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Enhancement of Glucosamine Hydrochloride on Thioredoxin Production by Synoviocytes from Knee Osteoarthritis *In vitro*

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Abstract

Background: Knee osteoarthritis (OA) is well known to be a common disease of the synovial joint in elderly peoples, which are characterized by progressive loss of articular cartilage with bony remodeling, periarticular muscles, among others. Although the etiology of OA is not well understood, oxidative stress responses are attracted attention as one of important mechanisms in the development of OA. Oral administration of dietary supplements, such as glucosamine hydrochloride (GH) and chondroitin sulfate are accepted to be effective treatment of OA, however, the therapeutic mechanisms of these dietary supplements are not fully understood. The present study was designed to examine the influence of GH on the production of thioredoxin (TRX), one of the most important endogenous anti-oxidative molecules, by synoviocytes from an OA patient using an *in vitro* cell culture technique.

Methods: Human fibroblast-like synoviocytes (1 x 10^5 cells/ml) were stimulated with 25.0 μM H_2O_2 in the presence of various concentrations of GH. After 24 h, TRX concentrations in culture supernatants were examined by ELISA. We also examined the effect of GH on transcription factor, signal transducer and activator of transcription factor 6 (STAT6), activation and TRX mRNA expression in synoviocytes 6 and 12 h after H_2O_2 stimulation, respectively. Moreover, we examined the influence of GH on TRX protein production by wheat germ cell-free protein synthesis system.

Results: Treatment of cells with GH caused an increase in the ability of cells to produce TRX after H_2O_2 stimulation, and the minimum concentration that caused significant increase was 0.75 μg/ml. Although the addition of GH at more than 0.75 μg/ml into cell cultures inhibited STAT6 activation and TRX mRNA transcription, GH increased translocation of mRNA to produce specific proteins.

Conclusion: These results strongly suggest that the ability of GH to increase TRX production from synoviocytes may account, at least in part, for clinical efficacy of GH on OA.

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Introduction

Osteoarthritis (OA) is well known to be a degenerative disease involving chondrocytes, cartilage and other joint tissues. Although OA is not a life-threatening disease, it is a leading cause of disability in the elderly peoples and economic burden owing to its clinical symptoms such as pain, stiffness, and loss of function in articulating joints, which develop slowly and worsen over time [1-3]. Although the details of OA pathogenesis are not fully understood, it is generally thought to be the complex interaction of mechanical stress and inflammatory responses and the catabolic-anabolic balance of the joint, synovium, matrix and chondrocytes [4]. It is reported that synovial fluid obtained from OA patients contained higher levels of matrix metalloproteinases (MMPs), which are responsible for the degradation and destruction of proteoglycan and collagen in articular cartilage, than those from healthy control and that the concentration of MMPs in synovial fluids are reflected the OA severity [1,5]. Furthermore, inflammatory mediators, such as IL-1β, IL-6 and TNF-α were elevated in both synovial fluid and serum from OA patients and were associated with the level of OA severity, especially joint space narrowing [6,7]. On the other hand, there is much evidence that reactive oxygen species (ROS), including nitric oxide (NO), play essential roles in the development of OA through their activities on apoptotic cell death and tissue destruction in the joint, which are associated with cartilage matrix degradation [8,9]. Under normal physiological conditions, there are several types of antioxidants such as superoxide dismutase and glutathione peroxidase, and prevent the development of oxidative stress responses [10]. Among these, thioredoxin (TRX) has attracted attention as an endogenous antioxidant protein. TRX is a 12-kDa oxidoreductase enzyme and functions as a scavenger of ROS and an inhibitor of ROS generation in many oxidative diseases such as diabetes, rheumatoid arthritis, and asthma [10].

Treatment of OA is divided into two categories, surgical and non-surgical approaches. Surgical treatments, including replacement of the affected joints, are considered as final procedures when non-surgical treatments failed to control the development of OA. Non-surgical treatment includes the use of non-steroidal anti-inflammatory drugs (NSAIDs), analgesics and intra-articular injection of either hyaluronic acid or corticosteroids [3,11,12]. Physical and occupational exercises are also used frequently to prevent the development and slowing structural joints progression [11] as non-surgical treatments. These treatments are known to relieve OA symptoms especially pain,

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stiffness, and inflammation, and improve functionally, but do not have structural-modifying effects on the pathological changes once triggered [4,11]. On the other hand, there are much evidence that oral administration of dietary supplements, especially chondroitin sulfate and glucosamine hydrochloride (GH) into OA patients could favorably modify the clinical conditions of the disease, including pain, stiffness, joint swelling and functional limitation [4,12,13]. Although the therapeutic mode of action of agents are reported to be owing to inhibition of MMP activity, NO production and degradation of glycosaminoglycan, as well as stimulation of hyaluronic acid synthesis in the joint [14], the precise mechanisms by which dietary supplements could modify the clinical conditions of OA are not well defined. The present study, therefore, was undertaken to examine the influence of GH on TRX production from synoviocytes in response to H_2O_2 stimulation *in vitro*.

