Original Article

EFFECT OF ZN AND ZN CONTAINING BIOACTIVE GLASSES ON EPITHELIAL MIGRATION IN AN *IN-VITRO* WOUND HEALING ASSAY

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ABSTRACT

BACKGROUND: Delayed wound healing is one of the common clinical manifestations due to moderate to severe zinc deficiency. This study was designed to observe effect of zinc and bioactive glasses on epithelial migration in in-vitro wound healing assays.

METHODS: A simple in vitro scratch method was used in this study. Penicillin-Streptomycin solution, L-glutamine, Trypsin, Ethylenediamietetraacetic acid (EDTA), Dulbecco's Modified Eagle's Medium (DMEM) in glucose, phosphate buffer saline (PBS), new born or fetal calf serum (FCS), Recombinant human EGF (Invitrogen), Ethyleneglycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), zinc in the form of zinc chloride (ZnCl) and silicon as silicic acid were used for experiments.

RESULTS: One pilot study and 7 experiments were conducted to observe role of Zn alone with concentrations of 10μM, 25μM, 50μM, 100μM and in combination with different concentrations of silicon. Zn (50μM) showed maximum wound closure (46.8%) however Zn (50μM) with addition of EGTA showed minimum closure of wound. The combination of Silicon and Zinc (Zn 25μM & Si 10μM) showed a wound closure of 45% and however has not shown enhanced epithelial migration as compared to controls; positive control showed 67% while negative control showed 35% of maximum closure.

CONCLUSION: This study has not shown enhanced migration of HaCat cell using different treatments with Zinc and/or Silicon.

KEY WORDS: Zn, Silicon, Bioactive glasses, epithelial migration, wound healing

INTRODUCTION:

Zinc the second most abundant micronutrient is found in all body tissues and fluids.[1,2] More than 20% of the world population is expected to have zinc deficiency due to low zinc intake. Zinc is significantly an important element, required from the embryonic development till death. It is a critical co-factor of more than 300 enzymes and is required for normal DNA replication and cell proliferation.[3] Zinc also influences chemotaxis of neutrophil, natural killer cells activity, phagocytosis by macrophages and neutrophils and generation of oxidative burst activity.[4]

Zinc is located in both the intracellular and extracellular matrix and in the dermis and epidermis in the form of protein complexes

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where zinc stabilizes the cell membrane, acting as an essential co-factor, playing a central role in cell mitosis, migration and maturation.[5] Reactive oxygen species are major cause of tissue injury in inflammatory environment. Multiple studies conducted on humans suggest that zinc has an important protective role against generation of free radical and oxidative stress.[6] A zinc enzyme, superoxide dismutase (SOD), is an important enzyme for skin health due to its antioxidant properties.[7]

Delayed wound healing is one of the most common clinical manifestations from moderate to severe zinc deficiency. In severe zinc deficiency, organ systems clinically affected are the epidermis, immune system, gastrointestinal, skeletal, central nervous and reproductive systems and thymus.[8] The requirement of zinc in the wound healing process has been demonstrated in multiple ways. Oral zinc supplementation has shown an increase in the rate of wound healing.[9] The topical application of Zinc to a wound will lead to a reduction in the formation of necrotic material and wound debris.[10] In a rat wound model, zinc levels were increased locally after wounding, thus suggesting its involvement in healing process.[11] Integrins are the cell surface proteins which mediate the cell–cell interaction and interaction of the cell with its surrounding environment. An expression of many integrins expressed by keratinocyte is altered by zinc treatment. The induction of integrin subunits such as α2, α3 αv and α6 is promoted by zinc supplementation which increases keratinocyte mobility in the proliferative phase of the healing process. [12]

Bioactive material induces precise biological response at the interface of material that results in the formation of a bond between material and tissue [13] and bioactive glasses are used for in situ tissue regeneration.[14] This study was designed to observe the effect of zinc and bioactive glasses on epithelial migration in an in-vitro wound healing assays. Bioactive glasses are silicon based so an effect of zinc alone, silicon alone, and both zinc and silicon in combination on epithelial migration was observed.

