The Effect of Different HCl Concentrations on Wound Healing of Bone Cell Monolayer

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Abstract- **Bone repair has been studied in order to understand the complex phases involved that result in full conformity of the original bone. Our body has the unique ability to heal a bone fracture without scarring. Bone repair is modulated by different stimulus including growth factors. Transforming Growth Factor-beta (TGF-β) super-family have been studied with strong evidence in vitro and in vivo that TGF-β has significant effects on bone construction and resorption by regulating the duplication and differentiation of chondrocytes, osteoblasts and osteoclasts. Dosage carriers of TGF-β1, 2 or 3 namely HCl was used in vitro to see the effects it has on closure rates using a model wound in cultured monolayers of MG63 bone cells. The wound healing time was investigated using 6.25µl, 12.5µl, 25µl and 50µl concentrations of HCl compared against control. The model wound was made on fully confluent monolayers of MG63 bone cells with an average wound width of 300µm ± 10-30µm. For each concentration of HCl and control after wounding, the wound width was measured over a 30hr period. The results showed that after the 30hr period, the 25µl and 50µl concentrations of HCl enhanced the speed of wound closure in vitro as compared to the control. The culture treated with 25µl concentration of HCl was fully confluent at 25hrs and the 50µl concentration of HCl showed the highest percentage of wound closure but taking the full 30hrs to become confluent. The 6.25µl and 12.5µl concentrations of HCl had slower closure rates than that of the higher concentrations with confluency not achieved at 30hrs. Results from un-treated flasks (control) showed slowest wound closure compared to treated flasks with HCl.**

Index Terms: **Bone monolayer repair; HCl; Transforming Growth Factor (TGF-β3); Wound Healing.**

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I. Introduction

Bone is unique in nature since it has the ability to heal similarly to its original form without the development of a fibrous scar [1,2]. The regeneration of bone goes under a complex set of four overlapping phases, which are characterized by specific molecular and cellular events [3,4,5]. The four phases include: the haematoma formation/inflammatory phase, the soft callus formation, the hard callus formation and the remodeling process. Bone has the ability to repair itself using different methods depending on the biophysical environment. Ultimitely bone synthesis is achieved through osetoblasts via woven and/or lamellar matrix. Investigations have shown that with an increased ageing population healthcare in the United Kingdom is set to cost over £900 million each year [6,7]. A large percentage of that will go to the 150,000 fractures each year in the United Kingdom due to osteoporosis alone.

There are many factors that have effect on bone repair including age, nutrients, hormones, and growth factors, etc. The transforming growth factor-Beta (TGF-β) superfamily has been accepted as the most popular stimulus with strong evidence in both in vitro and in vivo studies that TGF-β has detrimental effects on bone construction and resorption by regulating the duplication and differentiation of chondrocytes, osteoblasts and osteoclasts [8]. Other groups claimed that bone is formed in vivo by osteoblasts when TGF-β has been injected into the fracture site [9,10].

TGF-β has been identified in the fracture haematoma and surrounding periosteal mesenchymal cells with staining techniques [11]. This is thought to orchestrate the cascade of cellular events resulting in fracture repair. The three isoforms (TGF-β1, -β2, -β3) have been found [12] to express overlapping patterns in vivo and with nearly identical biological activities in vitro. TGF-β1 is more potent throughout adult development, whereas TGF- β3 is more potent in tissues with mesenchymal origin. The three isoforms signal through the same kinase receptor with different binding abilities [12].

In order to understand how TGF-β regulates bone repair and formation it is necessary for in vitro experiments using osteoblast cell lines, which are mainly derived from osteosarcomas, and share phenotype similarities to the osteoblast cell. In-vitro experiments have proven that TGF-β stimulates osteoblast chemotaxis, DNA synthesis, cell division, and promotion of bone matrix proteins [13].

