent crosslink. Furthermore, such reduced enzymatic crosslink formation was associated with a decreased expression of lysyl oxidase. These observations suggest that GC administration causes decreased strength of the Achilles tendon by hindering the formation of enzymatic crosslinks in maturing collagen fibers, thereby keeping them in an immature state.

In newborn animals, ligaments and tendons are composed only of uniform, thin collagen fibers, but the mixture of thick and thin fibers increases as they mature.[17] In mature ligaments with sufficient strength, a large part of the ligament cross-sectional area is occupied by thick collagen fibers. The strength of tendons and ligaments increases as the diameter of collagen fibers and the percentage of the cross-sectional area occupied by collagen fibers increase.[16] These changes occur as an adaptation to increasing exercise load in a growing animal. In the present study, GC administration resulted in decreased overall diameter of collagen fibers and a decreased number of thick collagen fibers, making thinner fibers prominent. These changes are the opposite to those that are observed in the normal maturation of tendons in animals. These observations support the implication from the biochemical analysis that GC administration keeps collagen fibers in an immature state.

The main limitation of the present study is its design as an animal study using rats, where the effects of aging and GC administration are not necessarily the same as those in humans.

CONCLUSION

The observations of this study suggest that systemic GC administration causes decreased strength of the Achilles tendon by decreasing its collagen content, thereby hindering the formation of enzymatic crosslinks and keeping collagen fibers in an immature state with smaller diameters. Systemic GC administration directly prevents the maturation of tendon collagen fibers and decreases tendon strength, regardless of the presence or absence of underlying disease.

DECLARATION OF CONFLICTING INTERESTS

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LEGENDS

Figure 1. Maximum tensile load

The maximum tensile load was significantly decreased in the steroid group.

Figure 2. Gene expression of type 1 collagen

Data are expressed as relative gene expression

The gene expression of type 1 collagen was decreased by 99% in the GC group.

Figure 3. Gene expression of lysyl oxidase

Data are expressed as a relative gene expression

The gene expression of lysyl oxidase was decreased by 83% in the GC group.

Figure 4. Collagen content of control versus GC group

Data are expressed as a percentage of the tissueôs dry weight

* Significant difference ($p < .05$)

Collagen content was significantly decreased in the steroid group.

Figure 5. Amount of enzymatic crosslinks (mature + immature)

* Significant difference ($p < .05$)

The amount of enzymatic crosslinks (mature + immature) was significantly decreased in the steroid group.

Figure 6. Pentosidine content

There was no significant difference in pentosidine content between the groups.

Figure 7. Electron microscopic images of collagen fiber cross-sections.

The mean collagen fiber diameter was 147.37 ± 15.1 and 169.7 ± 19.6 nm in the GC and control groups, respectively, being significantly smaller in the GC group.

Figure 8. Histograms of collagen fiber diameter

In the GC group, thick fibers (defined as a diameter of Ó260 nm) were decreased and thin fibers (defined as a diameter of 100 to 240 nm) were increased in number, compared with the control group.

Table 1. Quantitative changes in collagen crosslinks in the Achilles tendon of control versus GC group

DHLNL: dihydroxylysinonorleucine; HLNL: hydroxylysinonorleucine; Pyr: pyridinoline

Data are expressed as mol/mol of collagen and means ± SDs.

Data are expressed as mmol/mol of collagen and means ± SDs.

The amount of enzymatic crosslinks was significantly decreased in the steroid group, whereas no significant difference was found in pentosidine content between groups.

Table 2. Electron microscopy

Collagen fiber diameter, percent unit area occupied by collagen fibers, and collagen density were significantly decreased in the steroid group.

