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The Effects of Metformin and Hyaluronic Acid on Wound Healing in High Glucose Incubated Fibroblast Cells

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ABSTRACT: Chronic wounds in diabetes are common and serious health problems which also create significant economic burden in society. A comprehensive treatment has not yet been found in chronic wounds and new treatment recommendations are needed. The beneficial effects of metformin on wound healing was shown previously in healthy or diabetic animal models. Hyaluronic acid was also found to accelerate wound closure in clinical trials as well as animal models. Metformin and hyaluronic acid have been shown to be beneficial for wound healing in diabetes in in vivo studies, but their effect on wound healing under in vitro diabetic conditions have not yet been enlightened. In this study; we investigated the effects of metformin and hyaluronic acid on wound healing in mouse fibroblast cells under high glucose conditions. We used the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and scratch migration assays to examine fibroblast cell viability and migration in wound healing respectively. We showed that metformin and hyaluronic acid enhanced wound healing in fibroblasts incubated with high glucose by increasing cell viability, proliferation and migration. This is the first study to examine the effect of metformin, hyaluronic acid, or their combination on wound healing under high glucose conditions in vitro.

KEYWORDS: Fibroblast; hyaluronic acid; metformin; wound healing; diabetes.

1. INTRODUCTION

Chronic wounds frequently seen in diabetic patients create common and serious health problems [1]. The lifetime risk of developing foot ulcers in diabetic patients is 15-25%, of which 40-80% become infected severely. Due to the increase in diabetes cases in the population, the number of diabetes-related wounds is expected to increase which makes the impaired wound healing in DM; a major healthcare issue and a significant economic burden [1, 2].

Treatment of chronic wounds is still a problem today, as diabetes impairs the wound healing process. This impairment is a result of complex pathophysiology involving vascular, neuropathic, immune, and biochemical components [3]. Diabetes affects the process negatively in all of the standard wound healing phases of inflammation, proliferation/migration and remodeling [4]. Hyperglycemia seen in diabetes correlates with stiffer blood vessels which cause reduced perfusion and microvascular dysfunction, causing reduced tissue oxygenation and insufficient nutrient intake [5], also account for reduced leukocyte migration into the wound [3]. The hyperglycemic environment itself can increase inflammation [2]. Among the many factors that impair wound healing in diabetes [3], we focused our research on the effect of hyperglycemia.

In the standard wound-care practice for impaired wound healing, first of the aims is to prevent the development of an infection which can easily occur due to the loss of the innate barrier and to clean the area from non-viable tissue material. Following these initial phases, the treatment relies mainly on the use of topical dressings that, by providing the moist environment required for proper healing,

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facilitate the production of granulation tissue and epithelialization [2]. Today, becaplermin (0.1 % gel) which is a platelet-derived growth factor is the only FDA-approved drug used in the treatment of diabetic foot ulcer. Another aim is to ensure cell proliferation and migration which can be achieved with various pharmacological agents. New treatment recommendations are needed in the treatment of impaired healing wounds which have not yet found a comprehensive treatment.

Metformin is recognized worldwide as the first-line drug for the treatment of type II diabetes. Action mechanism of metformin is activation of AMP-activated protein kinase (AMPK) which regulates metabolic responses of cells [6,7]. Beside its regulatory role of metabolic responses of cells, metformin has immunomodulatory and anti-inflammatory properties [8,9], which may support the potential of metformin for the treatment of wound healing. Indeed, metformin has been tried before in wound healing studies [2]. Topically applied metformin accelerated healing of excisional wounds in rat skin by improving epidermis, hair follicles, and collagen deposition [10,11]. Furthermore; faster wound healing and increased angiogenesis has been found in db/db diabetic mice following two weeks of chronic and systemic administration of 250 mg/kg/day metformin [12]. Metformin (6 mg/g gel twice daily) has also been shown to be effective in patients with non-healing traumatic wounds or ulcers of lower extremity after a maximum 1-month treatment [13].