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TGF-β is difficult to manufacture due to its poor physical and chemical instability in aqueous solution and in powder form. When TGF-β is ready to be administered it is reconstituted into a liquid form before use. A number of problems can exist with TGF-β in liquid formulations, e.g. aggregation can take place under certain conditions in which TGF-β can loose its biological activity due to dimidiation and/or oxidation. Also, TGF-β has a high binding affinity for surfaces of containers which will reduce the percentage of TGF-β that will be administered reducing the actual dose delivered to the patient.

It is therefore very important for the growth factor to be delivered properly for ensuring a stable form and without losing its bioactivity. To overcome these problems HCl is added to the TGF-β to stabilize the solution and reconstitute it from the powder form. Upon cell culturing further HCl is added to TGF-β, which prevents losses allowing a final concentration ranging from 0.1ng/ml to 10ng/ml in most culture systems [14].

A physiological carrier is also added, e.g. Bovine Serum Albumin (BSA), which acts as a carrier for the TGF-β hindering adherence to surfaces of equipment. There are many different delivery systems that TGF-β can be administered to a wound site, e.g. by gradual release from a scaffold (usually biodegradable which releases TGF-β intermittingly) or it can be administered directly by injection to the wound site. Administration of TGF-β adjacent to the periosteum has been found to increase bone thickness and chondrogenesis [10,11]. TGF-β can be secreted from bone in its latent form, were TGF-β can be found in abundance. The latent form of TGF-β has a non-covalent association with LAP protein. LAP protein had a unique structure, in that it contained a set of leucine-rich repeats (LRRs) as well as a PSD-95/Dlg/ZO-1 (PDZ) domain; these domains are thought to mediate protein–protein interactions [15]. Activation of the TGF- β can be achieved through acidification with dissociation of the LAP protein that unmasks TGF- β binding receptors and hence activating the TGF- β mechanism [16].

However, it has been proposed [17] that TGF-β may not be the only reason for increasing the rate of wound repair. As mentioned earlier, in order for TGF-β to be administered exogenously it needs to be dissolved with HCl and BSA used as carriers of TGF-β. It is suggested that HCl also contributes to the rate of wound repair [1]. It is therefore important to investigate what role HCl actually plays on wound closure and that there are areas of bone injury in vivo that have been found to be acidic which also activate TGF-β [17,18,19].

II. Aims and objectives

Main objective was to investigate the effect of different HCl concentrations on wound closure in cultured monolayers of MG63 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

A. Cell Culture

Bone cells were cultured in a low glucose Dulbecco's Modified Eagle Medium (DMEM, 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 other experiments on wound closure [20,21]

B. Preparation of HCl Dilutions

This *in vitro* work examined and compared wound closure properties of TGF-β solvent "HCl" in various concentrations. 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. A stock solution of 32mM of HCl was made from a source of 2.5mM of HCl. 32mM concentration was the desired concentration to work with in this study, as it is the same concentration that TGF-β is dissolved in so that it can be administered exogenously. After wounding, cultures were then treated with 6.25μl, 12.5μl, 25μl and 50μl of HCl at concentrations of 20μM, 40 μM, 80 μM and 160 μM HCl. Also, the same method was applied for cell-cultured monolayer with no growth factor as control. Due to variations in wound width, normalization was performed for proper data analyse.

C. Data acquisition

After wounding, culture flasks were stored inside the incubator and wound width was imaged and measured every 5hrs over a 30hrs period. The whole procedure was repeated three times. Image J software was used in order to measure the distance between the wound edges. Ten 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.

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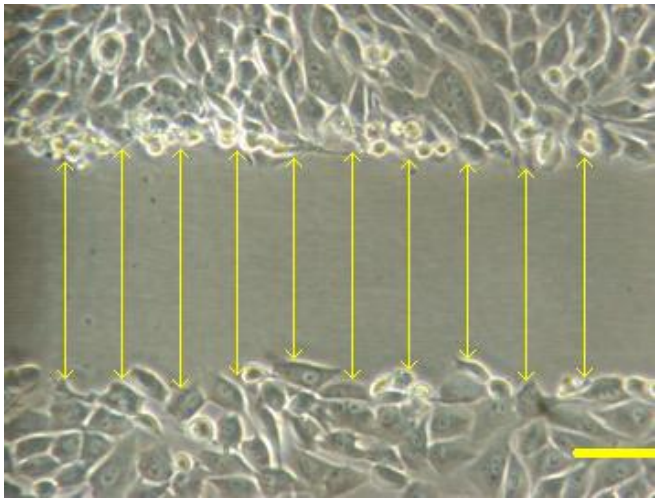

