CHAPTER III

RESULTS

3.1 Isolation of primary equine chondrocytes

Primary equine chondrocytes (Figure 3.1; B) were isolated from equine cartilage (Figure 3.1; A) obtained from a fetlock (metacarpo/tarsophalangeal) joint with no history of inflammatory joint diseases and without known infectious diseases. They were expended by culture in 25 cm^2 culture flasks.

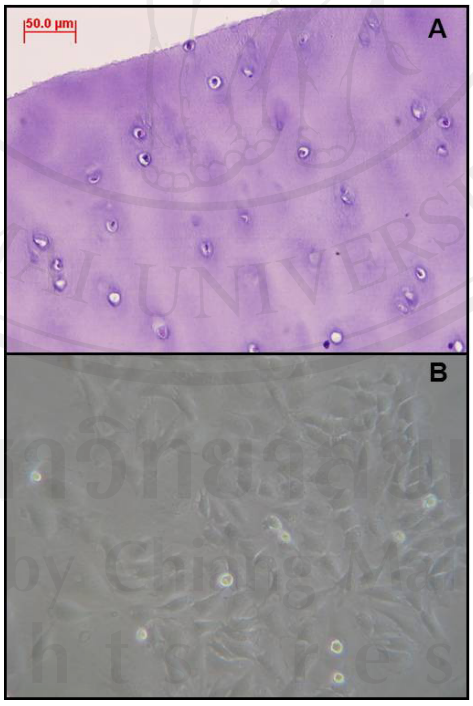

Figure 3.1 Equine chondrocytes embedded in cartilage tissue (A) and isolated equine chondrocytes in 25 cm^2 culture flasks (B).

3.2 Effect of TGF-β1 on HA synthesis in monolayer cultured equine chondrocytes

Equine chondrocytes were seeded and cultivated in 10% FCS-DMEM. After 48 hour, medium were replaced with 10%FCS-DMEM containing TGF-β1 at concentrations of 2.5, 5.0 and 10.0 ng/ml and incubated for 3 days. The HA levels in conditioned media of each treatments were measured using the competitive based enzyme-linked immunosorbent assay (ELISA) method. HA amount was normalized to the DNA content of each treatment. The result was shown in figure 3.2. It was found that equine chondrocyte monolayer cultures with TGF-β1 significantly enhanced HA level in dose dependent manner compared with control.

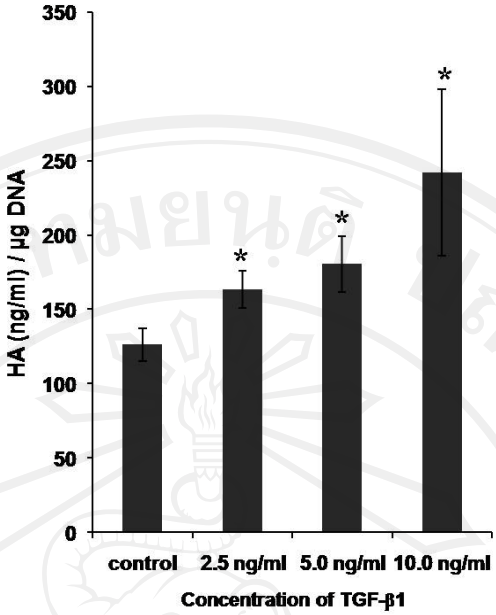

Figure 3.2 Effect of TGF-β1 on HA synthesis in monolayer cultured equine chondrocytes. Chondrocytes were treated with different concentrations (0, 2.5, 5.0, 10.0 ng/ml) of TGF-β1 for 3 days. HA amount was measured in conditioned media using the competitive based enzyme-linked immunosorbent assay (ELISA) method.

* denote a value that is significantly different (p<0.05) from control.

3.3 Time-effect and dose-effect of TGF-β1 on HAS-2 mRNA levels in monolayer cultured equine chondrocytes

For time-effect investigation, equine chondrocytes were treated with or without of 2.5 ng/ml TGF-β1 at the indicated time points. Cell lysates were harvested for mRNA extraction. HAS-2 mRNA levels were determined by semi-quantitative RT-PCR and normalized with the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) of each sample. The result showed that TGF-β1 up-regulated HAS-2 mRNA within the first 9 hours (Figure 3.3) with significant differences at 3 and 9 hours after treatment. Then the HAS2 mRNA was gradually decreased to lower than that was in control.

For investigation of dose-effect, equine chondrocytes were treated with TGF-β1 in different concentrations (0, 2.5, 5.0, 10.0 ng/ml) for 3 hours. The result clearly showed that TGF-β1 up-regulated HAS-2 mRNA in dose-dependent manner with significant difference at 5.0 and 10.0 ng/ml comparing to control.

