Fully-Reduced HMGB1 with Metformin Improves Nonunion Bone Fracture Healing in Diabetic Rats

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INTRODUCTION: Diabetes mellitus (DM) is a chronic metabolic disease that increases fracture risk, interferes with bone formation, and impairs nonunion fracture healing [1]. Fully reduced high mobility group box1 (frHMGB1) can enhance wound healing [2] and the activity of frHMGB1 can be inhibited by metformin (Met) which is a hypoglycemic drug commonly used for the treatment of DM [3]. However, Met has beneficial effects on bone tissue by enhancing fracture healing and reducing the risk of bone fracture in diabetic patients. The effect of frHMGB1 and Met on the bone fracture healing of the patient taking Met is largely unknown. In this study, we tested the hypothesis that injection of frHMGB1 with Met improves nonunion bone fracture healing in a diabetic rat model.

METHODS: DM was induced by injection of streptozotocin (STZ) into total 32 SD rats (**Fig. 1A**) and confirmed by blood glucose levels. The skin at tibia area was opened, tibia bones were fractured (**Fig. 1B**), and the muscle and skin wound were sutured (**Fig. 1C**). The wounded rats were divided into four groups with 8 rats/group and injected with Met (IP, 160 mg/kg) and/or frHMGB1 (injection to wound area, 250 µg/kg): Group 1: No treatment- (**Wound**); Group 2: Met daily (**Met**); Group 3: frHMGB1 weekly (**frHMGB1**); and Group 4: Met daily and frHMGB1 weekly (**frHMGB1+Met**). The rats were monitored every day after surgery and four rats from each group were sacrificed postoperative on day-28 and day-90. The nonunion bone fracture healing was examined by gross inspection, micro-CT image scout view, histochemical staining and immune staining. HMGB1 and interleukin1-β (IL1-β) levels were determined using ELISA kits. ***Statistical analysis*** – Data were analyzed by One-way ANOVA followed by Fisher's LSD for multiple comparisons. A p-value less than 0.05 was considered to be a significant difference between the groups. This study was approved by IACUC.

RESULTS: Two days after injection of STZ, the blood glucose levels were over 200 mg/dL in all the experimental rats confirming DM. At day-28 post-surgery, the untreated wound was still open, the tibia fractures were exposed, and red and swollen tissues were visible on the wound area (yellow arrow in **Fig. 1D**). At day-90 post-surgery, the infection was evident in the wound area with swollen muscle tissue (yellow arrow in **Fig. 1E**). Although Met-treated wound healed better than untreated wound, large red and swollen tissues were present at day-28 and day-90 (**Fig. 1F, G**). frHMGB1-treated wound had normal tissue color, indicating much faster healing than the other three groups (**Fig. 1H, I**). frHMGB1+ Met-treated wound (**Fig. 1J, K**) healed better than untreated wound (**Fig. 1D, E**) and Met treated wound (**Fig. 1F, G**), but slower than frHMGB1 treated wound (**Fig. 1H, I**). Micro-CT scout view images showed that unhealed bone fractures in wound group had a large gap between two thin fractured bones (**Fig. 2A, B**). In Met-treated group (**Fig. 2C, D**), the gap was much smaller, and the fractured bone was much thicker with higher bone density than those of untreated bone fractures (**Fig. 2C, D**). There was no gap in the frHMGB1 treated fractured bone area which was filled with high density of bone tissues (**Fig. 2E, F**). Although Met slowed the bone fracture healing in frHMGB1+Met treated rats, the healed bone tissue was much stronger than Met alone group and untreated group (**Fig. 2G, H**). The levels of inflammatory markers, HMGB1 and IL1-β that were increased in the serum of the untreated and frHMGB1-treated wounds were decreased by Met (**Figs. 2I, J**).

Histology analysis indicated that the untreated fractured tibia bone healed much slower than the other three groups, and H&E staining showed large unhealed wound area with little bone tissues in the fractured bone area (yellow dash line area in **Fig. 3A**). The fractured bone in Met-IP injection group (**Fig. 3B**) healed much better than wound only group as evidenced by smaller gap area (black dash line area in **Fig. 3B**). The local injection of frHMGB1 enhanced bone fracture healing (**Fig. 3C**) by recruiting cells to the wound area (yellow arrow in **Fig. 3C**). Although Met-IP injection slowed the healing, the healed bone tissue with high cell density and small gap area (**Fig. 3D**). Picro-Sirius red and Safranin O and Fast green (S&F) staining indicated that frHMGB1 enhanced bone fracture healing, produced more functional bone with collagen type I (red in **Fig. 3K,** yellow and red in **Fig. 3O**). High levels of collagen III in the wound area (green in **Fig. 3O**), indicated that these bones were newly formed tissues. Large empty areas were found in untreated wounds (**Fig. 3I**) and Met-treated wounds (**Fig. 3J**). More collagen III was found in the wound area treated with frHMGB1+Met (green in **Fig. 3P**), indicating that healing is in process.

DISCUSSION: Our results indicated that in diabetic rats, nonunion bone fracture occurred easily once they were injured. The frHMGB1 treatment enhanced nonunion bone fracture healing by promoting cell proliferation, fibroblast migration and collagen I and collagen III production. Met injection inhibited frHMGB1activity but may improve the healing quality. Future studies will focus on the histological and immune staining of rat bone tissue section to further investigate the effect of frHMGB1 on diabetic nonunion bone fracture healing, especially on regeneration of high-quality bone tissue with less scar tissue.

SIGNIFICANCE/CLINICAL RELEVANCE: frHMGB1 can enhance diabetic nonunion bone fracture healing but may form scar tissue. Met may help to form high quality bone tissue with reduced scar tissue formation.

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REFERENCES: 1) Olokoba, A., *et al.* Oman Med J: 2012;27(4):269-73; 2) Lee, G., *et al.* PNAS:2018;115(19): E4463-E4472; 3) Horiuchi, T., *et al*. J Biol Chem: 2017; 292(20):8436-46.