Hyaluronic acids (HA) are naturally occurring polysaccharides in connective, epithelial and nervous tissues in the body. HA plays a role in cell proliferation and migration which is two essential processes required for wound healing [14]. Formulations containing HA widely employed in regenerative medicine and in particular for topical and intradermal applications [15]. The healing effect of HA in burns, post-operative epithelial wounds and chronic wounds has been previously demonstrated in a meta-analysis of clinical studies which reported improved healing compared to conventional treatments or placebo [16]. HA has also significant benefit for increasing the healing rate of diabetes wound ulcers over standard treatments [16]. Moreover; it has been shown that significant improvement in wound closure, an increase in the number of fibroblasts and inflammatory cells and higher collagen I and collagen III levels in HA treated rats [17].

Studies are ongoing to understand diabetic wounds and why they persist for such long periods of time, and also to develop new and efficacious treatment strategies. Metformin and HA have been suggested as beneficial for wound healing in diabetes in previous in vivo studies however the effects of metformin, HA and co-treatment of metformin and HA on wound healing under in vitro diabetic conditions are not clarified yet. In this study; we investigated the effects of metformin and hyaluronic acid on wound healing assay in mouse L929 fibroblast cells which incubated with high concentrations of glucose. We found that metformin and HA increased wound closure in high glucose incubated fibroblasts, suggesting that metformin and HA are promising therapeutic agents for diabetic wounds alone or in combination.

2. RESULTS

2.1. Effects of increasing concentration of glucose on fibroblast viability over time

We firstly examined cytotoxic effect of increasing glucose concentration on L929 cells at different time points. Increasing glucose concentrations (10, 25, 50, 100 mM) did not affect significantly cell viability after 24 hours compared to control group which was incubated with 5 mM glucose (Figure 1A). The incubations with 25, 50 and 100 mM glucose concentrations decreased cell viability after 48 and 72 hours compared to control group (5 mM glucose; Figure 1B and 1C). The incubation with glucose at 100 mM concentration for 48 hours was chosen for further experiments in order to observe the exaggerated responses.

2.2. Effect of different concentrations of metformin and hyaluronic acid on viability of fibroblast incubated with high glucose

We examined the increasing concentration of metformin and HA on fibroblast viability. Metformin did not change cell viability which was decreased induced by high glucose incubation (100 mM, 48 hours) at 1 and 2.5 mM concentration. Only 5 mM metformin treatment restored the decreased cell viability induced by high glucose incubation. 5 mM of metformin was chosen as the treatment concentration for following experiment. Metformin concentrations of 10 and especially 50 and 100 mM were found highly cytotoxic. 0.05 % of hyaluronic acid treatment did not alter the decrease in cell viability induced by high glucose incubation whereas 0.1 % of hyaluronic acid was significantly

increased cell viability (Figure 2). 0.1 % of hyaluronic acid was chosen as the treatment concentration for following experiment.

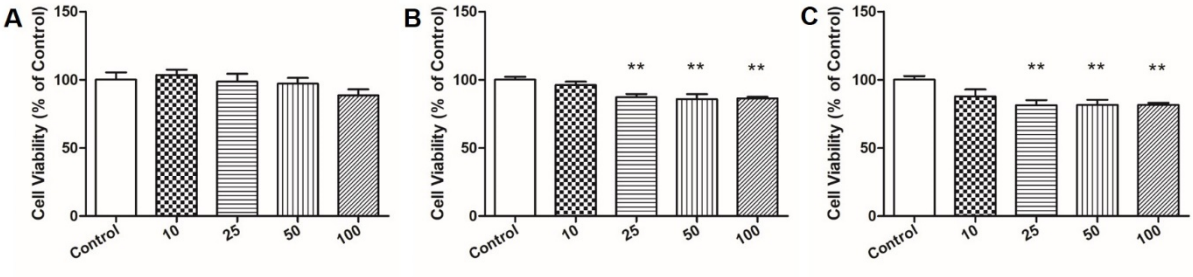
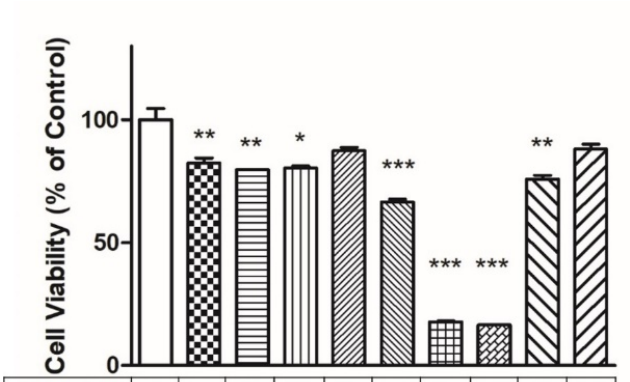


