The Anti-Scarring (Wound Closure) Properties of TGF-β3, BSA/HCl and HCl in Cultured Human Bone Cell Monolayer

F. Sefat, M. Youseffi, R.F. Berends, S.A. Khaghani, and M.C.T Denyer

Abstract- **Bone repair can be modulated by different stimulus including growth factors. TGF-β3 is a cytokine known to be associated with the scarless healing of skin and it is highly probable that it may play a role in the repair of other tissues. Thus the aims of this study were to investigate the effect of TGF-β3 on closure of a model wound in cultured monolayers of the MG63 human bone cells. This *in vitro* work examined and compared the anti-scarring (wound closure) properties of TGF-β3, and its dosage carriers, HCl and BSA/HCl. The wound healing response was investigated in TC grade culture flasks by creating a wound (with average scratch width of 300µm±10-30µm SD, 1.7-5µm SEM) on confluent monolayer of MG63 human bone cell. After wounding cultures were then treated with 50ng/ml TGF-β3 at concentration of 4mM HCl and 1mg/ml BSA and Distilled water. Also the same method was applied for cell cultured monolayer with no growth factor as control and with HCl/BSA and HCl only solutions. After wounding, wound width was measured every 5 hours over a 30-hour period. The results showed that TGF-β3 (with addition of HCl and BSA/HCl) enhances the rate of wound repair in a monolayer of MG63 bone cells. After careful observation it was observed that after 20 hours all the culture flasks treated with TGF-β3 (with 15.5% of wound remained open), HCl (with 16% of wound remained open) and finally BSA/HCl (with 17.7% of wound remained open) had resulted in faster wound healing compared to control (with 85% of wound remained open). These results indicated that wound closure in model MG63 wound with TGF-β3 (with addition of HCl and BSA/HCl) is higher than the control. TGF-β3, HCl alone and HCl/BSA all enhanced the rate of wound repair in relation to the negative controls.**

Index Terms: **Bone cell engineering, BSA/HCl, HCl, TGF-β3, Wound healing.**

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I. Introduction

Bone Tissue Engineering is a promising field in the area of medicine and involves principles of biology and biomedical engineering with the aim of developing a viable tissue substitute that can restore the function of human tissue [1]. Despite healing of soft tissues, bone healing has features of degeneration, and usually no scar can be find after healing. As soon as the fracture has been bridged by new bone, it remodels. Bone repair (wound healing of bone) can be manipulated by different stimulus such as growth factors, distraction osteogenesis and electrical stimulation. TGF-β3 is a cytokine produced by different cell types inside the body and influences a number of cell activity such as differentiating, stimulating mesenchymal stem cell (MSC) growth, acting as a chemotactic factor and also enhances bone cells and extracellular matrix (ECM) product secretion [2].

II. Aims and objectives

Main objective was to investigate the effect of TGF-β3 on wound closure in cultured monolayers of MG63 bone cells. The lab-based experimental work investigated and compared the anti-scarring (wound closure) properties of TGF-β3, HCl and BSA/HCl in cultured dish environment using cultured monolayers of human bone cells. Other cellular responses such as proliferation, differentiation and detachment have also been investigated along with different stages of cell behaviour and morphology during wound healing.

III. Materials and Methods

Bone cells were cultured in a low glucose culture medium known as Dulbecco's Modified Eagle Medium (DMEM, from SIGMA) containing various supplements such as L-glutamine (4mM), Penicillin-Streptomycin (5ml), Amphotericin or a fungizone (1ml), HEPES buffered culture medium and 'fetal calf serum' (50ml). The bone cells were cultured inside culture flasks and bathed in the culture media. The cells attached to form a layer at the bottom of the culture flasks. A 'wound' was made using a disposable long nosed plastic pipette. The tip was bent downwards so that it could be inserted into the flasks. The tip was then drawn across the cells on the cultured surface creating the wound. The scratch markings facilitated orientation while imaging and another wound was later applied at 90 degree angle to the initial scratch and pictures were also taken at the cross points. Thus

the same points were always photographed which gave more accurate data for analysis.