Figure 1. Measurement of wound closure width on cultured MG63 bone cells in the presence of different HCl concentrations and control, using 'Image J' software. (Error bar = 100 µm)

Image analysis consisted of a set of ten 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 5hrs for 30hrs. These wound width averages were plotted against time.

IV. Results

The laboratory experiments performed during this study showed that a higher concentration of HCl (25µl and 50µl) enhanced the speed of wound closure in in vitro environment. It was statistically found that after 30hrs of wounding the culture flasks treated with 25µl and 50µl concentrations of HCl resulted in faster rate of wound closure compared to the lower concentrations of HCl and control.

Fastest wound closure was achieved with 25µl concentration of HCl (percentage of wound remained open after 15hrs of culture was ~27%), closely followed by the 50µl (percentage of wound remained open after 15hrs of culture was ~15%) in descending order. The culture treated with 25µl concentration of HCl was fully confluent at 25hrs with the 50µl HCl showed the greatest percentage of wound closure but taking the full 30hrs to heal. Images of the wound closure process for the 25µl and 50µl HCl showed that after 5hrs the morphology of the cells at the wound edges had 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 seemed to be less dense and more spread out at the wound edges. After 15hrs 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 gap. As soon as the bridges formed in the wound site the wound was closed quite rapidly.

The 6.25µl and 12.5µl concentrations of HCl and control had slower closure rates compared to the higher HCl concentrations with full confluency not achieved even after 30hrs. Images for the wound closure process in the 6.25µl and 12.5µl HCl culture flasks (see Figure 2) showed that there were no changes in wound width even after 10hrs. Cell morphology changes could be observed only in the cells at the wound edges after 15hrs. These cells had elongated but without migration to the wound site. After 20hrs there were still huge gaps between the cells in the wound site, for the 6.25 and 12µl HCl.

The cells in the control flask showed even slower wound closure compared with the higher concentrations of HCl. The cells in the 6.25µl and 12.5µl HCl culture flasks did not seem to form bridges with cells from the opposite wound margin to stabilize the wound site until about 25hrs. Cells met each other after 25hrs and started making bridges but without complete closure. It was clear that wound remained open even after 25hrs. The percentage of wound remained open after 15hrs of culture was ~68% in the case of 6.25µl culture flask and~59% in the case of 12.5µl culture flask. Wound healing occurred very slowly in control culture flasks (percentage of wound remained open after 15hrs of culture was ~63%).

These findings clearly suggested that in in-vitro cultured human bone cell environment, HCl acts as a stimulant enhancing wound repair. Figure 2 shows all four wounded bone cell monolayers during 30hrs of healing with addition of different HCl concentrations and control.

These experiments showed that culture flasks treated with 25µl and 50µl concentrations of HCl did indeed speed up the wound closure process in a pure cell culture environment. HCl did not act as a growth stimulant but enhanced wound closure as compared to the control (See Figures 3a and 3b).

These results, however, showed that wound healing took place in all five culture flasks but at different rates. HCl additions with 25µl and 50µl concentrations caused model wounds to heal fully after 25hrs whilst wounds with 6.26µl and 12.5µl HCl concentrations and control remained open even after 30hrs. Figures 4a and 4b compare the % wound remaining open against time for all five conditions.

The rate of wound closure for each HCl concentration was calculated and a graph of wound closure rate vs. time was plotted, as presented in Figures 5a and 5b. It became apparent that the higher the HCl concentration the faster the rate of wound closure up to a peak value after which the rate decreased.