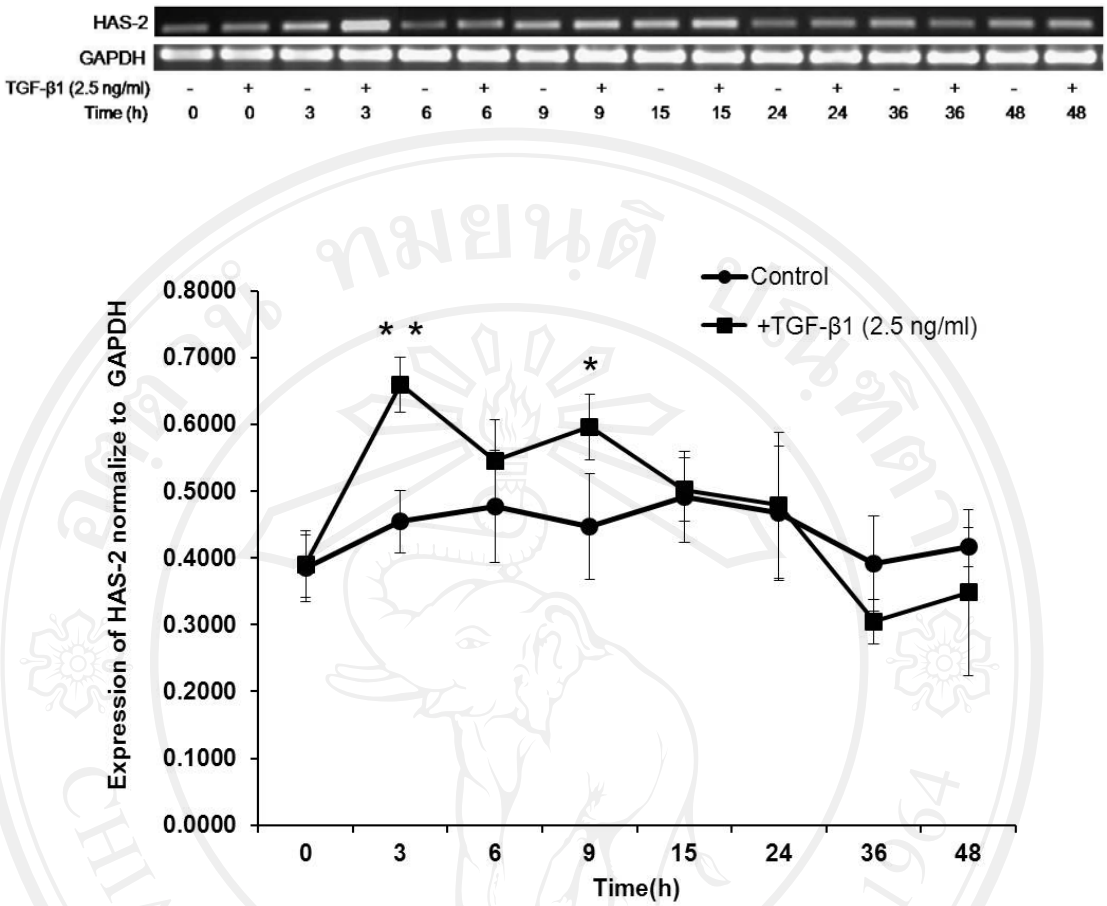

Figure 3.3 Time effects of TGF-β1 on HAS-2 mRNA levels determined by RT-PCR and normalized with GAPDH. Equine chondrocytes were treated with or without 2.5 ng/ml TGF-β1 at indicated time points.

* and ** denote a value that are significantly different (p<0.05 and p<0.01, respectively) from control.

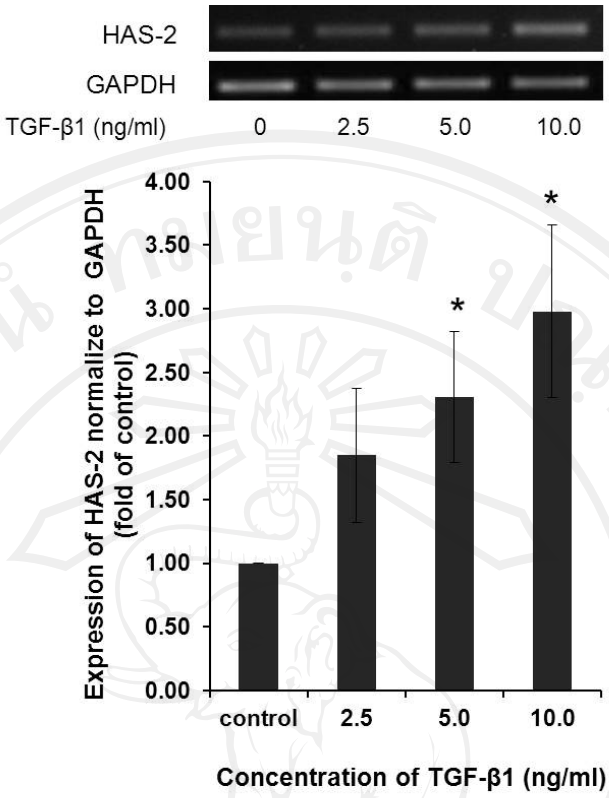

Figure 3.4 Dose effects of TGF-β1 (0, 2.5, 5.0, 10.0 ng/ml) on HAS-2 mRNA levels determined by RT-PCR and normalized with GAPDH. Equine chondrocytes were treated with TGF-β1 for 3 hour.

* denote a value that is significantly different (p<0.05) from control.

3.4 Effect of TGF-β1 on equine chondrocyte proliferation in monolayer culture

Equine chondrocytes were seeded in 24-well plate and incubated with 10% FCS-DMEM containing TGF-β1 at concentration of 0, 2.5, 5.0 and 10.0 ng/ml. Proliferation was measured using Alarmablue assay on day 0, 4 and 7 of culture. There was no statistic difference among concentrations of TGF-β1 at any time points although chondrocyte proliferation seemed to be slightly increased following TGF-β1 concentrations at day 7.