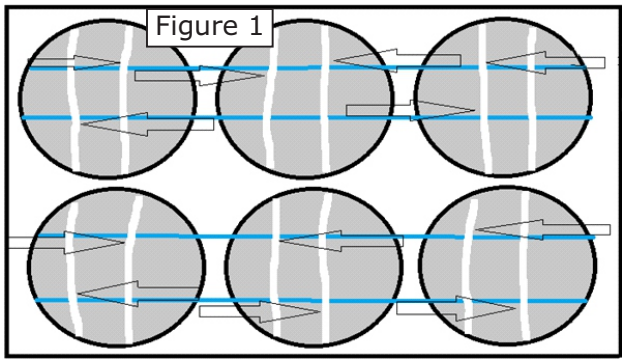
MATERIALS AND METHODS:

The study was conducted at Queen Mary, University of London. Materials used in this study were Penicillin-Streptomycin solution (5000 units penicillin and 5000 µg/ml streptomycin), L-glutamine, 5% Trypsin, 1mm EDTA, (Ethylenediamietetraacetic acid), Dulbecco's Modified Eagle's Medium (DMEM) 4.5g/l high in glucose, phosphate buffer saline (PBS), new born or fetal calf serum (FCS), Recombinant human EGF (Invitrogen), Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), zinc in the form of zinc chloride (ZnCl) and silicon as a silicic acid.

The cell line used in this study was the HaCat cell line (non tumoregenic immortalized human keratinocyte cell line greater than 140 passages). The cells were cultured in full serum containing medium which was DMEM (Invitrogen) (4.5g/l glucose) supplemented with 10% fetal calf serum, 5ml aliquots of peptostreptomycin (P/S) and 5ml aliquots of L-glutamine in T75cm^2 flasks. Cells were kept in a humidified chamber in an incubator with an atmospheric pressure of 5-10% carbon dioxide and in 95% air at 37°C. The cells were grown until confluent. Confluent cells were passaged using the usual procedure for passaging cells. In the whole study cell number was maintained constant. The cells were then grown in 6 well plates and in each well 0.5 x 10^6 cells were added. Each well contained total volume of 2ml serum containing medium (medium and cells). Then cells were incubated to become confluent.

A simple in vitro scratch method was used in this study. This is very simple and inexpensive method to study in vitro cell migration of different cell lines, and this method mimics in vivo cell migration during wound healing. Initially scratch was practiced using different tools to make a uniform and consistent scratch. Several different pipette tip sizes, appendrof, and aspirator tip were tested; every tool was tested twice to get a uniform and consistent scratch. The Pipette tip size of 200 µl was selected for making scratch. A few more experiments were performed to know whether speed or force while making the scratch affects the size of the scratch or not. While making a scratch, different speed and force were applied,

and it was found that moderate speed and the moderate force give smooth and a scratch of somewhat consistent width.



In each well of a 6 well plate two scratches were made and one image of each scratch was taken using confocal microscopy. The making of scratches and images taken are presented in diagram (Figure 1). Reference lines were drawn with a marker before plating out the cells on the backside of the plate. Each well had two reference lines. When the cells were confluent and ready for scratch, then scratches were made at right angle to the reference lines. Two scratches were made in each well and afterwards cells were washed once with PBS and then again supplemented with media and desired doses of different drugs were applied. After application of drugs, images were taken using a confocal microscope. From each well two images were taken whenever the experiment was performed. The arrow head shown in the diagram (Fig. 1) indicates the points where images were usually taken. During the whole study, images were taken at different points of time, at zero hour and at six hours. Images were read using software named as Tscratch.

A pilot study was conducted followed by the series of experiments with different doses of zinc alone and with combination of silicon to see the effect of these different doses on epithelial migration. Cells were washed with PBS once they reached confluence in 6 well plates. The control was zinc free serum containing media (DMEM). Zinc was added in serum containing media in the first experiment. Different doses of zinc were applied as 10μM, 25μM, 50μM and 100 μM. All Experiments with specific doses were repeated twice. In the second experiment two controls were used, one with 10% serum media and the other with 1% serum media. In the same pattern zinc with doses of 10μM, 25μM, 50μM, 100μM were applied, but cells were supplemented with zinc in 1% serum only. Another experiment was performed with a similar approach. We set the controls, one with 10% serum containing media and other with 1% serum, and supplemented the cells with zinc 50μM in low serum (1%) media alone, with zinc 50μM and EGTA in low serum (1%) media. The cells were also supplemented only with EGTA in low serum media (1%) and with EGTA in serum containing media (10%). In the fourth experiment, cells were supplemented with different doses of silicon (25μM, 50μM, and 100μM), and addition was made by using EGTA with and without silicon.