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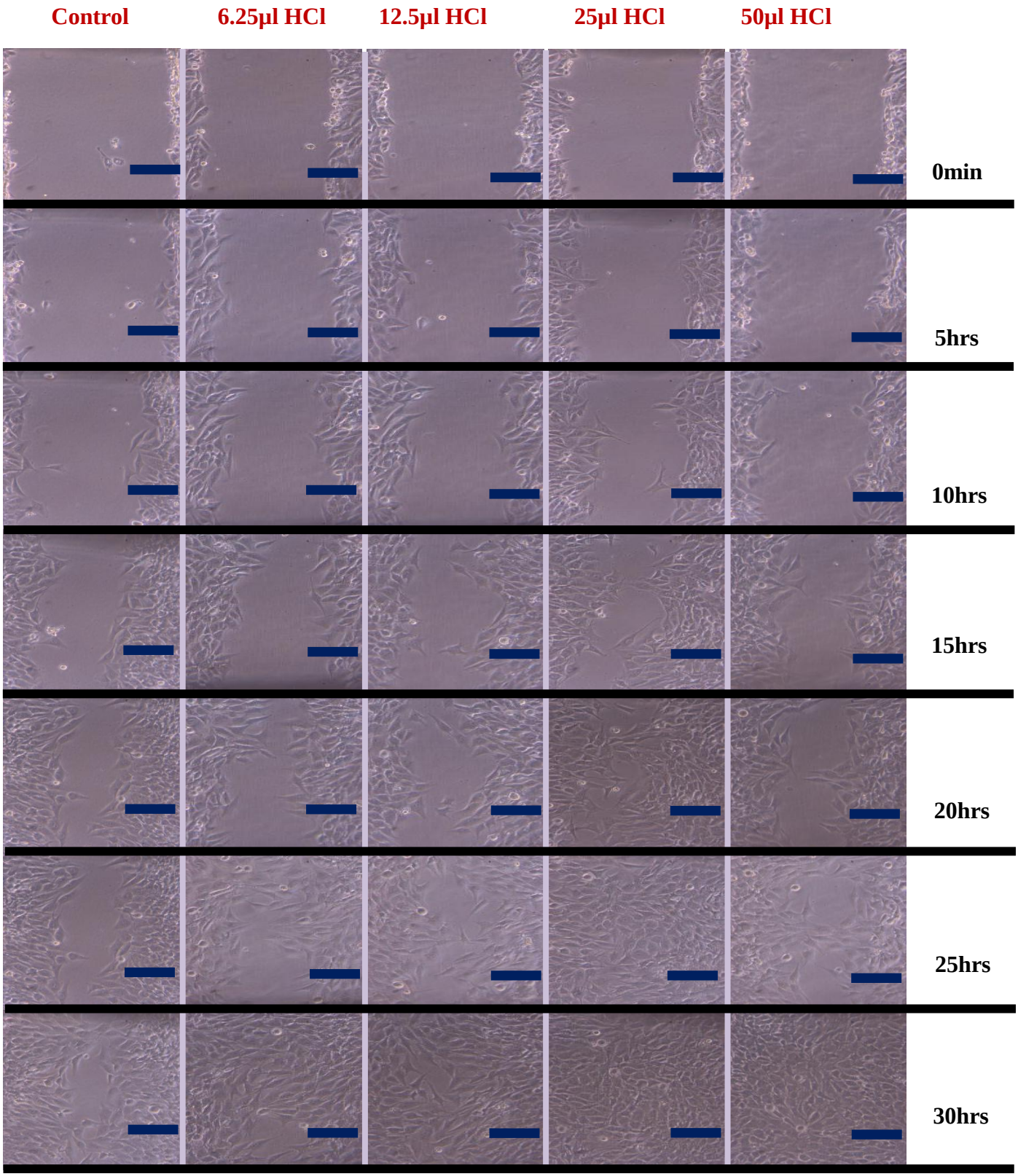

Figure 2. Images of the wound healing process for the bone cell monolayers with four different HCl concentrations and control for the period of 30hrs (Scale bar=100µm).