Materials and Methods

Reagents

GH was purchased from SIGMA-Aldrich Co. Ltd. (St Louis, MO, USA) as a preservative free pure powder. GH was dissolved in Synoviocyte Growth (SG) Medium (Cell Applications, Inc., San Diego, CA, USA) at a concentration of 10.0 mg/ml, sterilized by passing through 0.2 μm filters and stored at 4 °C until used.

Cell culture

Human fibroblast-like synoviocytes (HFLS-OA) obtained from the inflamed synovial tissues of an OA patient (Cell Applications, Inc.) was suspended in SG Medium at a concentration of 1 x 10^5 cells/ml and used as a target cell. To examine the influence of H_2O_2 on TRX production from HFLS-OA, 1 x 10^5 cells (1.0 ml) were introduced into 24-well culture plates in triplicate and stimulated with various concentrations of H_2O_2 in a final volume of 2.0 ml. After 24 h, culture supernatants were collected and stored at -40°C until used [10]. In the case of examining the influence of GH on TRX production from HFLS-OA, 1 x 10^5 cells (1.0 ml) were stimulated in triplicate with 25.0 μM H_2O_2 in the presence of 0.25 to 1.5 μg/ml GH in a final volume of 2.0 ml. After 24 h, the culture supernatants were obtained and stored at -40°C until used. To prepare cells for examining the influence of GH on transcription factor, signal transducer and activator of transcription factor 6 (STAT6) activation and TRX mRNA expression in HFLS-OA after H_2O_2 stimulation, 1 x 10^5 cells (1.0 ml) were stimulated in triplicate with 25.0 μM H_2O_2 in the presence of 0.5 to 1.5 μg/ml GH in a total volume of 2.0 ml for 6 h and 12 h, respectively. In all experiments, GH was added to cell cultures 2 h before stimulation.

Assay for TRX mRNA expression

Poly A^+ mRNA was separated from cultured cells with oligo (dT)-coated magnetic micro beads (Milteny Biotec, Bergisch Gladbach, Germany). The first-strand cDNA was synthesized from 1.0 μg of Poly A^+ mRNA using a Superscript cDNA synthesis kit (Invitrogen Corp., Carlsbad, Calif, USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was then carried out using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Forster City, Calif., USA). The PCR mixture consisted of 2.0 μl of sample cDNA solution (100 ng/μl), 25.0 μl of SYBR-Green Mastermix (Applied Biosystems), 0.3 μl of both sense and antisense primers, and distilled water to give a final volume of 50.0 μl. The reaction was conducted as follows: 4 min at 94°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. GAPDH was amplified as an internal control. mRNA levels were calculated by using the comparative parameter threshold cycle and normalized to GAPDH. The primers used for real-time RT-PCR were 5'-GCCTTGCAAAATGATTCAAGC-3' (Sense) and 5'-TTGGCTCCAGAAAATTCACC-3' (Antisense) for TRX [15], and 5'-TGTTGCCATCAATGACCCCTT-3' (Sense) and 5'-CTCCACGACGTACTCAGCG-3' (Antisense) for GAPDH [15].

Preparation of TRX specific mRNA

To prepare TRX specific mRNA for cell-free protein synthesis, the first-strand cDNA synthesized was amplificated with a Takara PCR Amplification kit (Takara Bio, Inc., Shiga, Japan) using specific primers for TRX in a final volume of 30.0 μl. The PCR conditions were as follows: 5 min at 94°C, followed by 30 cycles of 20 s at 94°C, 30 s at 58°C, and 30 s at 72°C. After measuring mRNA contents, samples were stored at -80°C until used.