After pilot study first experiment was carried out to see the response of HaCat cells to epidermal growth factor. HaCat cells were grown to confluence and then starved in low serum (1%) media overnight. Scratches were made as per protocol. The Epidermal growth factor (EGF) used was Recombinant Human EGF (Invitrogen) with the concentration of 10 ng/ml. 0.1μl of EGF was added in 10ml of medium.

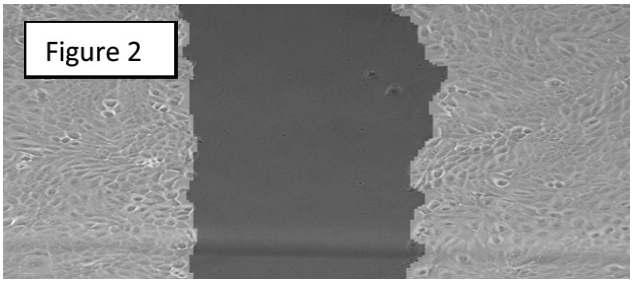
An experiment was designed with a similar protocol in order to observe the effects of silicon and zinc in combination. The control set was with low serum (1%) zinc and silicon free, and one control was with zinc 25μM (1%) only. The dose of zinc 25μM was kept constant with different doses of silicon (10μM, 25μM, 50μM, and 100μM.). Another experiment was designed to see the effect of Zinc 50μM in combination with different doses of silicon (10μM, 25μM, 50μM, 100μM). Two controls were set in this experiment as well, one was in low serum (1%) treatment free and other was with zinc 50μM only in low serum (1%).

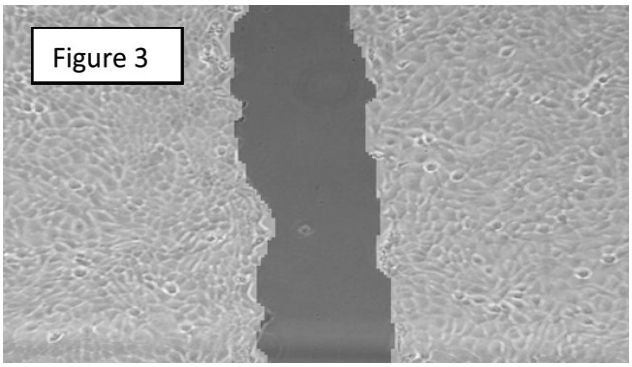
The scratches were made after the cells were confluent in 6 well plates. In each well two scratches were made and the image of every scratch was taken and each image was analyzed in Tscratch software. This program is a software tool for automated analysis of wound healing assays (or in vitro scratch assays); with extensive possibilities to assess and adjust analysis results in easy to use graphical user interface. This program can also perform statistical analysis of the result, present results in bar diagrams and text files and can be simply

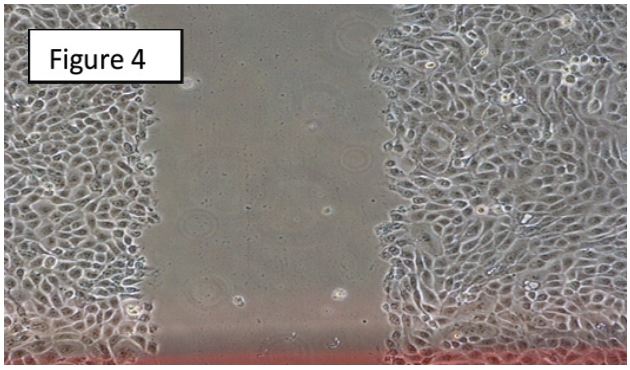
imported into a spreadsheet program. In this study this software was only used for image analysis and statistical analysis was done using Microsoft Excel. After loading the images in the program, analysis was run and the open image area was presented as the percentage of an image that was not considered as occupied by cells by the algorithm. The open image area could be manually adjusted to achieve maximum accuracy of the scratch. This percentage value was computed for each analyzed image and was written in the out-put text files. The scratch assay analysis was done in Microsoft Excel after getting the percentage from Tscratch. The difference in the percentage between time zero and time six hours was calculated and was expressed as a percentage of original amounts. Averages and standard deviations were calculated and presented in a graph for each experiment. This indicates that how much Hacat cells have migrated in six hours. After 24 hours of scratch we could not see the open area as scratches were healed completely. An aseptic protocol was followed for all procedures.