Figure 1: Effect of increasing concentrations of glucose incubation (10, 25, 50, 100 mM) on L929 cell viability after different time points. (A:24, B:48 and C:72 hours after). Results were given as % percentage of control group (5 mM glucose). One-way ANOVA following post-hoc Newman-Keuls was used for statistical analysis (** P<0.01 compared to control group; n=4).



Glucose (mM)	5	100	100	100	100	100	100	100	100	100
Metformin (mM)	-	-	1	2.5	5	10	50	100	-	-
HA (%)	-	-	-	-	-	-	-	-	0.05	0.1

Figure 2: Effect of different concentrations of metformin and HA incubation on L929 cell viability incubated with high glucose (100 mM) for 48 hours. Results were given as % percentage of control group (5 mM glucose). One-way ANOVA following post-hoc Newman-Keuls test was used for statistical analysis (*P<0.05, ** P<0.01 and *** P<0.001 compared to control group; n=3).

2.3. Effect of metformin and hyaluronic acid treatment on wound closure after fibroblast scratch assay

Finally; we examined the effect of metformin and HA on wound closure of fibroblasts. High glucose (100 mM) lead to a significant decrease (8.3 ± 0.9 %) in wound closure compared to that of control group (5 mM glucose; 12.33 ± 1.12 %) after 24 hours. It was further decreased (18.5 ± 0.67 %) compared to control group (28.9 ± 1.85 %) after 48 hours. Metformin treatment was significantly increased wound closure (24.8 ± 0.3 %) compared to high glucose after 48 hours, whereas it was not changed after 24 hours. Hyaluronic acid treatment was significantly increased wound closure (12.2 ± 0.5 % and 24.2 ± 2 % respectively) compared to high glucose after 24 and 48 hours. Metformin and hyaluronic acid combination treatment was also significantly increased wound closure (14.2 ± 0.9 % and 30.3 ± 2.6 % respectively) compared to high glucose after 24 and 48 hours. There was no difference between the effects of metformin-hyaluronic acid combination treatment group and metformin or hyaluronic acid treatment alone (Figure 3A, B).