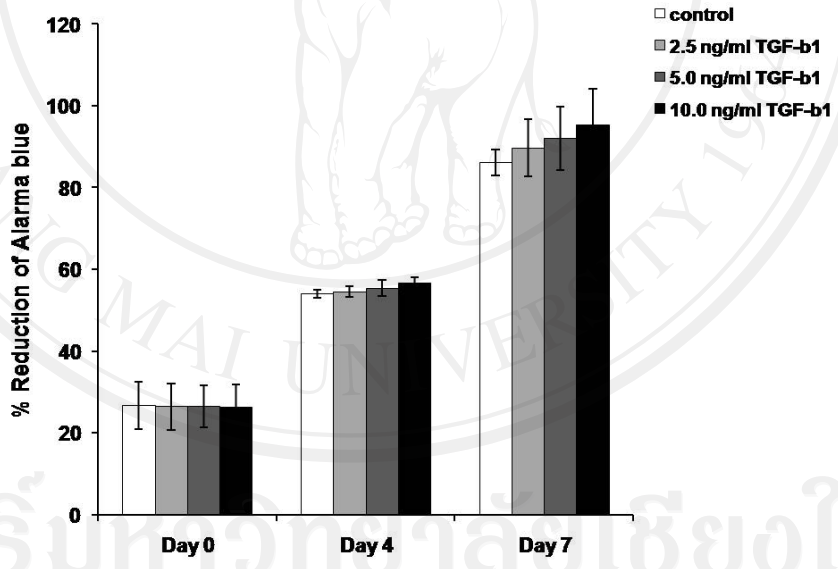

Figure 3.5 Effect of TGF-β1 concentrations (0, 2.5, 5.0 and 10.0 ng/ml) on equine chondrocyte proliferation estimated by Alamar blue assay on day 0, 4, 7 of culture.

3.5 Effect of TGF-β1 on HA synthesis in 3-dimensional cultured equine chondrocytes

Each gelatin scaffold was seeded with $1x10^6$ equine chondrocytes and cultivated in chondrogenic media (10% FCS-DMEM supplement with 10% ITS, 25 ug/ml ascorbic acid, 10^{-7}M dexamethasone) with or without TGF-β1 (10.0 ng/ml). The media were collected every two to three days for determination of HA amount. The constructs remained in culture for 21 days before being harvested. Figure 3.6 showed the constructs with and without TGF-β1 supplement. Constructs with TGF-β1 appeared to be more glossy and smoother than ones without TGF-β1.

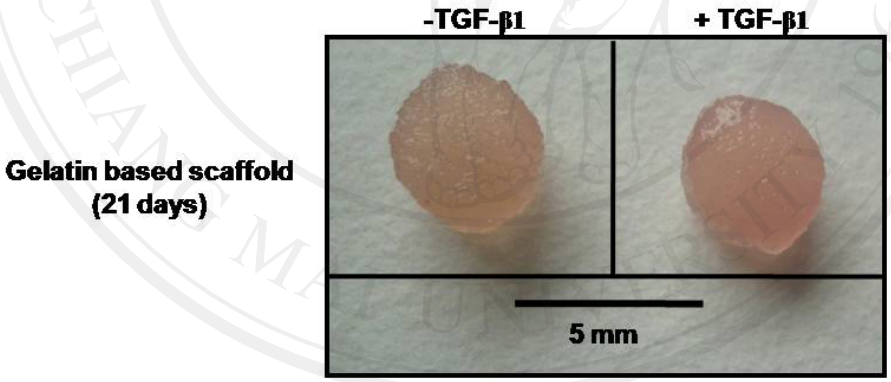

Figure 3.6 Gelatin based constructs of equine chondrocytes culture in chondrogenic media with or without 10.0 ng/ml TGF-β1 at 21 days.

HA amount in the conditioned media was determined using the competitive based enzyme-linked immunosorbent assay (ELISA) method. The result was shown in figure 3.7. HA amount in the culture media of 10 ng/ml TGF-β1 treated constructs was seemed to be higher than culture without TGF-β1 condition since day 3 until day 21; however, there was no significant difference.