385 Table 1. Quantitative changes in collagen crosslinks in Achilles tendon of control

386 versus GC group

	Control (n = 10)	GC (n = 10)	t test
DHLNL [a]	**0.399 ± 0.129**	**0.328 ± 0.123**	**p = .219**
HLNL [a]	**0.196 ± 0.092**	**0.061 ± 0.020**	**p = .001**
DHLNL+HLNL [a]	**0.595 ± 00.215**	**0.389 ± 0.129**	**p = .018**
Pyr [a]	**0.102 ± 0.036**	**0.069 ± 0.021**	**p =.021**
Enzymatic crosslinks [a]	**0.697 ± 0.225**	**0.458 ± 0.137**	**p = .010**
Pentosidine [b]	**1.783 ± 0.879**	**1.481 ± 0.998**	**p = .481**

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390 Table 2. Electron microscopy

	Control (n = 15)	GC (n = 15)	t test
Diameter	**169.684 ± 19.638**	**147.370 ± 15.077**	**p = .002**
Percent of area occupied	**80.492 ± 1.772**	**77.034 ± 2.952**	**p < .001**
Density	**33.133 ± 1.772**	**43.151 ± 8.364**	**p = .002**

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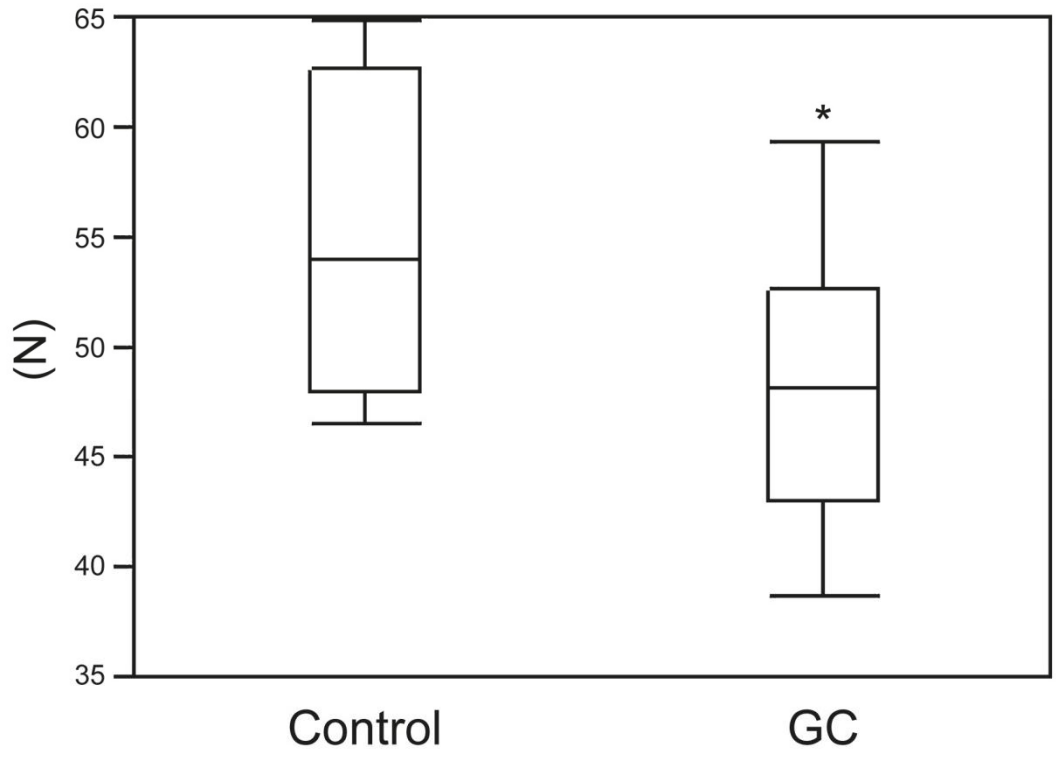

