EFFECTS OF COLLAGEN SYNTHESISE GENES ON CULTURED FIBROBLAST FROM HANWOO RELATED TO MEAT TENDERNESS

A.S.Sivakumar, J.S.Lee and I.H. Hwang

Department of Animal Science, Chonbuk National University, Jeonju 561-756, South Korea

Abstract – Meat tenderness is an important factor affecting the consumers' assessment of meat quality. It is associated with intramuscular collagen and is a major component of connective tissue protein which is a contributing factor to variation in meat tenderness. The aim of this study to investigate the mRNA expression of collagen related genes and proteolytic enzymes such as μ-calpain, m-calpain and calpastatin (the endogenous inhibitor of calpain) on cultured fibroblast from *longissimus dorsi* (LD) and *semimembranosus* (SM) muscles of Hanwoo cattle by real-time reverse transcription polymerase chain reaction (RT-PCR). Total collagen content was significantly higher in SM muscle fibroblast than LD muscle fibroblast. The mRNA expression of lysyl oxidase (LOX), Cystatin C (CYS), TRAP-1, NK-κB, TNF-α, Caspase-7 and caspase-7 had more in SM than LD muscle fibroblast whereas, the mRNA expressions of HSP-70 and HSP-90, PPAR-γ were significantly decreased in SM than LD muscle fibroblast. Additionally, the μ-calpain and m-calpain were significantly upregulated in LD than SM muscle fibroblast. These results indicate that the collagen biosynthesis genes were upregulated in cultured Hanwoo SM leads to more tough in SM muscle than LD muscle.

Key Words – connective tissue, meat quality, *longissimus dorsi, semimembranosus*

I. INTRODUCTION

Tenderness constitutes the most important factor that consumers evaluate when determining the acceptability of their eating experience [1]. Connective tissue, which is present in all the muscles, leads toughness in beef. The strength of connective tissue is derived by presence of collagen mainly of Types I and III which are the major components of intramuscular collagen. Collagen I is the most abundant collagen, being present in bone, tendon and skin, and collagen III is second most abundant and occurs particularly in tissues exhibiting elastic properties such as skin [2]. Typically, muscles of locomotion (i.e thoracic and pelvic) have more connective tissue (collagen) and are less tender. The lower tenderness is due to reduced calpain proteolytic system consisting of μ-calpain, and m-calpain are associated with greater activity of protease inhibitor calpastatin. Many studies reported on meat muscle tissue have shown that the calpain proteolytic system plays a vital role in tenderization [3]. Connective tissue may influence meat quality and depend on factors such as body location, age, sex and breed [4]. The collagen biosynthetic pathway production is very complex; about 34 genes are associated with collagen formation and may influenced by different muscle of same animals. Therefore, we hypothesized that was to determine *in vitro*, the genes which are involved in promoted and proliferation of collagen synthesis and collagen degradation process in LD and SM muscles fibroblast cells of Hanwoo cattle related to determination of meat tenderness in two different muscles of carcasses.

II MATERIALS AND METHODS

All chemicals and laboratory wares were purchased from Sigma- Aldrich Chemical Co. (St. Louis, MO, USA) and Falcon Lab ware (Becton-Dickinson, Franklin Lakes, Nj, USA), respectively.

Isolation of Intramuscular connective tissue fibroblasts from Hanwoo cattle: The fibroblast cells were freshly isolated from *Semimembranosus* (SM) and *Longissimus Dorsi* (LD) muscles of Hanwoo cattle (20-30 months old) by sequential enzymatic digestion and further purified by magnetic-activated cell sorting (MACS) with antibodies against fibroblast-specific protein-1 (FSP-1). Cell subsets obtained were cultured in parallel for further experiments.

Collagen extraction from fibroblast cellular layer: All the cells should be between passage numbers 7-9. $1x10^5$ cells were seeded into two T-75 culture flasks, one each for fibroblasts isolated from SM and LD muscle. The cells were allowed to grow for 7 days. During these 7 days the medium in the flasks should not be changed. At the end of 7th day, total collagen from the cellular layer was

obtained with the addition of a protein extraction solution after rinsing the culture flasks with PBS. This solution consisted of a protease inhibitor cocktail in PBS. Then cellular layer were harvested by scraping at 4°C. Total collagen from the cellular layer was obtained by sonication of the cellular layer extract (3 x 15 s, with 30 s intervals, 20 Hz, 4°C). The sonicated samples were submitted to a collagen precipitation reaction with 25% saturated ammonium sulfate, for 24 hours at 4°C, under constant agitation. Then collagens were isolated by centrifugation (40000 x g, 30 minutes, and 4°C). The discard the supernatant and the pellet were solubilized in 2 ml of 0.5 M acetic acid, consist the aliquots of collagen from the cellular layer, the collagen was estimated by the following method.