Cell-free protein synthesis

Cell-free protein synthesis was performed using Wheat germ cell-free protein synthesis core kits (Toyobo Co., Ltd., Osaka, Japan). The reaction mixture consisted of 2.0 μl of reaction buffer, 1.7 μl of creatine kinase (10 μg/ml), 1.0 μl of ribonuclease inhibitor (40 U/ml), 10.0 μl of wheat germ extract, 33.5 μl of specific mRNA (0.4 μg/μl) and distilled water contained various concentrations of GH to give a final volume of 50.0 μl. The reaction mixture (50 μl) was then introduced into each well of 96 well plates that contained 250 μl of reaction buffer. The plates were incubated at 26°C for 24 h and the solutions were stored at -40°C until used.

Assay for TRX

TRX levels in culture supernatants and protein synthesis solution were examined by the commercially available TRX ELISA test kits (BioVendor Lab. Med. Inc., Brno, Czech Republic) according to the manufacturer's recommendations. The minimum detectable level of this kit was 2.13 ng/ml.

Statistical analysis

The statistical significance between control and experimental groups was examined by ANOVA followed by Dunette's multiple comparison tests. The level of significance was considered at P value of less than 0.05.

Results

Influence of H_2O_2 stimulation on TRX production from HFLS-OA *in vitro*

The first experiments were undertaken to examine whether H_2O_2 stimulation could induce TRX production from HFLS-OA. HFLS-OA was stimulated with various concentrations of H_2O_2 for 24 h and TRX levels in culture supernatants were examined by ELISA. As shown in Figure 1, H_2O_2 stimulation caused a significant increase in the ability of cells to produce TRX, which was firstly observed at 12.5 μM and was peaked at 25.0 to 50.0 μM. However, H_2O_2 at 75.0 μM and more exerted inhibitory effects on TRX production: TRX levels in culture supernatants significantly decreased and the levels of TRX were nearly identical ($P > 0.05$) to that observed in control.

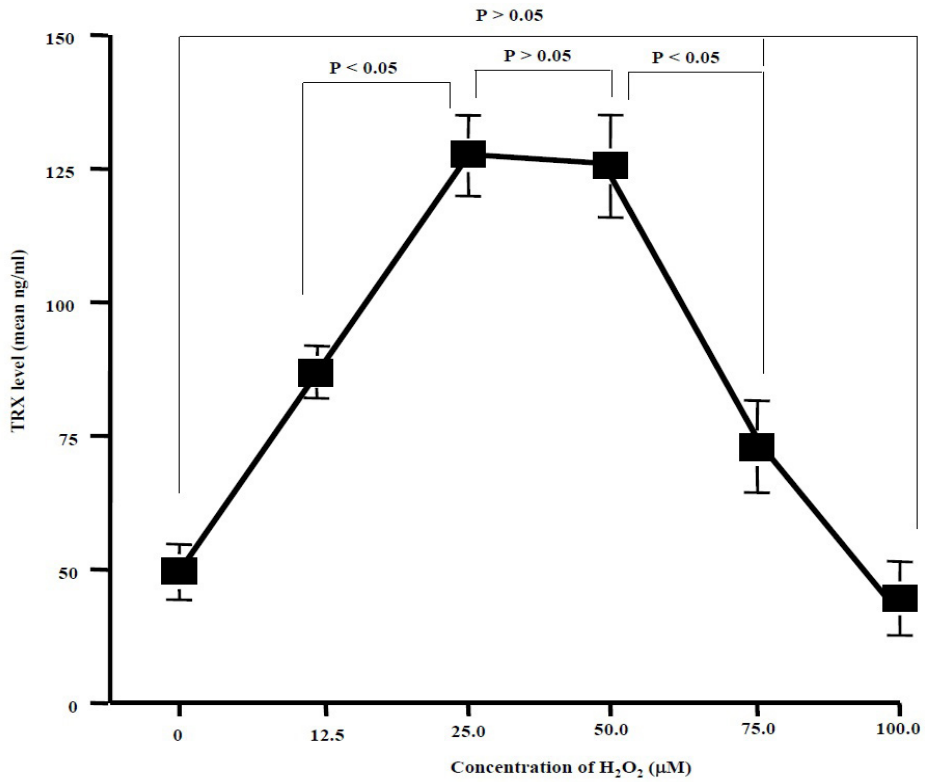

Figure 1: Influence of H_2O_2 on thioredoxin (TRX) production from HFLS-OA *in vitro*. Human fibroblast-like synoviocytes (HFLS-OA) at 1 x 10^5 cells/ml were stimulated with various concentrations of H_2O_2. After 24 h, TRX levels in culture supernatants were examined by ELISA. The data are expressed as the mean ng/ml ± SE of triplicate cultures. The experiments were done twice with similar results.