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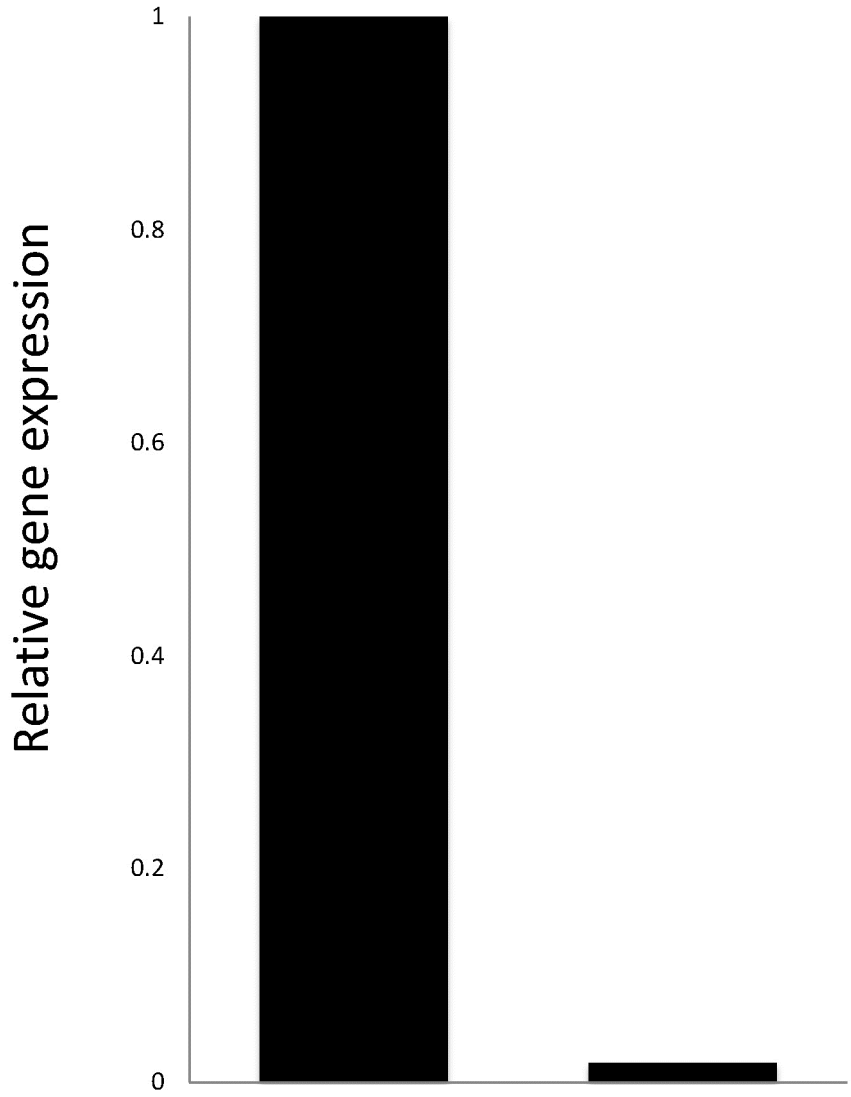

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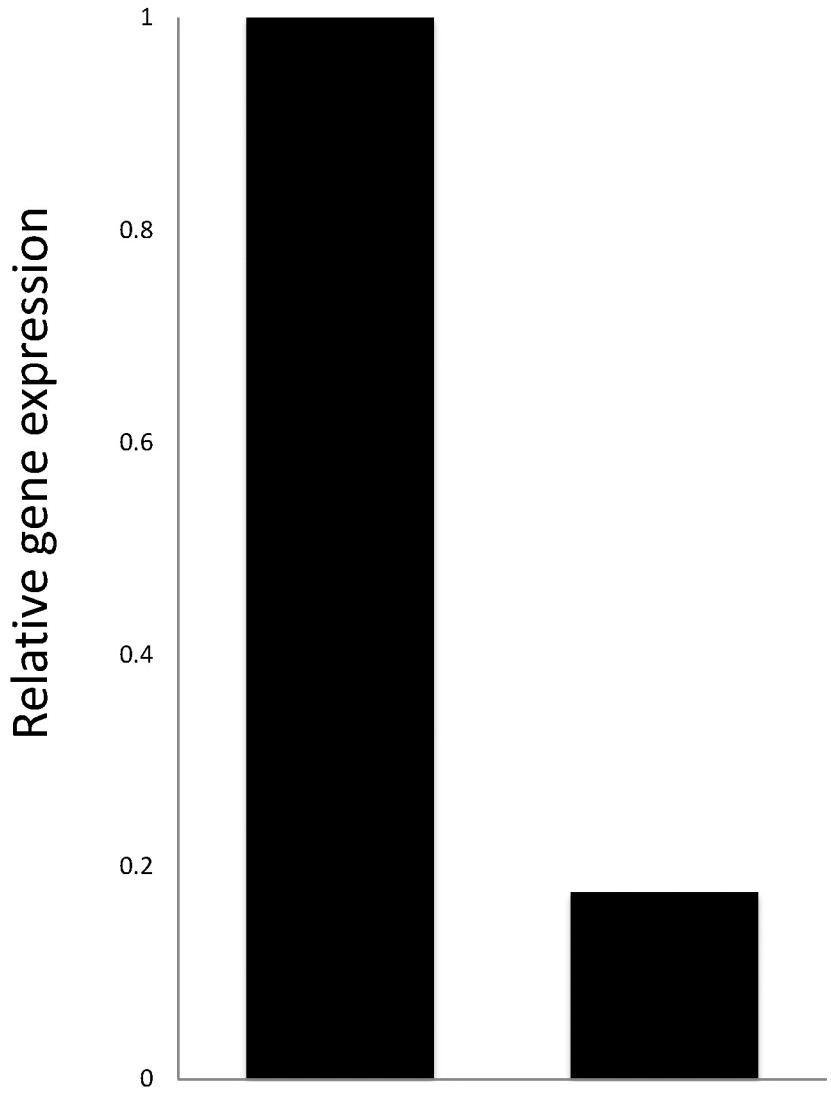