A 'test experiment' was performed in order to determine the time frame of wound closure in the wounded models. This was similar to another experiment on wound closure using NIH/3T3 fibroblast monolayers [3] for which wound closure was achieved after ~300 minutes. In their study, cell monolayers were wounded with "the corner of a piece of Mylar film which is commonly used in copy machines". An average scratch of 300µm was produced which is equal to three to four times bigger than cell widths and wound closure was completed after ~300 minutes. During our 'test experiment' with TGF-β3, it became clear that complete wound closure was not achieved during this time frame and thus the time frame for our experiment was set to 30 hours with data collection every five hours.

This *in vitro* work examined and compared the anti-scarring (wound closure) properties of TGF-β3, and its dosage carriers, HCl and BSA/HCl. The wound healing response was investigated in TC grade culture flasks by creating a wound (with average scratch width of 300µm±10-30µm SD, 1.7-5µm SEM) on confluent monolayer of MG63 human bone cell. After wounding cultures were then treated with 50ng/ml TGF-β3 at concentration of 4mM HCl and 1mg/ml BSA and Distilled water. Also the same method was applied for cell cultured monolayer with no growth factor as control and with HCl/BSA and HCl only solutions.

After wounding, culture flasks were stored inside the incubator and wound width was imaged and measured every 5 hours over a 30-hour period. Image J software was used in order to measure the distance between the wound edges. Six vertical lines at semi-random horizontal distances were drawn and the distances between the intersections of the lines with the wound edges were measured as shown in Figure 1.

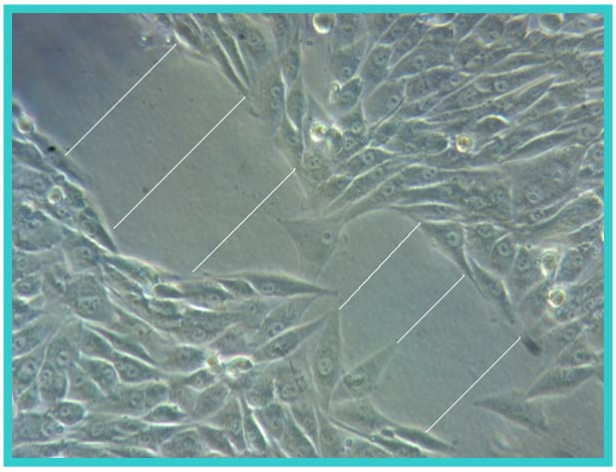

Figure 1. Measurement of wound closure width on cultured MG63 bone cells in the presence of TGF-ß3, HCl, BSA/HCl and control, using 'Image J' software.

Image analysis consisted of a set of six measurements of the wound width for each image. There were some images in which the wound was completely closed, but in few others there were still some gaps where cells had moved across to cover the wound. For these cases measurements of the gaps were also taken. For each image six separate sets of photos were taken along the marker line. This meant that two averages were needed. The first average was the average wound width within an image. The second was the average of the 'wound width averages' for each of the culture flasks. Measurements were taken every five hours for 30 hours. These wound width averages were plotted against time.

IV. Results and Discussion

The laboratory experiments performed during this study showed that TGF-β3 does indeed speed up the wound closure process in a pure cell culture environment. It was observed that after 20 hours of wounding all the culture flasks treated with TGF-β3, HCl and BSA/HCl had resulted in faster wound healing than the control. Fastest wound closure was achieved with TGF-β3, closely followed by the HCl and BSA/HCl in descending order with very little difference. These findings proved that in in-vitro cultured human bone cell, TGF-β3 acts as a growth factor enhancing wound repair and stimulant for wound closure.

Figure 2 shows all four wounded bone cell monolayers together during 30 hours of healing with addition of HCl, BSA/HCl, TGF-β3 and control. The average scratch width produced for all different culture flasks were 300µm±10-30µm SD, 1.7-5µm SEM.