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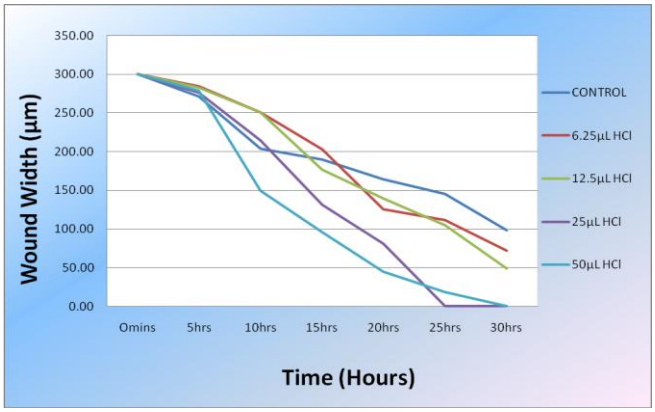

Figure 3a. Graph of wound closure width against time for wounded bone cell monolayers with addition of different HCl concentrations and control.

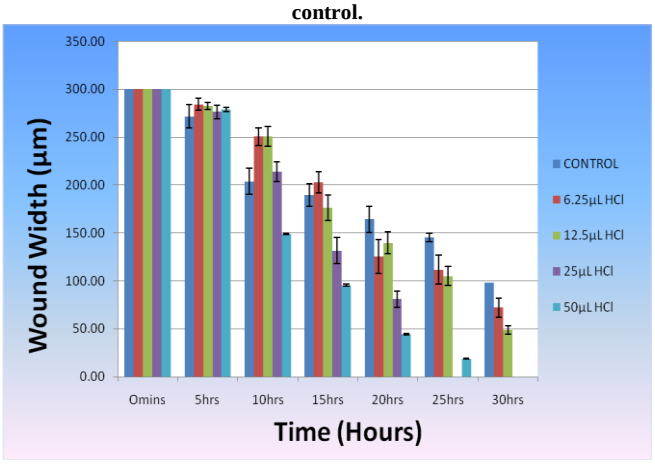

Figure 3b. Bar chart showing the comparison in wound width against time for addition of different HCl concentrations and control.

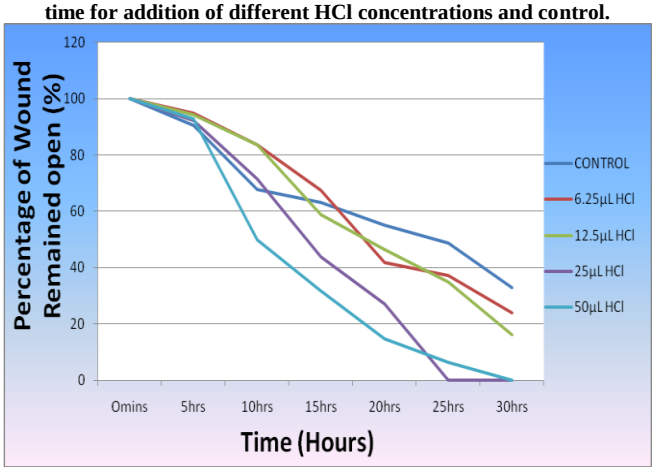

Figure 4a. Graph of % wound closure with time for wounded bone cell monolayers with addition of different HCl concentrations and control.

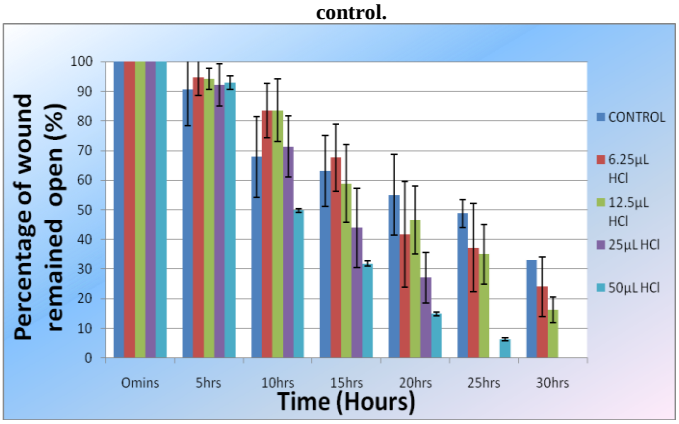

Figure 4b. Bar chart showing the comparison in % wound closure with time for addition of different HCl concentrations and control.