RESULTS

In pilot study, ranges of initial parameters were tested and it was observed that cell is monolayer and not over confluent as the scratch would be without flap of cells. It was also noted that while making a scratch the speed and force applied to wound cells have to be moderate. After reading the image, Tscratch shows the open area in the scratch; the open image area at time zero is 36% (Figure 2), while open image area at time six hours is 22% (Figure 3). It was also found during pilot study that pipette tip of 200µl gives scratch of uniform width with clear margins and the width of the scratch was easily captured. The red line in the images shows the reference line. (Figure 4)





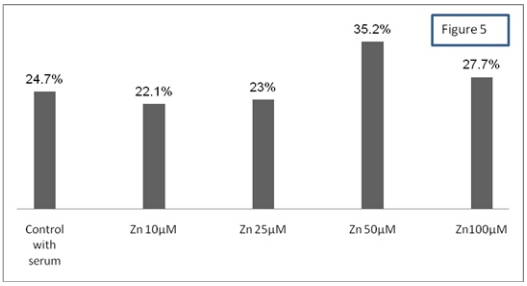


Experiment 1

The first experiment was done to see the migration of HaCat cells after scratch. EGF was added after growing cells in low serum media for 24hrs. EGF was a positive control for the whole study. When calculation was expressed as percent (%) of original amount, cells showed 67% (n=4) movement (wound closure).

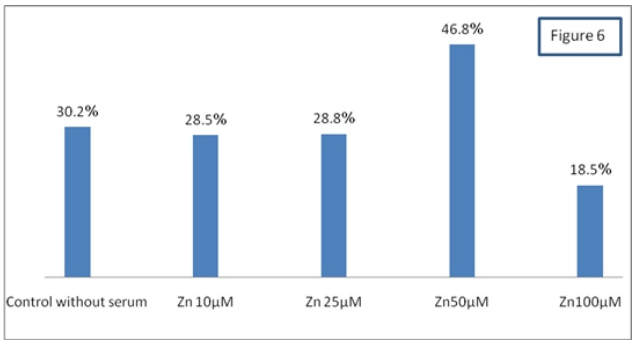
Experiment 2

Figure 5 represents the analysis of a scratch assay with a control and different concentrations of Zinc using 10µM, 25µM, 50µM and 100µM. All the concentrations of Zinc and the control were tested in serum containing media. Zn with 50µM concentration showed maximum wound closure.



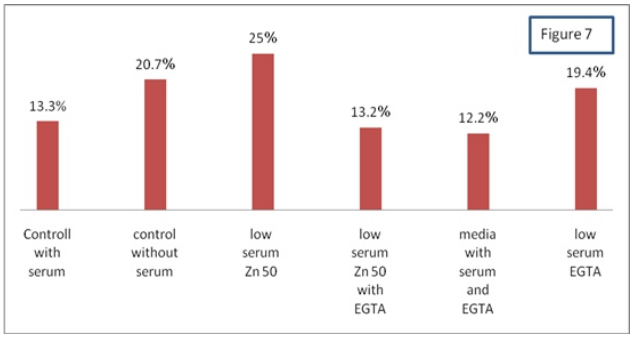
Experiment 3

Figure 6 is the graphical presentation of the scratch assay analysis with a control and with different concentrations of zinc such as 10μM, 25μM, 50μM, 100μM. The control and all the concentrations of zinc were investigated in 1% serum. Zn with 50μM concentration in low serum showed maximum wound closure.



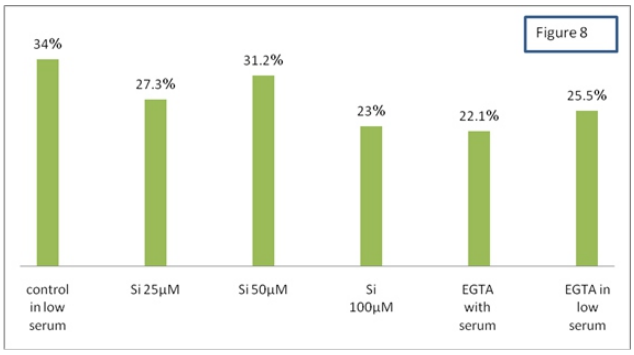
Experiment 4

Figure 7 demonstrates the analysis of scratch assay done with negative control using 10% serum and 1% serum. The concentration of zinc was kept 50μM with 1% serum. Zinc with concentration of 50μM in 1% serum was tested with the addition of EGTA. The effect of EGTA was tested alone in serum containing media and also in 1% serum. Zn (50μM) showed maximum wound closure, but addition of EGTA (chelating agent) with zinc 50μM showed minimum closure as zinc added to cell was chelated by EGTA