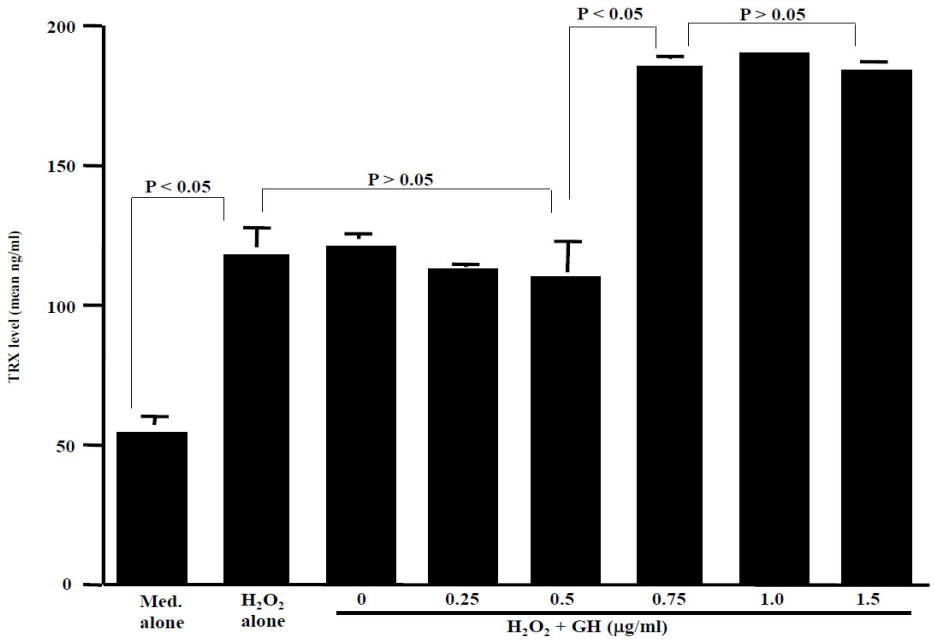

Figure 2: Influence of glucosamine hydrochloride (GH) on thioredoxin (TRX) production from HFLS-OA induced by H_2O_2 stimulation *in vitro*. Human fibroblast-like synoviocytes (HFLS-OA) at 1 x 10^5 cells/ml were stimulated with 25.0 μM H_2O_2 in the presence of various concentrations of GH for 24 h. TRX levels in culture supernatants were examined by ELISA. The data are expressed as the mean ng/ml ± SE of triplicate cultures. The experiments were done twice with similar results.

Influence of GH on H_2O_2-induced TRX production from HFLS-OA *in vitro*

The second experiments were designed to examine the influence of GH on TRX production from HFLS-OA after H_2O_2 stimulation. HFLS-OA was stimulated with 25.0 μM H_2O_2 in the presence of various concentrations of GH for 24 h and TRX contents in culture supernatants were examined by ELISA. As shown in Figure 2, GH at more than 0.75 μg/ml, but not less than 0.5 μg/ml, significantly increased TRX contents in culture supernatants.

Influence of GH on transcription factor activation and TRX mRNA expression in HFLS-OA after H_2O_2 stimulation

The third experiments were carried out to examine possible mechanisms by which GH could increase the ability of HFLS-OA to produce TRX after H_2O_2 stimulation. To do this, we firstly examined the influence of GH on transcription factor, STAT6, activation in HFLS-OA. HFLS-OA was stimulated with 25.0 μM H_2O_2 in the presence of various concentrations of GH. After 6 h, STAT6 activation was examined by ELISA. As shown in Figure 3, GH at more than 0.75 μg/ml, but not 0.5 μg/ml, caused significant suppression of STAT6 activation, which was increased by H_2O_2 stimulation. We then examined the influence of GH on TRX mRNA expression in HFLS-OA after H_2O_2 stimulation. HFLS-OA were stimulated with 25.0 μM H_2O_2 in the presence of various concentrations of GH. After 12 h, mRNA expression for TRX was examined by real-time RT-PCR. As shown in Figure 4, GH at 0.75 μg/ml and 1.0 μg/ml, but not 0.5 μg/ml, caused significant suppression of TRX mRNA expression, which was increased by H_2O_2 stimulation. The final experiments were undertaken to examine the influence of GH on TRX production in cell-free protein systems. As shown in Figure 5, the addition of GH at 0.5 μg/ml into cell-free proteins systems did not increase TRX synthesis: TRX levels in the solutions were nearly identical ($P > 0.05$) to those observed in control. However, GH at 0.75 μg/ml and 1.0 μg/ml significantly increased the ability of wheat germ extract to produce TRX as compared with control.