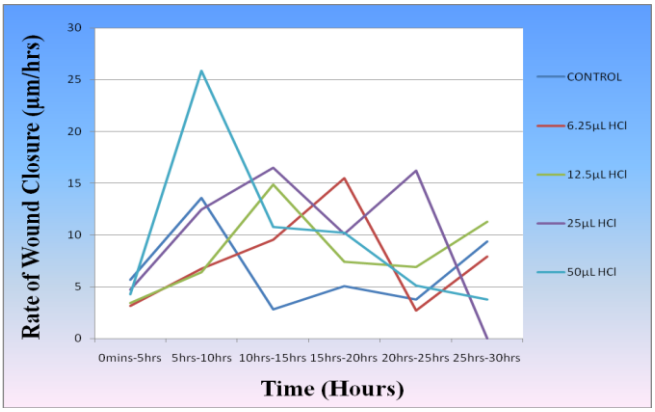

Figure 5a. Rate of Wound closure for different concentrations of HCl.

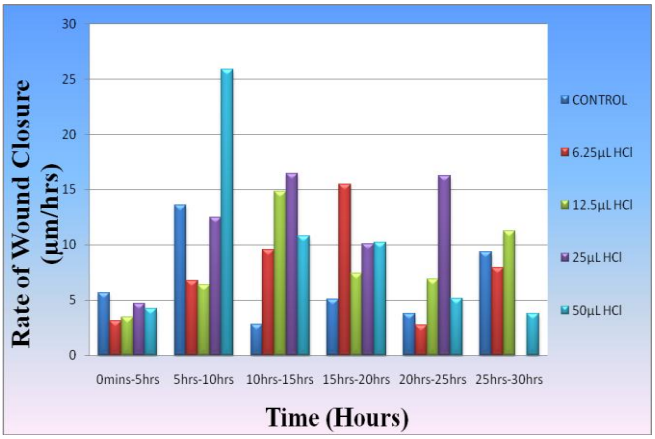

Figure 5b. Rate of Wound closure for different concentrations of HCl.

Three different stages in cell behaviour and morphology could be distinguished for the culture flasks treated with different HCl concentration and control flasks: spreading, migration and bridge formation of the cells. This confirmed earlier observations in bone cell cultures [21,22]. In the case of higher concentrations of HCl cells proliferated, replicated and grew well. Image analysis revealed that proliferation seemed to take place not directly at wound edges, but further away from them. This could be due to certain chemicals released by the cells during wound infliction. These chemicals 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 in half.

V. Discussion

From observation and upon initial scratch it was clearly seen that there were a number of dead cells appeared at the edges of the wound. It took a while for these dead cells to become detached. At 5 to 10hrs the MG63 bone cells at the edge of the wound became elongated and larger than the original existing bone cells and this could be seen more clearly in the higher concentrations of HCl.

HCl addition clearly changed the morphology of the MG63 bone cells. Replication occurred with the cells becoming linear to the wound edge. Dendritic like projections appeared from the elongated cells with some of the bone cells appearing to have as many as four projections. The bone cells, which

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had close neighboring cells, showed fewer projections so that they could easily connect. The less stranded bone cells had more projections trying to find a connection with other bone cells especially in the higher concentrations of HCl. After 15hrs and 20hrs it could be seen that at certain points along the wound width, peaks started to form so that bridges were made across the width of the wound to meet cells on the opposite side of the wound. This is a crucial part of the healing process when contact bridging has been accomplished. This closed the area of the wound further with the cells being able to make contact more easily in a smaller space. Cells such as 3T3 fibroblasts migrate into a model wound site and known to replicate exponentially (1 to 2, 2 to 4, 4 to 8, etc.) until contact inhibition takes place which then slows the rate of replication [23].