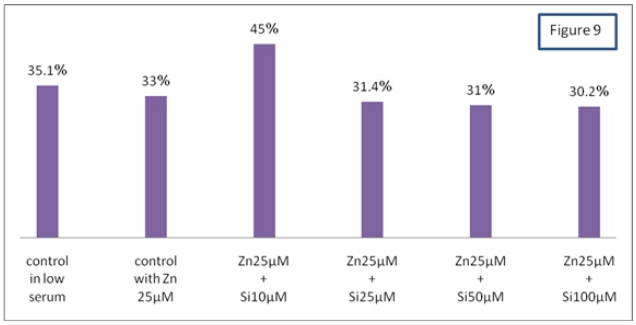
Experiment 5

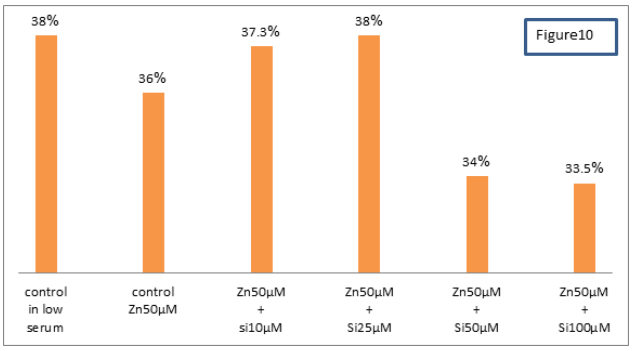
Figure 8 is the graphical presentation of the analysis with negative control in 1% serum and different concentrations of silicon 25μM, 50μM, and 100μM. EGTA was also tested with 10% serum containing media and with 1% serum containing media. In this experiment our control in low serum showed maximum closure.



Experiment 6

Figure 9 demonstrates the scratch analysis with control in low serum (1%). In this experiment concentration of zinc (25μM) was kept constant, while the concentration of silicon was changed as silicon 10μM, 25μM, 50μM, 100μM. All these concentrations were tested in 1% serum. Maximum closure was shown by dose of zinc 25μM + silicon 10μM.





Experiment 7

Figure 10 shows the graphical presentation of experiment in which the concentration of zinc was kept constant as zinc 50μM, while the concentration of silicon was changed as silicon 10μM, 25μM, 50μM, 100μM. All these

concentrations were tested in 1% serum. Different doses applied to cells in this experiment showed similar results to our control.

When cells were treated with EGF, cells responded to EGF significantly (67%, n=4) as compared to negative control (15% to 35%) in the study. The negative control showed epithelial movement on average of 15% to 35%. Cells were deprived of serum to get rid of excessive growth factors, and the scratch assay was also performed in low serum (1% serum). Zn 50μM (in 1% serum) showed on average 46.8%, while Zn 100μM showed least closure of 18.45%. As Zn 50μM proved to be a better dose of zinc among all used, effect of Zn 50μM and a chelating agent EGTA in combination and alone was observed; it was found that combination of Zn 50μM and EGTA decreased the rate of closure of scratch, while other doses did not show significant effects. Silicon is the major component of bioactive glasses, so another experiment was done to see its effect on cell migration. Among all treatments of silicon tested, no dose of silicon showed significant effect on cell migration as the control showed a better closure rate than silicon dose. When zinc & silicon were tested in combination (as in bioactive glasses zinc and silicon are important constituent) Zn 25μM + Si 10μM combination showed 44.7% closure

DISCUSSION:

Zinc, the second most important trace element found in human body, is required for a wide range of functions.[15-18] Several studies have been conducted to see its effects on wound healing.[9,10,11,19] This study was designed to assess the effect of zinc, silicon, zinc & silicon, and the effect of zinc containing bioactive glasses on epithelial migration in a sample wound healing assay. An immortalized cell line of human keratinocytes was used in this study allowing the assessment of the migration of human keratinocytes. In-vitro scratch assay was used in the study. The cells were treated with EGF and showed a positive response while they did not demonstrate significant response with zinc only, silicon only and a treatment in combination of both.