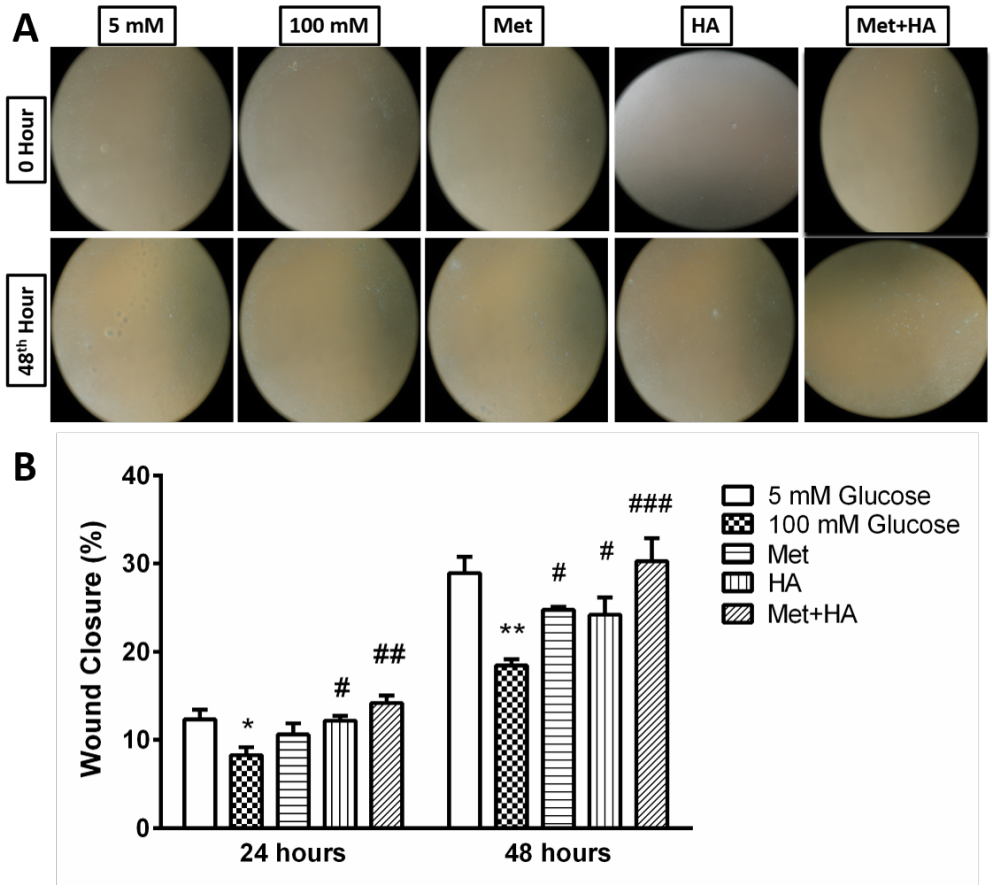


Figure 3: (A) Representative images of fibroblasts incubated with 5 mM and 100 mM glucose, 5mM metformin (Met) , 0.1% hyaluronic acid (HA) and Met+HA combination before and 48 hours after scratch assay. (B) Effect of Met, HA and Met+HA treatments on wound healing of fibroblasts incubated with high glucose (100 mM), 24 and 48 hours after scratch assay. Wound closure was calculated as % percentage of initial wound area. One-way ANOVA following post-hoc Newman-Keuls test was used for statistical analysis (*P<0.05, ** P<0.01 compared to control group and # P<0.05, ## P<0.01 and ### P<0.001 compared to high glucose; n=3).

3. DISCUSSION

The wound healing process is a physiologic, highly complex process that occurs when the skin is damaged and as a consequence its barrier function is compromised. Different subsequent steps occur during this process, and usually three phases are recognized: inflammatory, proliferative, and remodeling [3]. Diabetes affects the process negatively in all of the standard wound healing phases [4]. Hyperglycemia damages the microvasculature of small vessels that bring oxygen and nutrients to the wound [5]. As a result of excess glucose, the vessels' walls stiffen, reducing blood flow and permeability of erythrocytes and migration of leukocyte, which are essential for developing new skin tissues [2, 3,21] since the fibroblasts, keratinocytes and immune cells need nutrients and oxygen carried by blood. It is known that the hyperglycemic environment itself can increase inflammation [2]. Hyperglycemia may also affect cell proliferation and migration in the proliferative and remodeling phase of healing.

Diabetes-induced impaired migration and proliferation of keratinocytes and fibroblasts has been reported in animal models [22]. It is also known that high glucose impairs the proliferation and migration of human gingival fibroblasts [23]. There are different concentrations of glucose ranging from 10 to 100 mM and incubation periods to mimic hyperglycemia on fibroblasts in vitro conditions [18, 21, 24-29]. The first aim of our study was to examine the effect of increasing glucose concentrations on the viability of L929 fibroblast cells over time in our experimental conditions in order to mimic the diabetic conditions in vitro. In this study; glucose incubations higher than 25 mM decreased cell viability of fibroblasts compared to the group incubated with 5 mM glucose after 48 hours of incubation, but no difference was found in cell viability at any glucose concentration after 24 hours. Similarly, there are

several studies showing that fibroblasts tolerate high glucose concentrations [18, 21, 25,28]. Glucose incubation time and concentration can be suggested as at least 48 hours of incubation and 25 mM concentration in order to mimic harmful effects of hyperglycemia in vitro for fibroblast cells. Therefore, high glucose concentration for following experiments in our study was chosen as 100 mM in order not to miss any minor changes in the treatment groups. The incubation time was selected as 48 hours, which is the shortest time found to be as significant on the viability test.