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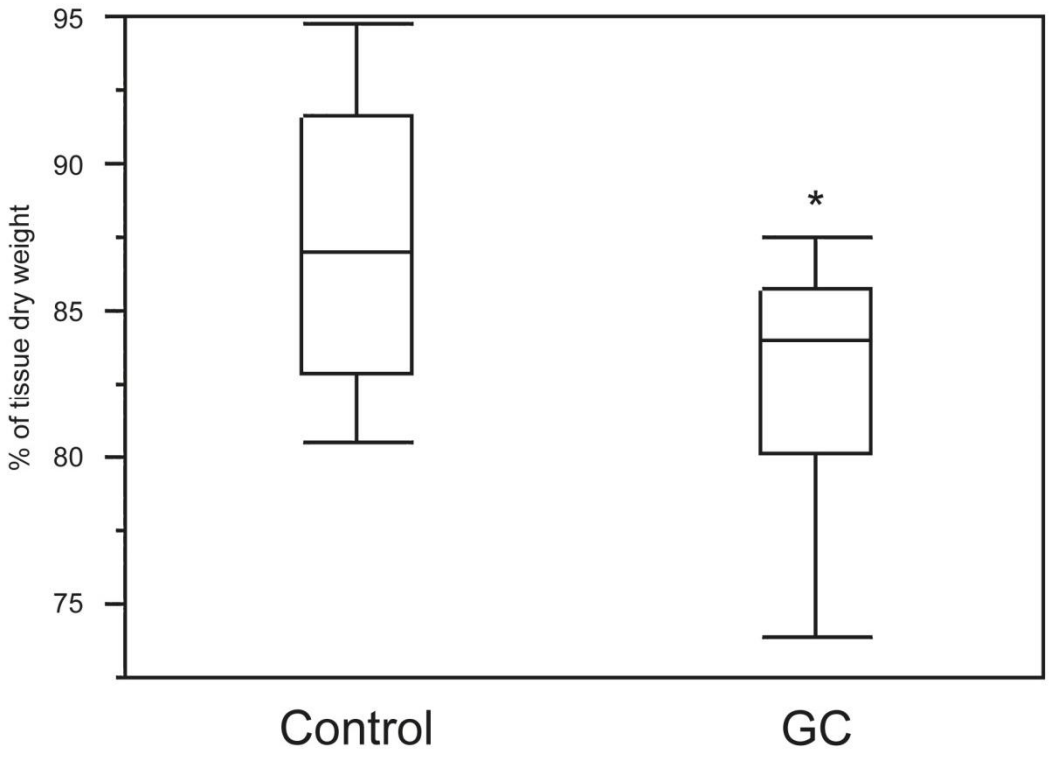