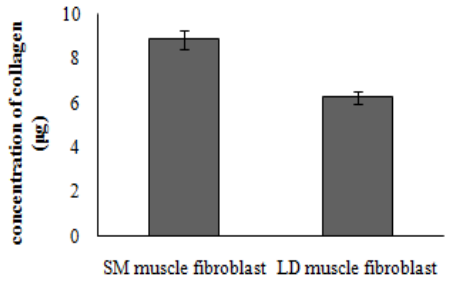
Estimation of collagen with Sirius-Red. Colorimetric detection was performed by Sirius red (Sigma-Aldrich Chemical Co., Saint Louis, EUA) according to the manufacturer's protocol (Keira et al., 2004).

RT-PCR: mRNA expression of selected genes which are involved in collagen synthesis/degradation such as LOX, BMP-1, CYS, TRAP, TNF-α, and NF-kB (inflammation marker), caspase-7, caspase-9 (apoptotic marker) Hsp-70, Hsp-90 and PPAR-γ.

III RESULTS AND DISCUSSION

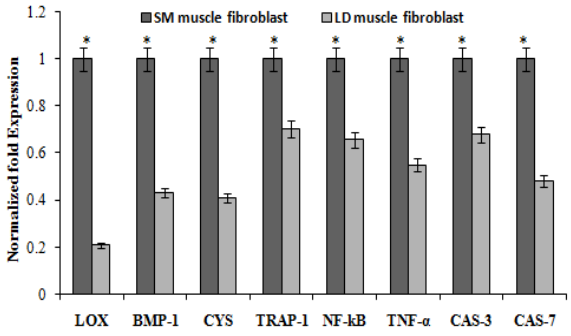
Figure 1 shows collagen concentration in SM and LD muscle fibroblast. The SM muscle fibroblast had significantly ($p<0.05$) higher collagen content than LD muscle fibroblast. Stolowski et al., 2006 reported that the LD and GM muscle contained lowest collagen content compared to SM, BF and ST muscles and demonstrated the highest WBS force values and lowest WBS force value in muscle containing lowest total collagen. Hanwoo ST and SM muscles had higher WBSF values because of higher total collagen content than LD muscle possessing first class quality of meat [5]. The mRNA expression of LOX, BMP-1, CYS, TRAP-1, NF-κB, TNF-α, CAS-3 and CAS-7 were significantly up-regulated in SM muscle fibroblast compared to LD muscle fibroblast. LOX is known to be essential for ECM maturation such as collagen type I and collagen type III [6]. LOX is mainly involved in collagen synthesis, deposition and maturation is also greatly expressed in Brangus and Angus than Brahman cattle.

Figure 1. concentration of collagen in SM and LD muscle fibroblast cells



This indicated that the collagen becomes mature in Angus and Brangus breeds than Brahman cattle. The LOX and BMP-1 mRNA expression positively correlated with WBS and palatability [7]. Increased LOX enzyme activity can lead to excessive accumulation of collagen [8]. BMP-1 a metalloproteinase which promotes the collagen synthesis, [7] proposed that the BMP-1 is responsible for pro-collagen maturation and collagen synthesis. It is greatly expressed in Brahman-influenced steers and reduction in meat tenderness.

Figure 2. mRNA expression collagen synthesis related genes

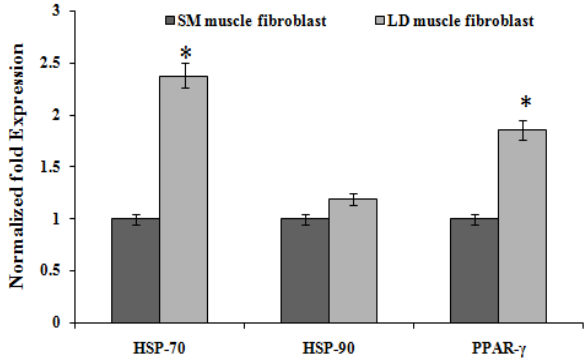


BMP may directly stimulate the collagen synthesis in dermal fibroblasts during wound healing via stimulating or activation of TNF-α to synthesize the collagen [9]. Cystatin C is a cysteine protease inhibitor abundantly secreted by cells and embedded in the extracellular matrix [10]. Increased activity of CYS or mRNA expression leads to collagen turnover and reduction in meat tenderness. CYS is moderately correlated with WBS values, sensory panel tenderness and connective tissue scores, soluble and insoluble

collagen content, and total collagen content. The mRNA expression of CYS greatly expressed in Half-Blood and Brahman genetic groups, their collagen fibrils could have more mature collagen than Angus and Brangus genetics groups. Half-Blood and Brahman steers had greater WBS values and tended to receive decreased panel tenderness scores than Angus and Brangus steers [7].