Images for the wound closure process in the control culture flask show that there is no change in wound width even after 10 hours. Cell morphology changes can be observed only in the cells at the wound edges after 15 hours. These cells have elongated but without migration to the wound site. After 20 hours there were still gaps between the cells in the wound site. The cells in the control flask did not seem to form bridges with cells from the opposite wound margin to stabilize the wound site until about 25 hours. Cells meet each other after 25 hours and started making bridges but without complete closure. It was clear that wound remained open even after 25 hours and that after 15 hours ~90% of the wound still remained open. Wound healing occurred very slowly in control culture flasks and ~61.1% of wound remained open after 30 hours.

Images for the wound closure process in the HCl culture flask show that after five hours the morphology of the cells at the wound edges have changed and that the cells had elongated, spread, replicated and migrated into the wound site perpendicularly to the wound axis. The vertically elongated cells look different (more elongated) to the cells a distance away from the wound edges (more rounded). The cells seem to be less dense and more spread out at the wound edges. After 15 hours the cells had migrated into the wound site and elongated in order to meet cells from the opposite side of the wound. These cells then formed bridges to connect the wound edges and close the wound. As soon as the bridges formed in the wound site the wound was closed quite rapidly.

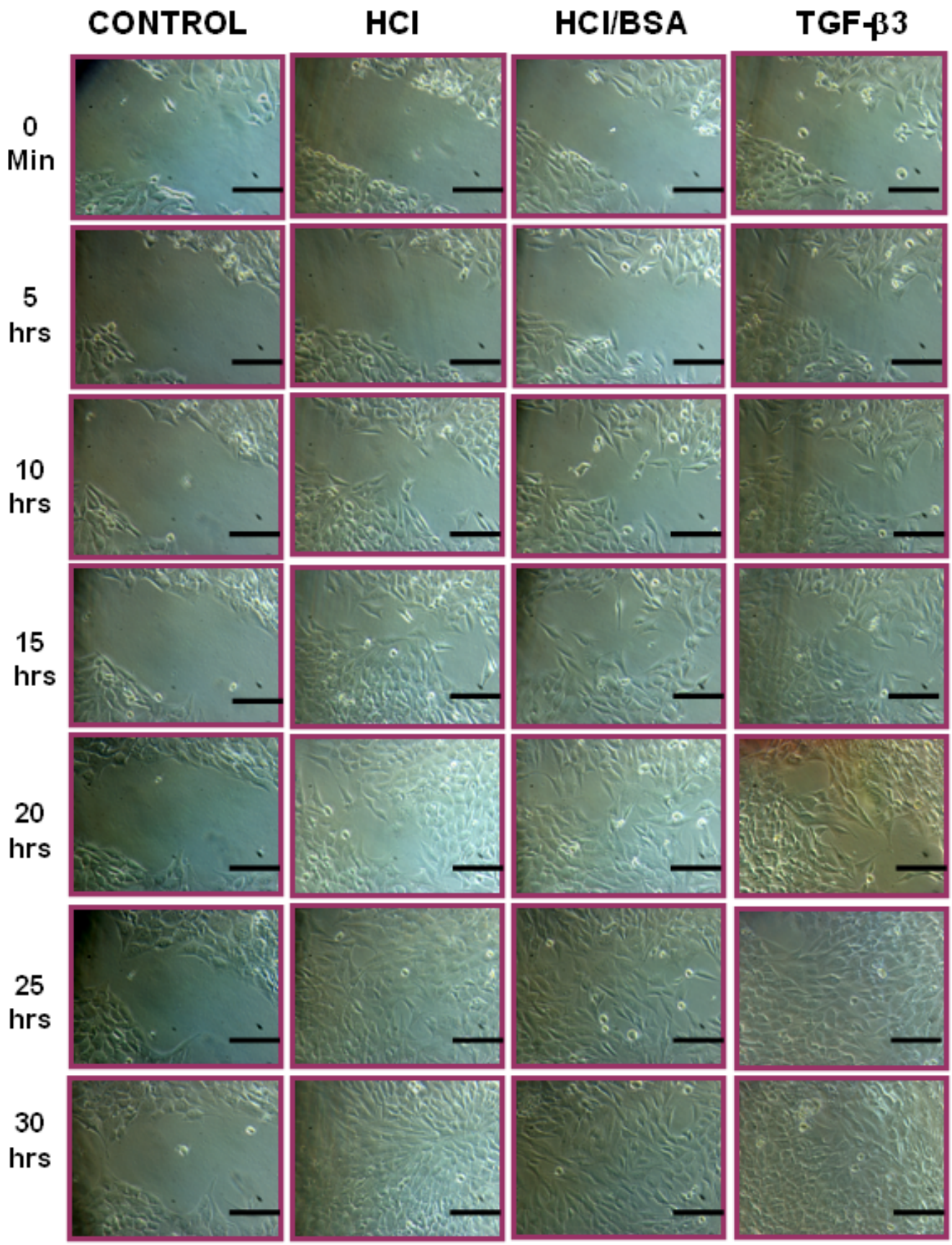

Figure 2. Images of the wound healing process for the bone cell monolayers with 50ng/ml of HCl, BSA/HCl, TGFβ3 and control for the period of 30 hours (Scale bar=100μm).

During bridge formation the cells a distance away from the wound edge aligned behind those bridges to organize the cell structure. After 25 hours the wound was closed and the cell morphology at the wound site was quite different from the morphology of the other cells. At the wound site the cells were more elongated whereas the cells away from the wound were more rounded and denser. There were still small gaps between cells at the wound site, which will eventually be closed. It was measured that after 20 hours the culture flasks with HCl showed high percentage of wound closure (only 15.5% remained open). By comparison between HCl and control it became clear that after 30 hours the wound in the control remained open by ~87% whereas HCl addition showed almost complete closure.