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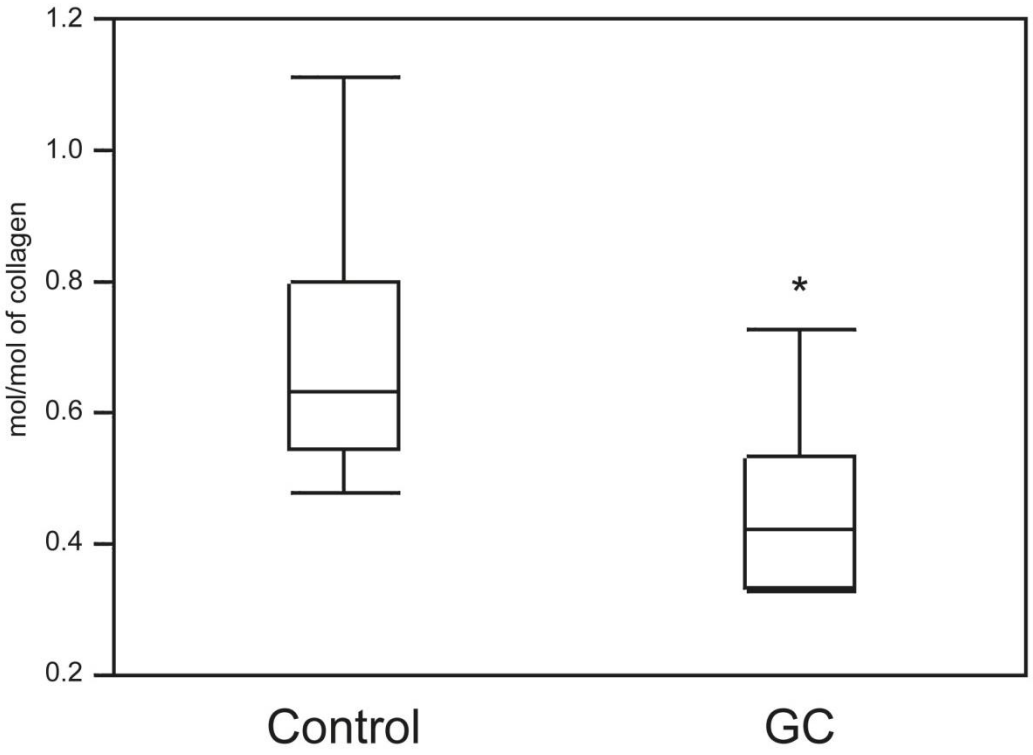

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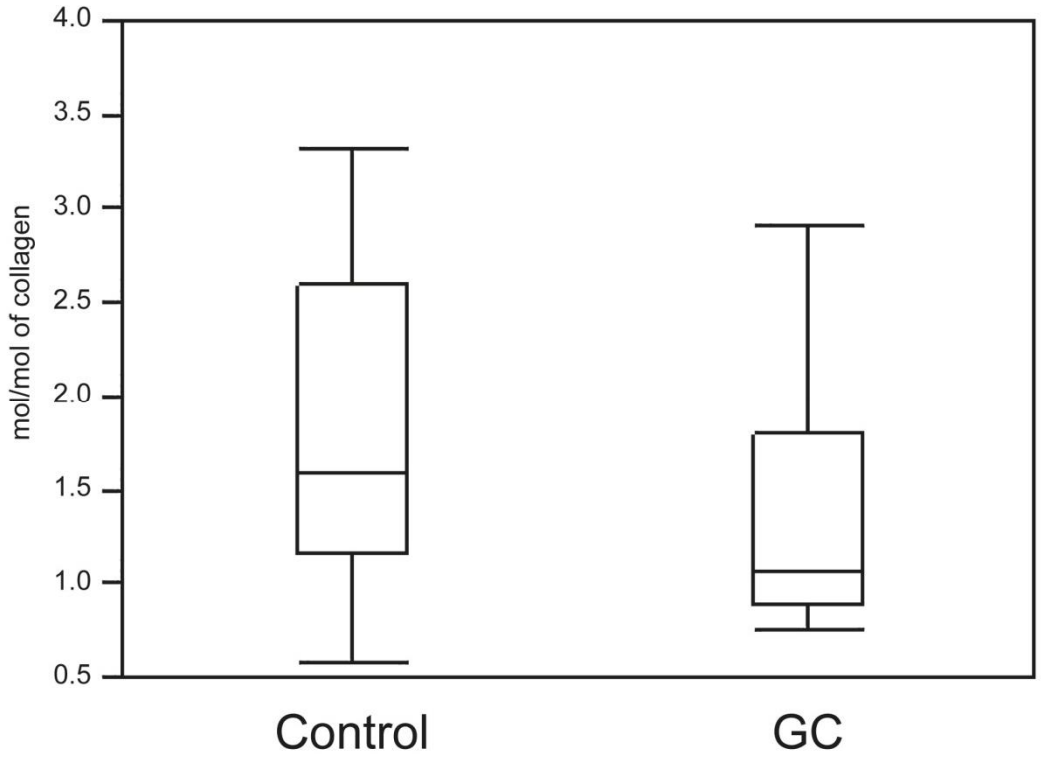