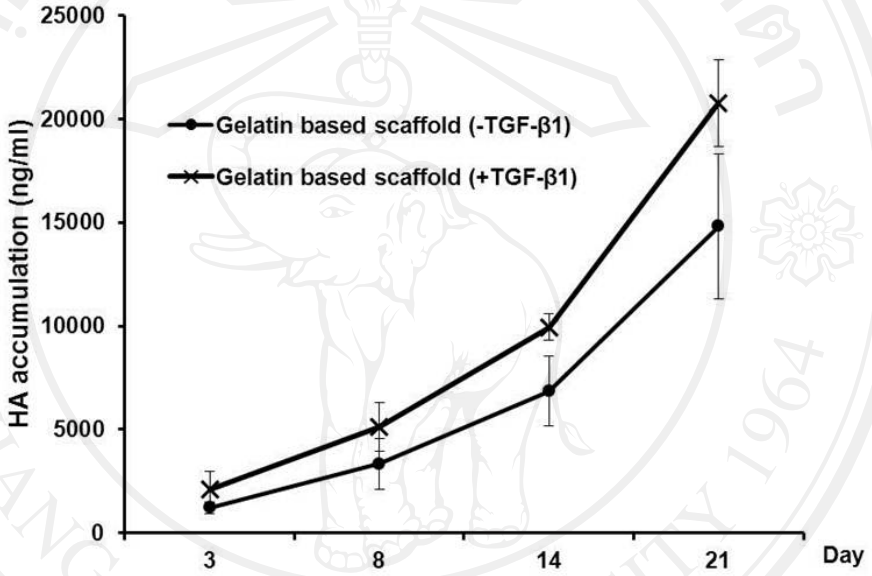

Figure 3.7 HA amount in media of equine chondrocyte seeded gelatin constructs with or without TGF-β1 condition. The scaffolds were cultivated for 21 days and media were collected every two to three days. HA amount in condition medium were determined using the competitive based enzyme-linked immunosorbent assay (ELISA) method.

After 21days, constructs were harvested and one construct from each treatment was digested with papain. Aliquots of the papain digest were analyzed for HA, uronic acid and DNA contents.

HA content of each cultured constructs were determined using the competitive based enzyme-linked immunosorbent assay (ELISA) method. They were normalized by the DNA content of each construct as shown in figure 3.8. The results showed that the HA content of cultured construct with TGF-β1 for 21 days was significantly higher than construct culture without TGF-β1 condition.

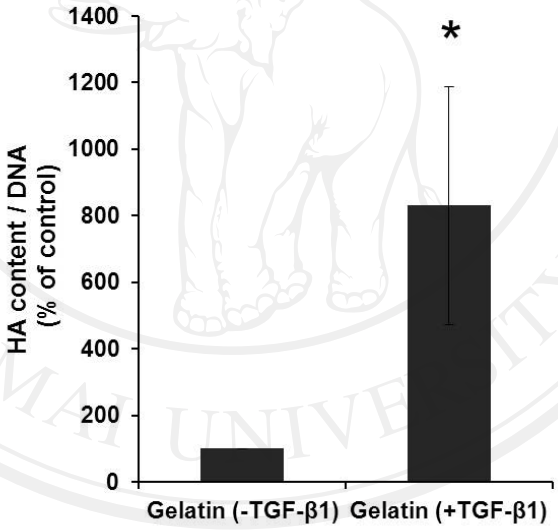

Figure 3.8 HA content of constructs were determined at day 21 using the competitive based enzyme-linked immunosorbent assay (ELISA) method and normalized by the DNA content of each construct.

* denote a value that is significantly different (p<0.05) from control.

Uronic acid content was also measured by colorimetric assay using carbazole method for confirmation of HA synthesis. The contents from each sample were normalized with the DNA content of each constructs. The result was shown in figure 3.9. Similar to HA content, the uronic acid content of construct cultured with TGF-β1 was significantly higher than construct culture without TGF-β1 condition.

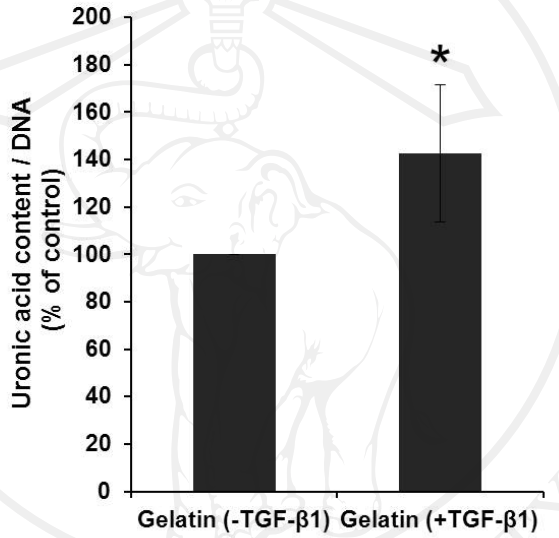

Figure 3.9 Uronic acid content of equine chondrocyte seeded gelatin constructs determined by colorimetric assay using m-hydroxydiphenyl and normalized with the DNA contents.

* denote a value that is significantly different (p<0.05) from control.

3.6 Effect of TGF-β1 on equine chondrocytes proliferation in 3-dimensional culture

Proliferation of equine chondrocytes culture in gelatin based scaffolds was evaluated using the Alamar blue assay and was shown in figure 3.10. The difference of chondrocyte proliferation between TGF-β1 treated and untreated samples was not observed during the first 7 days. But at day 14 and 21 chondrocyte proliferation was significantly lower in 3-D culture under TGF-β1conditions when compared to control.