Our results showed that bone cells replicate in non-linear way and divide forming daughter cells that form a complete and new wound margin. The daughter cells then form a component of the wound margin and are subject to a partial contact inhibition and then divide again to form a new wound margin. It also appeared that bone cells migrate to the centre of the wound. After 25 and 30hrs some proliferation occurred until the wound had completely healed and confluent. Contact inhibition took place naturally as also seen in the body in which cells stop replicating as they come into contact with each other as confluency occurs. At higher concentrations of HCl it was noticeable that bone cells looked longer and elongated with long tentacles and had more projections than that of the control. It became clear that HCl changed the morphology of the MG63 bone cells and encouraged proliferation, and accelerated the bridging process thus, the wound healed more rapidly. Our results suggest that wound healing took place in all concentrations of HCl but at different rates. The 25µl concentration of HCl fully healed after 25hrs whilst others remained open even after 30hrs. The 6.25µl and 12.5µl concentrations of HCl failed to close the wound after 30hrs with 24% and 16% of the wound remained open, respectively.

The 25µl concentration of HCl closely followed the 50µl with respect to the rate of wound closure up to the point of 20hrs at which wound closure occurred with accelerated healing. The greatest rate of wound closure was found between 5-15hrs by approximately 48% closure. This was slower for the control. The 50µl concentration of HCl had the quickest rate of repair up to 20hrs but took 30hrs for the wound to become fully confluent, with a large rate of closure between 5-15hrs at ~61% indicating that this concentration increased wound closure as compared to the control. The values for standard error indicated that there was some variation in scratch width measurements for the control and all the concentrations of HCl apart from the 50µl HCl with more uniform scratch width.

It is interesting to notice that the greatest percentage of wound closure was between 5 and 15hrs for the 25µl and 50µl concentrations of HCl. This indicates that the bone cells are working hard to make a connection across the wound width by successful bridging, with additions of HCl accelerating the process.

It can be speculated that some of the hydrogen ions from the HCl bind to the serum proteins and cause a change in amino acid molecular structure and hence its function as a protein. The hydrogen ions bind to the negative sites on the amino acids making the protein positively charged. When this occurs the proteins molecular structure is altered which encourages changes in the folding of the amino acid chains in the protein. The difference in folding is believed to influence the ability of the cells function. The serum protein is believed to become a signaling molecule, which encourages cell differentiation and proliferation for bone repair cells enhancing healing.

Hydrogen ions may have detrimental effects on the function of proteins, and that the HEPES act as buffer in the media and hence take out the hydrogen ions. It was observed in our investigation that different concentrations of HCl did not affect the pH level of the media indicating that HCl was being sequestered away. However, this is inconclusive as the pH value was not properly evaluated through testing. Observation of the colour of the media indicated to the naked eye that the pH value was not affected. Indeed after careful consideration of wound edge presence of some leading cells were visible. These leading cells are very different to other cells at the wound edge as they are larger, spreading, active and clearly dragging smaller cells behind them. These leader cells are a kind of “active leading cells” which have developed active “ruffling lamellipodium” and, in fact, have lost their osteoblast-like morphology due to their elongated shape. However, these leader cells have indeed maintained cell-cell contacts with each other and their followers through their common integrin-ligand bindings, i.e. via cell adhesion receptors that mediate cell-cell and cell-to-ECM interactions. Forming bridges is not the only mechanism during the healing process and the other clear mechanism is that of the “Motility of the border cells at the wound edges which crawl and spread into the free space by invading the wound site both collectively and individually. These two mechanisms occur at the same time. The number of bridge formation had increased with time but their occurrence and distribution is very heterogeneous. It has become more evident that during scratching and therefore cell removal the intra-cellular content is released into the media and also the neighboring cells may become partially damaged and permeable due to this harsh injury and tearing off of the adhesion cell junctions. Therefore, a sudden reflux of extracellular matrix to these cells may potentially trigger their migration state. A free edge may also trigger a motility response. Also for migration to start the initial cell density has a significant effect and cell division only helps to densify the monolayer.