Images for the wound closure process in the BSA/HCl culture flask show that after five hours the morphology of the cells at the wound edges have changed and that the cells had elongated, spread, replicated and migrated into the wound site perpendicularly to the wound axis. The vertically elongated cells looked different (more elongated) to the cells a distance away from the wound edges (more rounded). The cells looked less dense and more spread out at the wound edges. After 15 hours, the cells looked migrated into the wound site and elongated in order to meet cells from the opposite side of the wound similar to HCl culture flask. These cells then formed bridges to connect the wound edges and closed the wound. As soon as the bridges are formed in the wound site the wound closed quite rapidly. During bridge formation the cells a distance away from the wound edge aligned behind those bridges to organize the cell structure.

After 25 hours the wound was closed and the cell morphology at the wound site was quite different from the morphology of the other cells. At the wound site the cells were more elongated whereas the cells away from the wound were more rounded and denser. There were still small gaps between cells at the wound site, which are expected to close eventually. HCl and BSA/HCl culture flasks showed very similar results with high percentage of wound closure (only ~15-18% of wound remained open). Fast healing occurred in BSA/HCl culture flasks and only 10% of the wound remained open after 25 hours.

Images for the wound closure process in the TGF-β3 culture flask show that after five hours the cells had already elongated and started to migrate perpendicularly to the wound axis. Thus the surface area of the cells at the wound edge had increased due to cells replicating and spreading into the wound site.

After 10 hours the cells had already covered a great part of the wound site. Cells from both wound edges had elongated in order to contact the opposite cells and form bridges. Cells had migrated along these bridges and a distance away from the wound edge they were aligned to reorganize the cell structure. These cells had elongated in order to fill the gaps created by the migrated and elongated cells at the wound edges.

After 20 hours, cells covered the wound site, but there were still some gaps between cells. It was observed that after 20 hours all the culture flasks with TGF-β3 showed high percentage of wound closure (only ~16% remained open). Elongation and bridging of the cells inside and outside the wound site was also observed and wound healing was almost completed after 25 hours.

The laboratory experiments showed that TGF-β3 does indeed speed up the wound closure process in a pure cell culture environment. HCl and BSA/HCl did not act as a growth stimulant in a pure cell culture environment but they seem to enhance wound closure as compared to the control. This result proved that TGF-β3 helped to speed up the wound healing process as shown in Figures 3a and 3b, and almost similar to HCl and BSA/HCl additions.