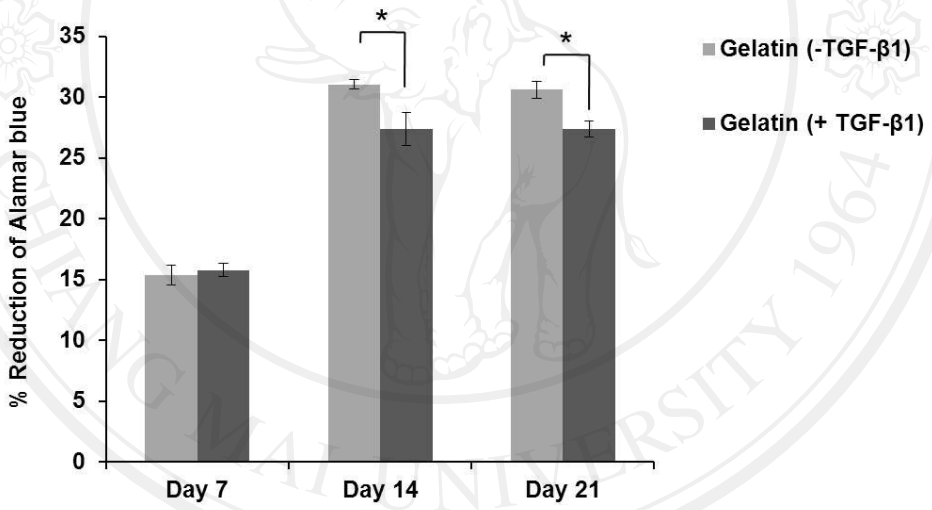

Figure 3.10 Proliferation of equine chondrocytes culture in gelatin based scaffold measured by Alamar blue assay. The constructs were incubated in plain chondrogenic medium and medium with 10.0 ng/ml TGF-β1.

* denote a value that is significantly different (p<0.05) from control.

The proliferation of equine chondrocytes culture in gelatin based scaffold was confirmed with DNA content as shown in figure 3.11. The DNA content was measured from aliquots of the papain digest and represent as μg DNA using the Hoechst dye 33258 assay. This result showed that DNA content was significantly lower in construct culture with TGF-β1 when compared to control. This result suggested that TGF-β1 decreased proliferation of chondrocytes culture in gelatin based scaffolds.

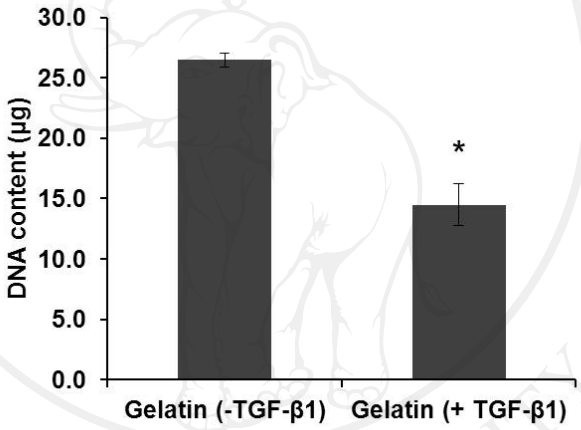

Figure 3.11 DNA content of each cultured constructs was determined by the Hoechst dye 33258 assay. The constructs were incubated in chondrogenic medium supplement with 10.0 ng/ml TGF-β1 compared with control.

* denote a value that is significantly different (p<0.05) from control.

3.7 Scanning electron micrographs and H&E staining of gelatin based scaffold seeded with equine chondrocytes

The equine chondrocytes were cultivated in 3-D gelatin scaffolds (Figure 3.12; A) nourished with chondrogenic media with or without TGF-β1 for 21 days. At the end of the experiment, the construct was observed by scanning electron microscope (SEM) as shown in figure 3.12. The appearance of TGF-β1 treated scaffolds was obviously different from control. The construct cultured without TGF-β1 showed porous surface lacking of extracellular matrix synthesis (Figure 3.12; B, D, F). In contrast, the porous appearance was not appeared in the construct cultured with TGF-β1 (Figure 3.12; C, E, G). But they formed a continuous layer of hyaline cartilaginous-like tissue throughout the surface of gelatin based scaffolds.

The sections of constructs aged 21 days were stained with H&E. The result was shown in figure 3.13. Chondrocytes randomly located throughout the constructs with no regards of TGF-β1 treatment. Interestingly, the construct cultured with TGF-β1 showed accumulation of cartilaginous-like extracellular matrix which formed as a continuous layer at the surface of scaffolds (Figure 3.13 D, E, F) while this layer was not observed in construct cultured without TGF-β1 (Figure 3.13 A, B, C).