Metformin is the first-line drug and has been safely used for several years in the treatment of diabetes. Recently, it has been proposed for wound healing in several studies [2]. Local administration of metformin was found beneficial on wound healing of both young and aged skin by its angiogenetic effect [11]. Also, metformin found to thicken the epidermis and to increase the hair follicles and collagen deposition in young rodents which contribute to wound healing [11]. Moreover, it was alleged that its immunomodulatory and anti-inflammatory effects may also help wound healing [8,9]. Local metformin treatment has been shown that promotes M2 macrophage polarization to accelerate the wound healing through inhibition of NLRP3 inflammasome activation which was regulated by AMPK/mTOR signaling pathway beside altered the IL-1β and IL-10 expressions [10]. Furthermore; systemic administration of metformin was found beneficial in diabetic wounds by increasing angiogenesis in a db/db diabetic mouse model [12]. Metformin has also been found to be effective in patients with non-healing lower extremity traumatic wounds or ulcers [13]. However, the effect of metformin under high glucose conditions has not been investigated yet in vitro. We showed that metformin (5 mM) was significantly increased cell viability of fibroblasts which was decreased by high glucose, but metformin at 10, 50 and 100 mM was found to be quite cytotoxic to fibroblasts as it was found in a previous study in hepatocellular carcinoma cell line [30]. Furthermore, we also showed that there was an increased wound closure in metformin-treated fibroblasts compared to the group which was incubated with high glucose only, after 48 hours. Accelerating cell viability and wound healing in fibroblasts can be suggested as the reason for the curative effect of metformin. On the other hand, in our study, metformin could not accelerate wound closure significantly in short treatments such as 24 hours as in line with the results of a previous study [31] performed with ovarian cancer cells. The 24-hour metformin treatment period may not be sufficient for cell migration in fibroblasts.

Hyaluronic acid plays a role in cell proliferation and migration which is two essential processes required for wound healing [14]. HA containing formulations in particular for topical and intradermal applications promotes wound healing [15]. The healing effect of HA on different types of wounds, including chronic ones compared to that of conventional treatments or placebo has been previously demonstrated in a meta-analysis of clinical studies [14,16]. Not only in these wounds but also in diabetic wounds, HA has significant benefit by increasing the healing rate over standard treatments [16]. In preclinical studies; a significant improvement in wound closure has been shown by increasing the number of fibroblasts, inflammatory cells and collagen levels in HA-treated rats [15,17]. Studies examining the effect of HA under in vitro conditions are lacking in the literature similar to metformin. In this study, we showed the beneficial effect of HA (0.1 %) in cell viability of fibroblasts under high glucose stress. HA treatment also increased wound closure in scratch migration assay starting from 24 hours. The combination treatment of metformin and HA in wound healing assay was carried out for the first time in this study to understand whether a synergistic effect would be observed. Combination treatment did increase wound closure which was decreased by high glucose incubation but not more than metformin or HA treatments alone.

One of the main causes of chronic wounds is diabetes mellitus. Hyperglycemia seen in diabetes is the main factor that hinders wound healing. The increase in the migration and proliferation of fibroblasts and keratinocytes has been suggested previously as an approach to accelerate wound healing. The use of only fibroblasts in this study can be considered as a limitation of the study because keratinocytes, which is planned to be used in further studies, could provide as much evidence as fibroblasts regarding reepithelialization process. However, since the fibroblasts are the most used cell types in literature in wound healing assay, it was also useful to compare the results with previous findings and in terms of examining the effects separately.

4. CONCLUSION

Various agents that increase the migration and proliferation of cells have been suggested for wound treatments until now. Metformin and hyaluronic acid were suggested as one of the agents that have been found beneficial and may increase the migration and proliferation of fibroblasts. In our study,

we showed that metformin and hyaluronic acid increased wound healing by enhancing the viability, proliferation and migration of fibroblasts which were incubated under high glucose conditions. To the best of our knowledge, this is the first study to examine the effect of metformin, hyaluronic acid or their combination on wound healing in hyperglycemia conditions mimicked in vitro assays. Explaining the effects of metformin and hyaluronic acid at the cellular level may help them to be included in the formulations used in wound healing in the future.