Discussion

OA is well known to be a common disease of the synovial joint in elderly individuals and characterized by progressive loss of articular cartilage with bony remodeling, periarticular muscles, among others, which develop slowly and worsen over time [1,2]. Since the precise mechanisms of OA progression is not fully understood, treatment focuses mainly on alleviating the symptoms of the diseases, especially pain, rather than modifying the disease process [4,11]. On the other hand, several reports clearly show that oral administration of chondroitin sulfate and GH for long periods (e.g. approximately 1 year or more) into OA patients attenuate the progression of OA including joint space narrowing and joint swelling [12-14]. However, the therapeutic mode of action of these nutraceuticals is not well defined. The present study, therefore, was undertaken to examine the influence of nutraceuticals on the ability of synoviocytes to produce TRX, which is one of the important endogenous antioxidant molecule in the development of diseases induced by oxidative stress, including OA [9,10], through the choice of GH and an *in vitro* cell culture technique. The present study clearly showed that GH increases the ability of synoviocytes to produce TRX in response to H_2O_2 stimulation, when the cells were treated with the agent at more than 0.75 μg/ml. After oral administration of GH at a dose of 1500 mg once a day, which is recommended therapeutic dose of OA, into

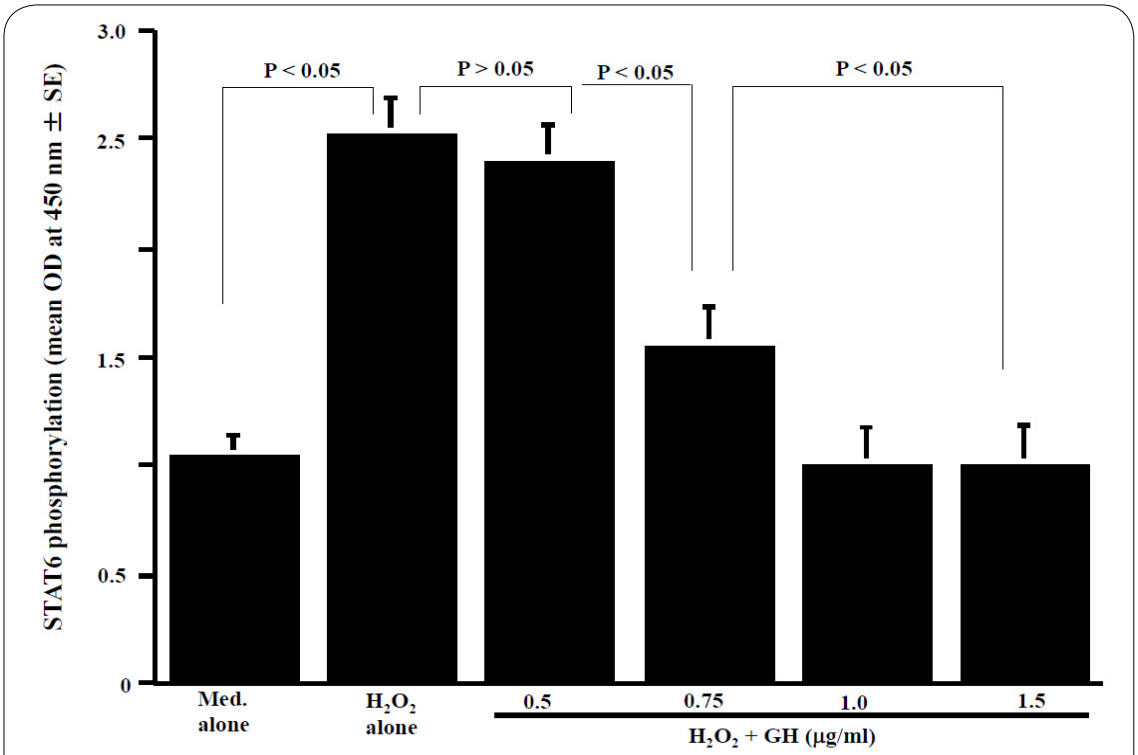

Figure 3: Influence of glucosamine hydrochloride (GH) on STAT6 activation in HFLS-OA *in vitro*. Human fibroblast-like synoviocytes (HFLS-OA) at 1 x 10^5 cells/ml were stimulated with 25.0 μM H_2O_2 in the presence of various concentrations of GH. After 6 h, STAT6 activation was examined by ELISA. The data are expressed as the mean OD at 450 nm ± SE of triplicate cultures. The experiments were done twice with similar results.