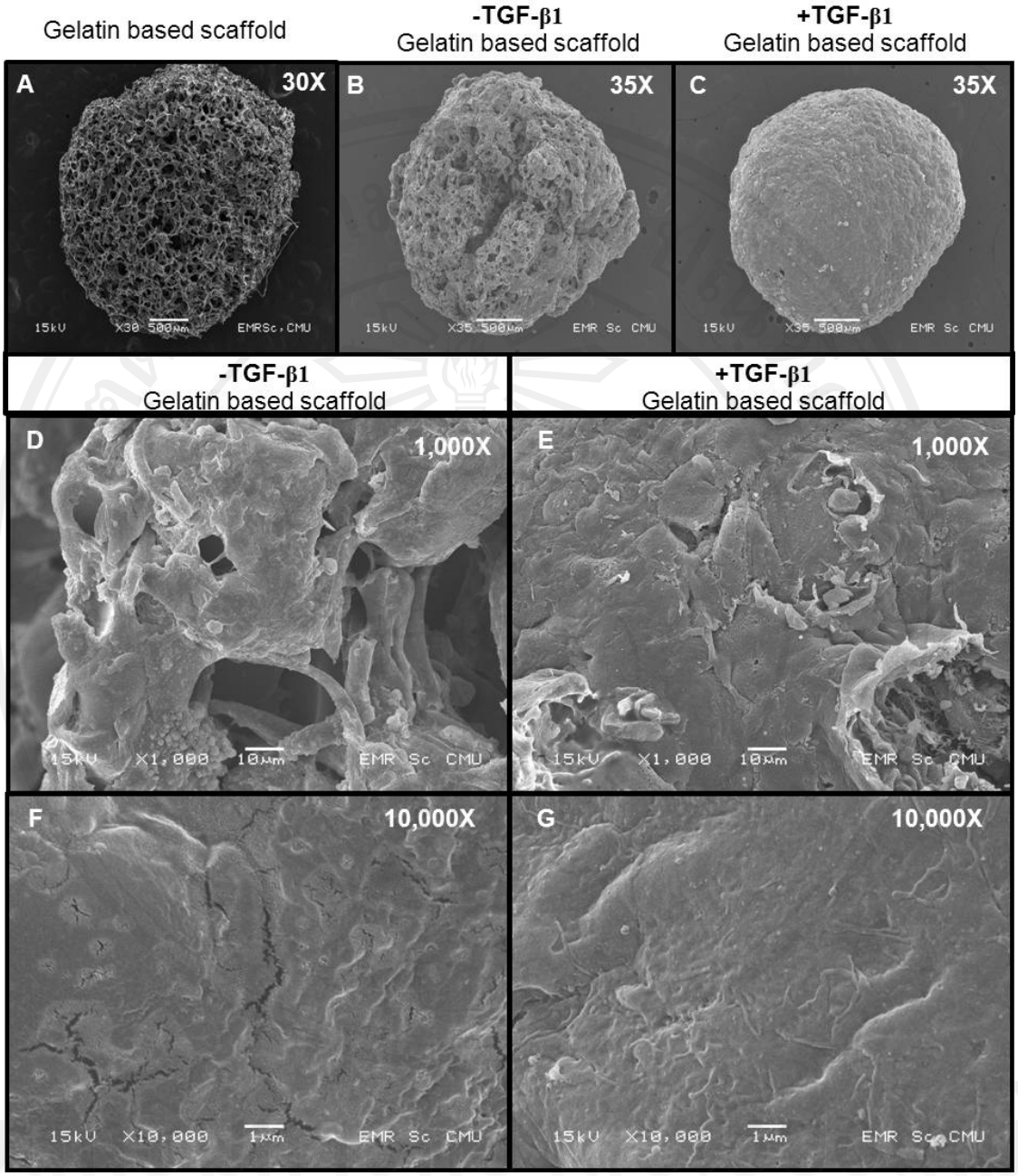

Figure 3.12 Scanning electron micrographs of gelatin based constructs embedded with equine chondrocytes culture in chondrogenic medium with (C, E, G) or without (B, D, F) TGF-β1 for 21 days.