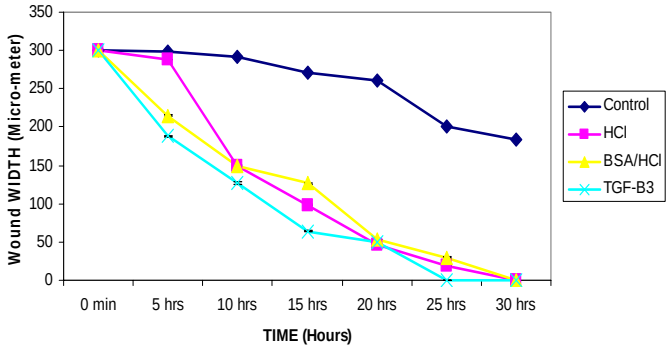

Figure 3a. Graph of wound closure width against time for wounded bone cell monolayers with addition of HCl, BSA/HCl, TGFβ3 and control.

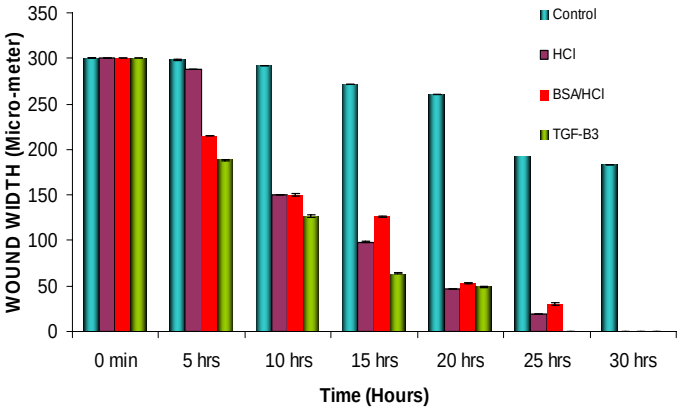

Figure 3b. Bar chart showing the comparison in wound width with time for control, HCl, BSA/HC and TGFβ3 additions.

Our results suggest that wound healing took place in all four culture flasks but at different rates. TGFβ3 additions caused model wounds to heal fully after 25 hours whilst control wound remained open even after 30 hours.

Figures 4a and 4b compares the % wound remaining open against time for all four conditions, with control showing the minimum healing, whereas growth stimulators resulted in faster and almost complete healing particularly with TGFβ3 addition.

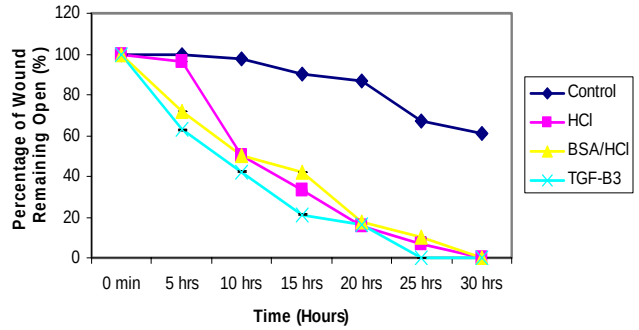

Figure 4a. Graph of % wound closure with time for wounded bone cell monolayers with addition of HCl, BSA/HCl, TGFβ3 and control.

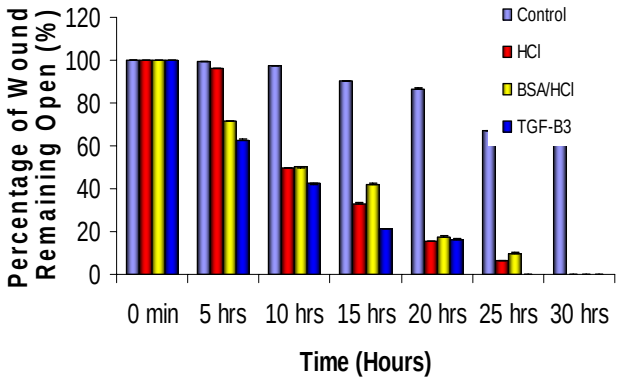

Figure 4b. Bar chart showing the comparison in % wound closure with time for control, HCl, BSA/HCl and TGFβ3.