By analyzing the graphs of % wound closure vs. time backed up by the % rate of wound closure vs. time it is evident that these results show a non linear free edge progression of cells. By careful consideration it can be concluded that these leading cells do not originate from the initial wound edge and may have originated from the back rows (2^{nd}, 3^{rd} or 4^{th}) and thus are not affected by the damaged cells at the border. Therefore, these leader cells were actually within the monolayer and came towards the border by flowing to the free space and hence no changes in their phenotype and only elongated and became more spread and very active. Their dynamic behaviour can be related to the graph of % rate of wound closure vs. time and hence they seem to progress at

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mainly a constant velocity but different constant velocities. Different gradient but constant velocity depends on the HCl content. Also, the more active leading cells move faster as compared to the other migratory border cells. Bridges or leading cells migrated in certain direction, most likely perpendicular to the wound edge. Also, as soon as reaching the opposite monolayer edge, they lost their highly active characteristics and hence the rate of wound closure decreased.

VI. STATISTICAL ANALYSIS

Following the scratching of the bone cell monolayers (approximately 300µm ± 10-30µm SD, 1.7-5µm SEM) with ~5hrs of incubation, the wound width showed different rate of healing according to different treatment. This rate was significantly different for HCl treated cells with higher concentrations of HCl (25µl and 50µl) compared to lower concentrations (6.25µl and 12.5µl) and control. However, the wound width showed significant enhancement in the rate of wound closure for 25µl HCl ($P < 0.001$) and 50µl HCl ($P < 0.001$). As time of the incubation increased (for instance 25hrs of incubation), the rate of wound closure became gradually similar to each other in the case of 25µl HCl ($P < 0.05$), 50µl HCl ($P < 0.05$) and control ($P < 0.001$).

By comparing all the treatments with respect to time required for complete wound closure, treatments with higher concentrations of HCl demonstrated significant difference in wound repair compared to the control and lower concentration of HCl with $P < 0.001$ for 6.25µl and $P < 0.001$ for 12.5µl. Comparative results were given as means ±SE. The significant values of the difference were tested by one-way ANOVA followed by the Bonferroni adjustment. Error bars = 95% CI.

VII. CONCLUSIONS

Bone repair is a complex system with interconnecting phases through cell management with many factors affecting the promotion of a successful bone repair. Many areas are investigated to promote bone repair and increase the rate of closure. In vitro studies allow us to understand how cells behave and react to different parameters. However, in vitro testing could be inconclusive until properly tested in vivo making sure that the correct biological environment is present.

This study showed the potential to yield some advancement in wound closure rates. It was found that higher concentrations of HCl, in particular 25µl and 50µl, promoted higher rate of wound closure compared to the control. The 50µl concentration of HCl showed the largest rate of closure between 5-10 hrs (43%). The imaging results also showed that HCl changed the morphology of the bone cells. The bone cells had elongated bodies with long tentacles and had more projections than those of the control. This enhanced the wound closure with easier connecting ability and bridging effects.

The HCl concentrations of 25µl and 50µl gave the greatest rate of wound closure between 5 and 15hrs. This indicated that the bone cells were making more contact by forming bridges which enhanced the healing process. Once bridging had occurred the rate of wound repair decreased at ~15hrs up to confluency. The HCl concentration at 25µl showed the wound to bridge at 15hrs and fully confluent at 25hrs, concluding that early bridging is an important factor, and enhances the rate of wound closure. The signaling pathways between the bone cells were enhanced with the correct concentration of HCl. The serum proteins present in the media act as signaling molecules due to the surface charge changes from the release of hydrogen ions via the HCl. The availability of hydrogen ions is essential for these signaling pathways to accelerate wound repair through administration of HCl.

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