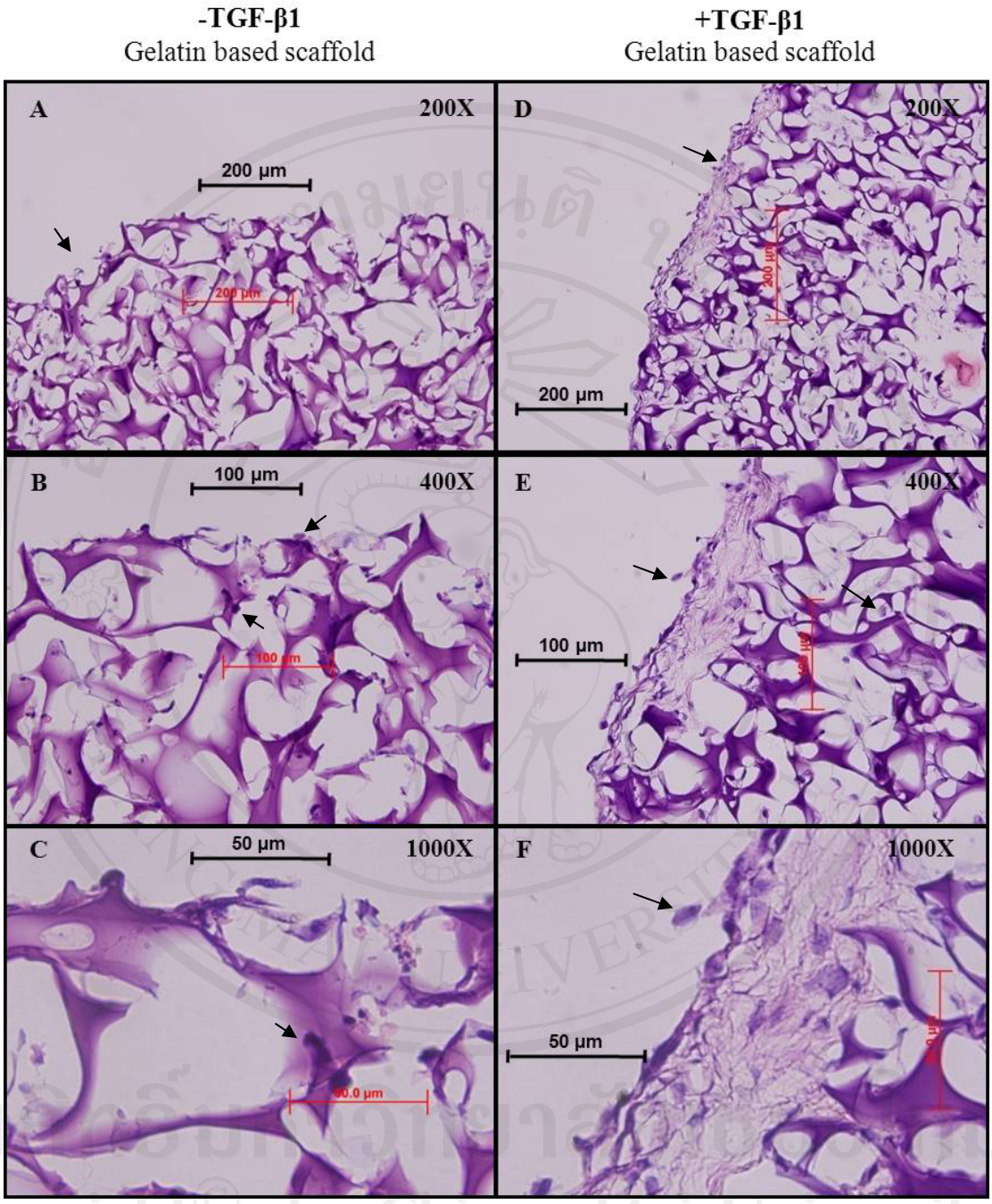

Figure 3.13 Hematoxylin and eosin stained section of gelatin based constructs seeded with equine chondrocytes. The construct cultured in chondrogenic medium with (D, E, F) or without (A, B, C) TGF-β1 for 21 days.

3.8 Histological and immunohistochemical analysis of cultured construct

To investigate HA synthesis immunohistochemistry was performed on sections of scaffolds embedded with equine chondrocytes at day 21 using primary antibody (biotinylated-HA binding proteins; B-HABPs) which specific binds to HA molecules. The result was shown in figure 3.14. The construct cultured with TGF-β1 (figure 3.14 D, E, F) was stronger stained as seen in brown color when compared to construct cultured without TGF-β1 (figure 3.14 A, B, C). The surface layer of the construct with TGF-β1 also appeared positive for this method (figure 3.14 D, E, F) confirming HA composition. This result suggested that TGF-β1 promoted HA synthesis in 3-dimensional equine chondrocyte culture.

Also, sections of the constructs were stained with alcian blue for proteoglycans investigation. Figure 3.15 demonstrated that the constructs in chondrogenic media supplemented with TGF-β1 had much stronger stain of Alcian blue (figure 3.15 D, E, F) when compare to constructs without TGF-β1. And the outer layer of the constructs treated with TGF-β1 appeared to have alcian blue positive ECM. This suggested composition of HA and other proteoglycans within the layer.