Three different stages in cell behaviour and morphology could be distinguished for the TGF-β3 treated and control flasks: spreading, migration and bridge formation of the cells. This confirmed earlier observations in bone cell cultures [4]. In the TGF-β3, HCl and BSA/HCl flasks cells proliferated, replicated and grew well whereas in the control flasks the cells did not replicate well as fast as others. Image analysis revealed that in the TGF-β3, HCl and BSA/HCl flasks proliferation seemed to take place not directly at wound edges, but further away from them. This could be due to certain factors released by the cells during wound infliction. These factors could inhibit or block cell signalling and hence prevent cell proliferation at wound margins. However, this is only a hypothesis as the scratching had mostly just torn the cells away rather than cutting them. Applied wound scratches to the cell monolayers were not straight but uneven. This resulted in great variations in wound width and throughout different culture flasks. Normalization of the data was performed to analyze and compare the results.

V. STATISTICAL ANALYSIS

Following scratching bone cell monolayer (approximately 300 μm±10-30μm SD, 1.7-5μm SEM) at 5 hours of incubation the wound width showed different rate of healing according to different treatment. This rate was significantly different for HCl treated cells compared to un-treated (control). However, the wound width showed significant enhancement in the rate of wound closure for BSA/HCl ($P < 0.001$) and TGF-β3 ($P < 0.001$). As time of the incubation increased (for instance 20 hours of incubation), the rate of wound closure became gradually similar to each other in case of TGF-β3 ($P < 0.05$), HCl ($P < 0.05$) and HCl/BSA ($P < 0.001$).

By comparing all the treatments with respect to time required for complete wound closure, all three treatments demonstrated significant difference in wound repair compared to control. TGF-β3 appeared to heal at a faster rate than HCl and BSA/HCl.

Comparative results were given as means ±SE. The significant values of the difference were tested by one-way ANOVA followed by the Bonferroni adjustment.

TGF-β3, HCl and HCl/BSA demonstrated to significantly ($P < 0.001$) enhance the rate of wound closure compared to the control. Error bars = 95% CI.

VI. CONCLUSIONS

The laboratory experiments performed during this study showed that TGF-β3 does indeed speed up the wound closure process in a pure cell culture environment. It was observed that after 20 hours all the culture flasks treated with TGF-β3, HCl and BSA/HCl had resulted in faster wound healing than the control. Fastest wound closure was achieved with TGF-β3, closely followed by the HCl and BSA/HCl in descending order with very little difference.

The control culture flasks did not perform well with 86.8% of the wound remained open after 20 hours. In contrast, the HCl treated culture flask had only 15.5% of the wound still open. This indicates that HCl had significant effect on wound closure. Also, with BSA/HCl addition the % of wound remained open was 17.7% after 20 hours. This effect can be seen quite clearly for both HCl and BSA/HCl and HCl that causes faster healing compared to BSA/HCl.

The TGF-β3 treated culture flask had 16% of the wound still open after 20 hours but the remaining wound healed very fast after 25 hours and the percentage of wound remaining was almost zero which meant that wound healed completely after 25 hours. These results indicate that addition of TGF-β3, HCl, and BSA/HCl show good wound healing process and that these additions act as growth stimulators.

In conclusion, it can be said that TGF-β3 did have a positive effect on wound closure in a pure cell culture environment whereas control did not have similar effects. TGF-β3, HCl alone and HCl/BSA all enhanced the rate of wound repair indicating that TGF-β3 does not act alone in the wound repair system, but instead functions synergistically with signalling pathways that are dependent on the availability of hydrogen ions. Such a mechanism would depend on signalling molecules undergoing a conformational change on binding hydrogen ions. This is not a new concept, one only has to think of haemoglobin's affinity for oxygen as a prime example, but it is potentially a concept that has been overlooked in the wound repair system.

This result is important as it shows that healing occurs in control flask but in longer period of time. This work concurs with what was found in the literature [4]. However, no clinical research has yet been undertaken on TGF-β3 thus this work is a first step to evaluate TGF-β3, HCl and HCl/BSA presence in relation to wound closure and the healing process for bone cell monolayers.

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