TRAP-1 plays an important role in the collagen synthesis in fibroblast. TRAP-1 can up-regulate the collagens synthesis by regulating TGF-β through the activation of Smad 3 pathways [11] and this is agreement with our results (Fig.2). TGF-β1 allows the expression of extracellular matrix gene to increase the synthesis and deposition of collagen [12 & 13].

Figure 3. mRNA expression collagen degradation related genes



The activation of NF-kB promotes the collagen synthesis through the activation of MMP-9 in cardiac fibroblast. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) inhibits the MMP-9 production and ammonium pyrrolidine dithiocarbamate (PDTC), responsible for specific inhibitor of NF-κB resulted in powerfully inhibited the MMP-9 production and significantly reduced collagen synthesis in fibroblast. They concluded that the activation of NF-κB promote the cardiac fibroblasts (CFs) and collagen synthesis [14]. In our current results, the mRNA expression of NF-κB was significantly increase in SM muscle fibroblast cell than LS muscle fibroblast cells (Fig.2), which indicates that NF-κB might be involved in collagen formation. In other results of our study the mRNA expression of caspase-3 and caspase-7 were significantly up-regulated in SM muscle fibroblast when compared to LD muscle fibroblast (Fig.2). Caspase-3 was involved to increased deposition of collage in fibroblast [15]. However, in contrast to HSP-70, HSP-90 and PPAR-γ is not master gene for collagen synthesis. However, some studies have suggested that these genes are negatively involved in collagen synthesis. Based on the findings of the current study, it is more likely that HSP-70 and HSP-90 down regulated the synthesis of collagens (Fig. 3), which means the mRNA expression of both the genes, were down regulated in SM muscle compared to LD muscle fibroblast cells. HSP-70 and HAP-90 are not associated with collagen synthesis [16]. PPAR-γ involved in collagen degradation process, [17] reported that Ajulemic acid inhibits the collagen synthesis through the activation of PPAR-γ. At the same time our present results clearly showed that the mRNA expression of PPAR-γ significantly ($p<0.05$) decreased in SM than LD muscle fibroblast (Fig.3).

Figure 4. mRNA expression calpain and calpastatin

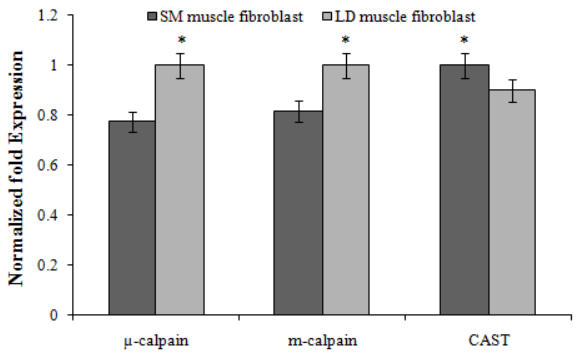


Figure 4 represents that in the mRNA expression of calpastatin on LD and SM muscle fibroblast, the expression was little higher in SM muscle fibroblast than LD muscle fibroblast. Our study performed with [18] who reported that the calpastatin activity and WBS value significantly higher in SM porcine muscles than LD porcine muscles at all postmortem time. In our study, current resents demonstrated that the mRNA expression of μ-calpain and m-calpain were significantly up-regulated in LD muscle when compared to SM muscle fibroblast cells. The activity of native m-calpain was similar in the LD muscles and SM muscle whereas the activity of the autolyzed form of the enzyme was lower in LD muscle compared to SM muscle [19]. Previous results from other scientist have suggested that μ-calpain, and not m-calpain, is involved in the tenderization process of meat [20].

IV CONCLUSION

The candidates genes of collagen synthesis and calpastatin are highly expressed in SM muscle fibroblast than LD muscle fibroblast whereas decreased the expression of collagen degraded genes and calpain (μ&m) in SM than LD muscle fibroblast. Based on our results we conclude that measurement of collagen concentration and mRNA expression of collagen related genes would provide meaningful explanation in sensory tenderness or shear force in LD and SM muscle from Hanwoo.

ACKNOWLEDGEMENTS

It should be acknowledged that this work was carried out with the support of Cooperative Research Program for Agriculture Science & Technology Development (Project No PJ010170) and a grant from the Next-Generation BioGreen 21 Program (Project No PJ011101), RDA, Republic of Korea

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