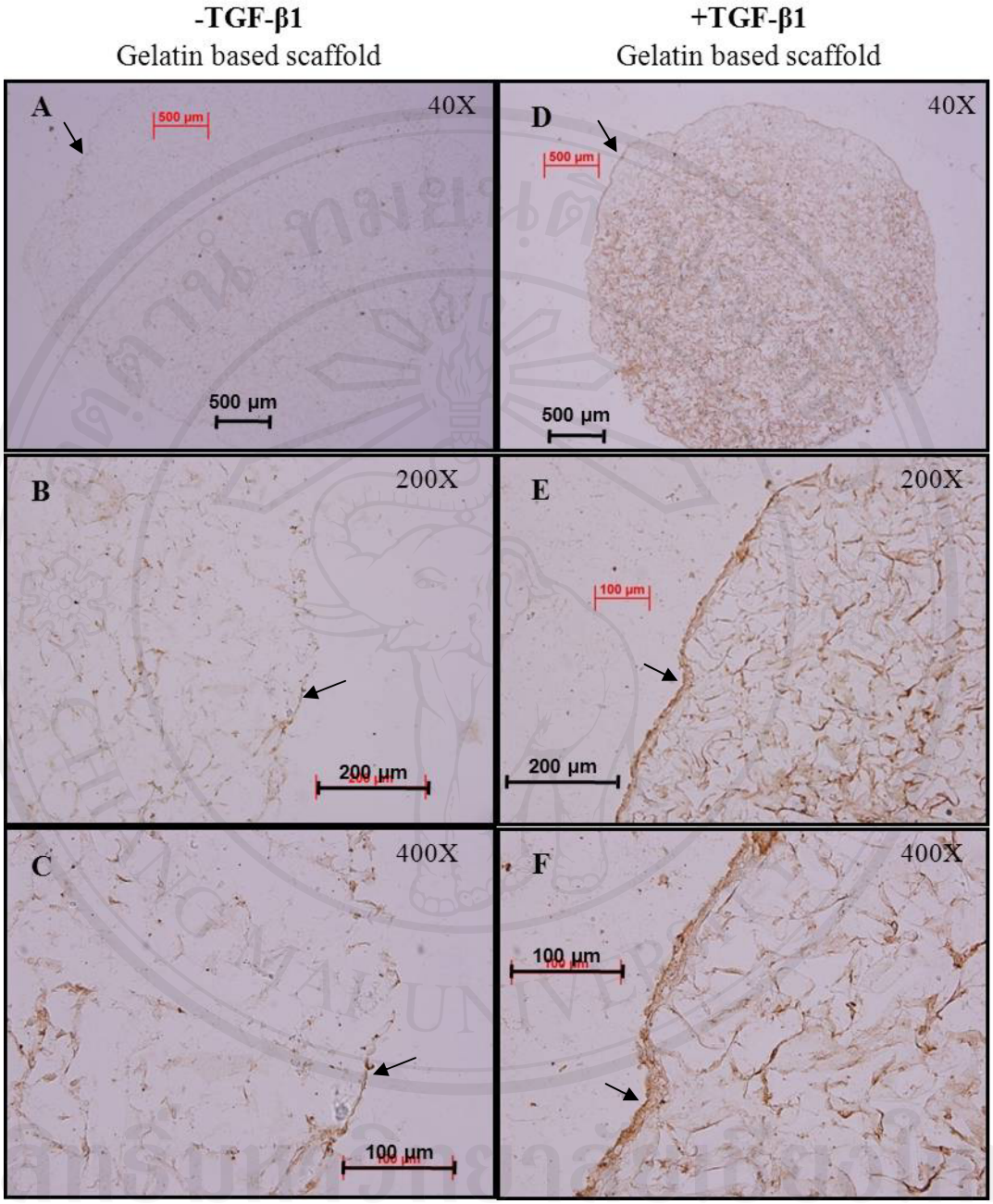

Figure 3.14 Immunohistochemical analysis of gelatin based scaffold for HA investigation. Constructs were cultivated in chondrogenic medium supplement with TGF-β1 (D, E, F) and without TGF-β1 (A, B, C) for 21 days.

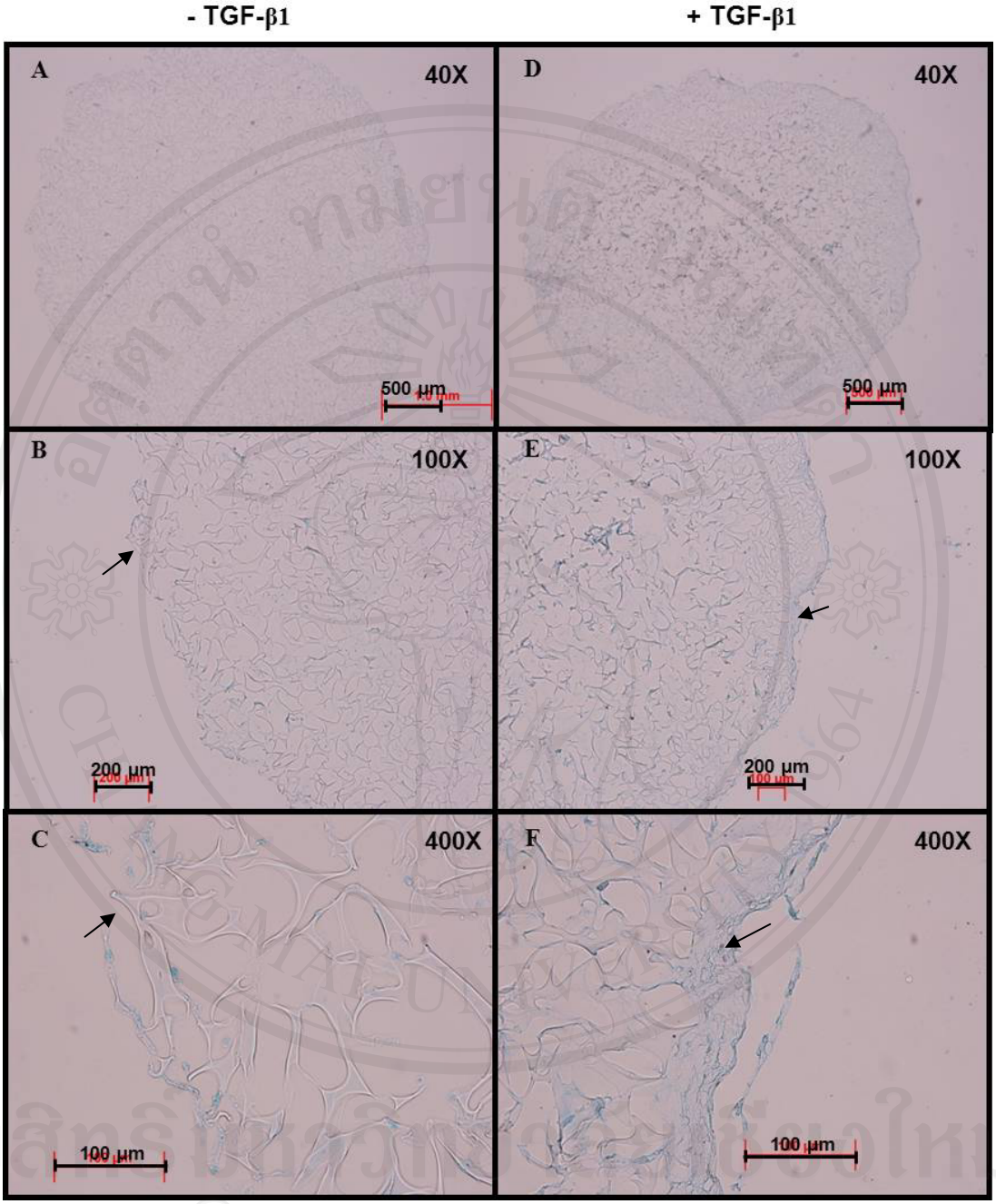

Figure 3.15 Histological analysis of constructs using Alcian blue staining for proteoglycans investigation. Constructs were seeded with equine chondrocytes and cultivated in chondrogenic medium with (D, E, F) or without (A, B, C) TGF-β1 for 21 days.