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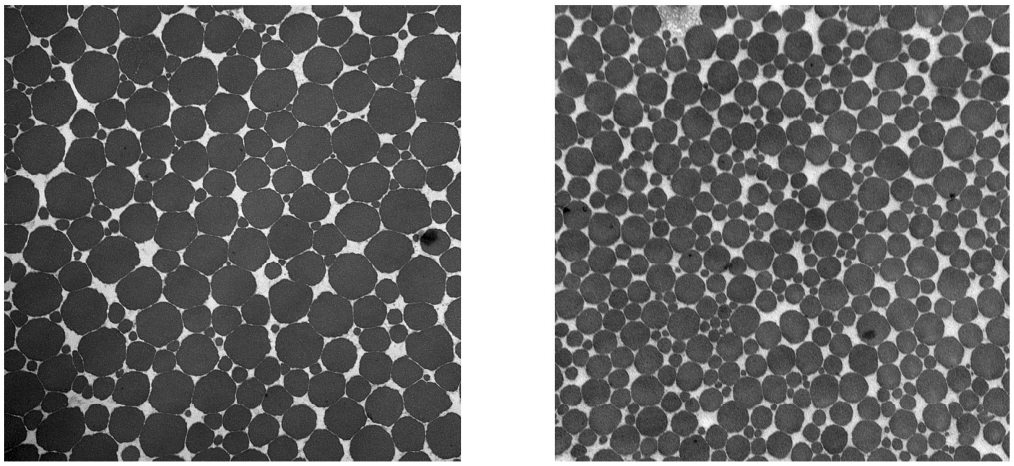
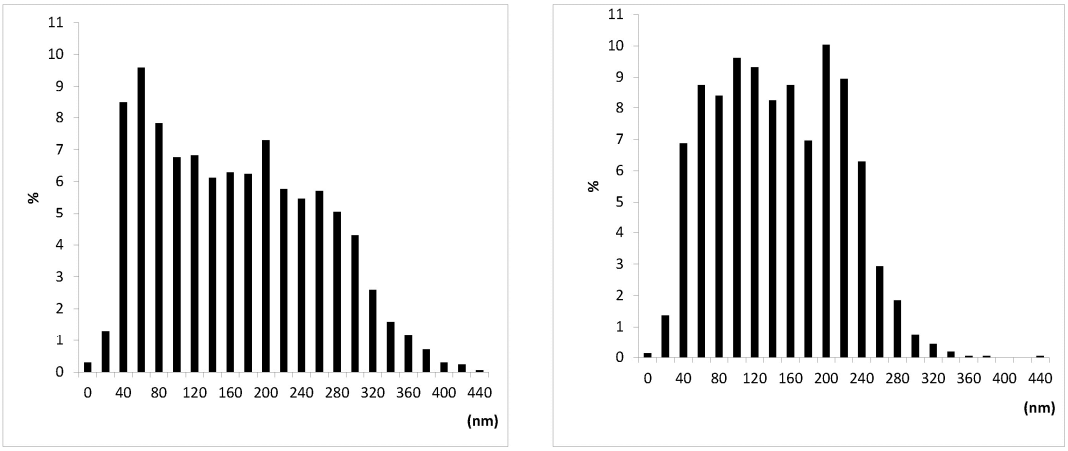


Figure 7.



Control GC group

Figure 8.