healthy volunteers for 14 days, plasma GH levels peaked at 1.1 to 1.8 μg/ml three hours after administration [16,17]. It is also observed that synovial fluid obtained from healthy human, who were orally administered GH at 1500 mg once a day for 14 days, contained 0.8 to 0.9 μg/ml GH [16]. Furthermore, it is reported that GH was rapidly absorbed, reached steady state after three hours and the maximal synovial fluid concentration was up to 25% higher compared to plasma levels after oral intake of 1500 mg [17]. From these reports, the

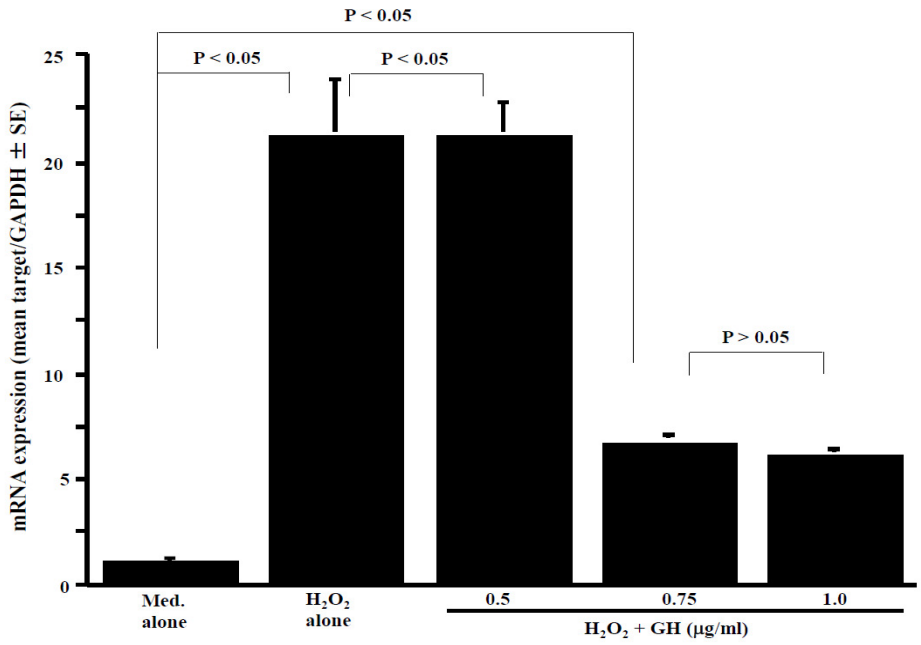

Figure 4: Influence of glucosamine hydrochloride (GH) on thioredoxin (TRX) mRNA expression in HFLS-OA *in vitro*. Human fibroblast-like synoviocytes (HFLS-OA) at 1 x 10^5 cells/ml were stimulated with 25.0μM H_2O_2 in the presence of various concentrations of GH. After 12 h, TRX mRNA expression was examined by real-time RT-PCR. The data are expressed as the mean ratio calculated as Target gene/GAPDH of triplicate cultures. The experiments were done twice with similar results.