5. MATERIALS AND METHODS

5.1. Experimental Groups

Fibroblasts are well-established cells in wound healing studies [1]. L929 mouse fibroblast cell line was used in our experiments. Dulbecco's Modification Eagle's Medium (DMEM) (Wisent Inc. 319-013-CL) supplemented with 10 % fetal bovine serum (FBS), 2 % L-glutamine and 1 % penicillin-streptomycin was used as medium for all groups [18]. Control group was incubated with only DMEM which already contains 5 mM glucose which approximates normal blood sugar levels *in vivo*. Glucose concentration at 10 mM is known as pre-diabetic as well as higher glucose concentrations is considered as diabetic in in vivo studies [19]. Increasing high glucose groups were created adding related amount of glucose to their medium. Metformin was dissolved in sterile distilled water and added to the medium of increasing concentrations. Hyaluronic acid was supplied as 0.2 % sterile injectable form and diluted with DMEM to the end concentrations.

5.2. XTT Assays

L929 cells were seeded into 96-well plates and incubated with medium for 24 hours (5 % CO_2, 37 °C) to form a confluent monolayer. After 24 hours of incubation, the medium was aspirated from the surface of cells. First, cells were incubated with increasing glucose concentrations for 24, 48 and 72 hours. After 24, 48 and 72 hours of treatments, the medium removed from wells. 100 µl of fresh medium and 50 µl of the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) /Phenazine methosulfate (PMS) solution was added to each well and the plates were incubated for 4 hours in the incubator (5 % CO_2, 37 °C). Then 100 µl of each well was transferred to the corresponding well of a new well-plate. The absorbances was measured with a microplate reader at 450 nm (reference wavelength 630 nm). The results were normalized to the control group. In another group of experiment; cells were seeded into 96-well plates and incubated with increasing concentrations of metformin and HA under high glucose conditions. After 48 hours of incubation, the medium was aspirated and XTT assay was performed as described above.

5.3. Scratch Migration Assays

The migration of fibroblasts was analyzed by the scratch migration assay as previously described [20]. Briefly, L929 cells were seeded in 24-well plates and incubated with DMEM at 37 °C and 5 % CO_2 for 24 to 48 hours to permit cell adhesion and the formation of a confluent monolayer. After 90% confluence was achieved, an artificial gap so called as "scratch" approximately 0.2-0.4 mm in width, was created with a 200 µL sterile pipette tip. The medium along with any detached cells and debris was immediately removed. Cells were incubated with high glucose and high glucose accompanying metformin, HA and combination treatments for 48 hours. Images were taken by an inverted microscope equipped with a digital camera in order to follow cell migration and morphological changes of cells. Images were taken just after the "scratch" was created which was accepted as initial wound area and images were taken at the 24^{th} and 48^{th} hours of incubation as well. The area of the scratch was measured by Image J 1.53e software (National Institute of Health). Wound closure was quantified as wound area relative to the initial one.

5.4. Statistical Analysis

One-way ANOVA following post hoc Newman-Keuls tests were used for statistical analysis. Sample size was determined at least 3 for each group with 0.8 power and 0.05 type I error. Data were represented as mean ± standard error of mean (SEM). $P<0.05$ was accepted as statistically significant. GraphPad Prism 5.0 software was used for statistical analysis (San Diego, USA).

Author contributions: All authors equally contributed to the article as first authors and approved the submitted version. I.K. and M.D. equally collected and analyzed data and wrote the paper.

Concept – I.K., M.D.; Design – I.K., M.D.; Supervision – I.K., M.D.; Resources – I.K., M.D.; Materials – I.K., M.D.; Data Collection and/or Processing – I.K., M.D.; Analysis and/or Interpretation – I.K., M.D., Literature Search – I.K., M.D.; Writing – I.K., M.D.; Critical Reviews – I.K., M.D.

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