Numerous factors were observed that could have caused negative effect on cell migration. Flaps of cells were avoided while performing scratch, but sometimes we got flaps of cell which may have caused hindrance in cell migration. Cells were incubated for 24 hours in serum containing media. They were monolayer and confluent, but when cells were deprived of serum for another 24 hours they were over confluent and dense. When scratch was performed even with moderate force and velocity there were flaps of cells sometimes. The area denuded from cells was sometimes larger than other scratches. Cells were removed from the incubator for taking pictures, while taking pictures migration rate might have reduced due to different conditions. Constant favorable pH value is vital for cell growth while acidic nature of Zinc reduced responsive capabilities of the cells. Cells were assessed after six hours of wounding; response might get better if cells were assessed after eight to twelve hours whereas there were no signs of scratch when cells were observed after 24 hrs.

In general in-vitro scratch method is used to study the migration of different cell lines. This study observed the effects of zinc and zinc containing bioactive glasses on an in-vitro wound healing assay. The method used to see the migration of Hacat cells was in vitro scratch assay. This in vitro scratch method is also used for studies on the primary cells. In a study on human epidermal keratinocytes (HEK) isolated from different individuals to see the migration during healing, [20] it was found that genetic variation alters the migration rate of HEK during wound healing. In a study done to see the migration rate of dermal fibroblasts (L929) and human keratinotocytes (HaCat) and their contribution to wound repair, using an in-vitro scratch method, it was found that L929 migrate faster than HaCat cells and contribute more to the wound repair process. [21]

Zinc promotes epithelial repair during wound healing. It has been found that zinc concentrations were higher than normal after ten days of wounding. [11] Metallothienien (MT which are the reservoirs for zinc) expression is upregulated in healing wounds than in unwounded normal skin. [22] Upregulation of MT expression can be induced in vivo by exposure to zinc. [23] In in-vitro treatment of keratinocyte with zinc chelator reduces the expression of MT

and also the cell proliferation.[24] The repair of ulcerated skin is also enhanced by local application of zinc.[25] Extracellular zinc either applied or released after injury will activate zinc sensing receptor ZnR which will trigger signaling that lead to the epithelial repair. [26] In this study it was found that HaCat cells treated with zinc 50μM showed better closure rate, so it might trigger signaling of ZnR leading to epithelial repair. In another study where it was evaluated that human keratinocyte migration was most stimulated with magnesium gluconate whereas migration of the HaCat cells was most stimulated with zinc gluconate. [12] In order to see the effect of zinc Carnosine (ZnC) on migration, a study was conducted on human colonic carcinoma cell line HT29 and the canine epithelial kidney cell line (MDCK). Different doses of zinc Carnosine were tested and it was found that ZnC at 100μM induces maximal cell migration, whereas doses more than 100μM showed no effect on migration. [27]

Zinc is used extensively in dressings and is also used in creams for diaper rashes and minor cuts. Zinc has a significant antibacterial role as it inhibits the growth of several bacterial species. In many studies it has been observed that zinc shows beneficial effects in the treatment of acne, possibly through anti inflammatory effects. [28] Gram-positive bacteria appear to be more sensitive to zinc than gram-negative bacteria. It is also observed that zinc oxide inhibits [29] the attachment and growth of S. aureus in-vitro.[19, 30]. Zinc also depresses the growth of E. coli.[31] From these studies it can be inferred that zinc possesses antimicrobial activities against common wound flora.

Bioactive glasses containing zinc are beneficial for bone formation, as there is data that suggest that zinc helps in bone formation.[32] Some data also suggest that zinc-containing bioactive glasses can affect the bone-associated cellular responses including cellular proliferation and synthesis of matrix.[33] In this study different doses of zinc were used to evaluate the dose which induce maximum migrating and promote wound closure. It was found that among all zinc 50μM shows (46.8%) better effect on migration of cells. The intention during this study was to test the potential effect of bioactive glasses on epithelial migration on wound healing assay whereas our results using the simple scratch assay did not support a role of zinc and/or silicon in enhanced wound healing. Hence testing on zinc containing bioactive glasses was not conducted.

It was observed that HaCat cells did not show enhanced migration whether cells were treated with zinc alone or silicon alone or in combination of both. The combination of Silicon and Zinc (Zn 25μM & Si 10μM) showed maximum of 45% closure. Silicon, the major constituent of bioactive glasses, has not shown enhanced migration as compared to controls, and so bioactive glasses were not tested to see epithelial migration. All the results of this study were compared with controls. The positive control showed a maximum of 67% closure and negative control showed a maximum of 35% closure while Zn 50μM showed 46.8% closure.

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Often your utterances and expressions of your face leak out the secrets of your hidden thoughts.

When you get ill does not get nervous about it and try as much as possible to be hopeful.

Hazrat Ali (Karmulha Wajhay)