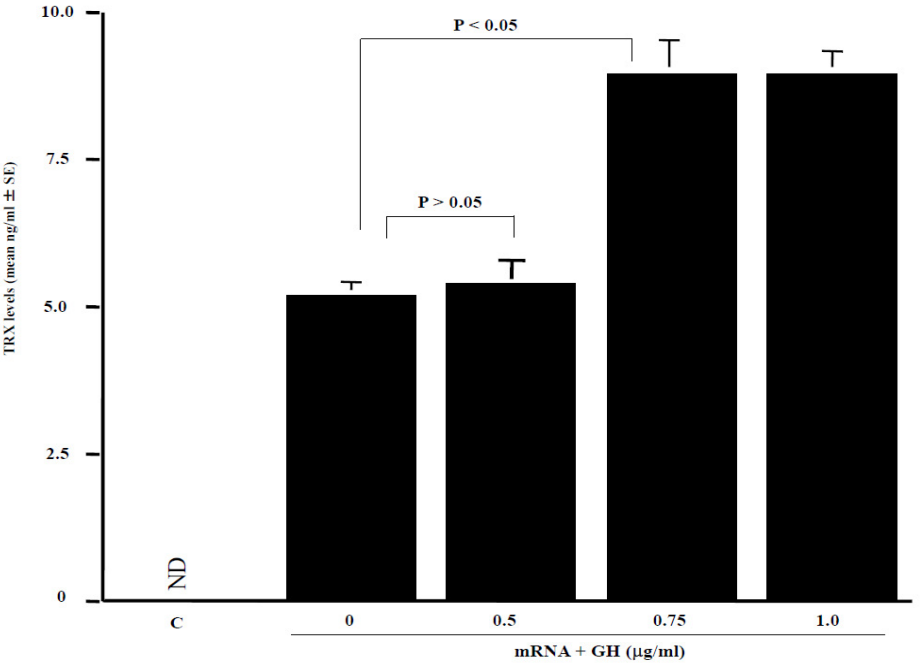

Figure 5: Influence of glucosamine hydrochloride (GH) on thioredoxin (TRX) production in cell free protein synthesis systems. TRX levels in samples were examined by ELISA. The data are expressed as the mean ng/ml ± SE of triplicate cultures. ND: not detected (below 2.13 ng/ml). The experiments were done twice with similar results.

present results showing the enhancement of GH on TRX production from synoviocytes after H_2O_2 stimulation may reflect the biological activities of GH *in vivo*.

TRX, a well-characterized protein with a highly conserved active site, is reported to play a variety of redox-related roles in organisms from bacteria to Man [18,19]. Intracellular TRX also plays essential roles in the scavenging of ROS and regulation of the activation of transcription factor such as nuclear factor (NF)-kB and activator protein (AP)-1 [19]. In addition to intracellular functions, TRX has been shown to inhibit inflammatory cell chemotaxis induced by chemokine stimulation [19]. Furthermore, TRX inhibits down-regulation of L-selectin precursor (CD62L) expression on inflammatory cells, especially neutrophils, after LPS stimulation via the suppression of p38 mitogen-activated protein kinase phosphorylation [19], suggesting that TRX exerts a suppressive effect on inflammatory cell activation. Taken together, the present data may be interpreted that this activity of GH constitutes, at least in part, of the therapeutic mode of action of GH on OA.

Forkhead transcription factor 3 (FOXO3) is reported to be an essential transcription factor in the up-regulation of TRX mRNA expression [20]. It is also reported that AMP-activated protein kinase (AMPK) plays a pivotal role in phosphorylation of FOXO3 [21], which is responsible for the promotion of TRX transcription in nuclei, indicating that the AMPK-FOXO3 pathway is essential for TRX mRNA expression [20,21]. Together with these reports, the present results showing the inhibitory effect of GH on TRX mRNA expression may be, in part, due to the suppressive effect of GH on AMPK-FOXO3 pathway activation. This speculation may be supported by the observation that GH exerts a suppressive effect on transcription factor, STAT6, activation at more than 0.75 μg/ml. On the other hand, the present results clearly show that treatment of synoviocytes with GH caused an increase in TRX protein production in spite of the suppression of its mRNA expression induced by H_2O_2 stimulation. The reasons for this discrepancy are not clear at present. The process of protein synthesis in cells requires two different steps: in the first step, transcription, mRNA is synthesized from DNA in the nucleus. mRNA formed then travels through nuclear membrane into cytoplasm where it binds to mRNA-binding site on ribosome and starts protein synthesis, which is called translation. The final experiments, therefore, were undertaken to examine whether pretreatment of synoviocytes with GH could increase the translocation activity of TRX mRNA. Cell free protein synthesis assay revealed that the addition of GH at more than 0.75 μg/ml could increase TRX synthesis. These results strongly suggest that there is the possibility that GH could increase the translation of TRX mRNA, resulting in an increase of TRX levels in culture supernatants. Further experiments are required to clarify this point.

Conclusion

The present results strongly suggest that GH causes an increase in the ability of synoviocytes to produce TRX, explaining the favorable modification of clinical symptoms of OA through the suppression of oxidative stress responses in the area of joint tissues.

Competing Interests

The authors declare that they have no competing interests.

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