Characterization of Femur, Mandible and Bone Marrow-derived Mesenchymal Stromal Cells from Streptozotocin-Injected Mice

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Abstract

Streptozotocin injection is simply shown to promote hyperglycemic condition in several species. In the present study, we aimed to characterize the femoral and mandibular bone features of streptozotocin-induced diabetic mice. In addition, the characteristics of bone marrow-derived mesenchymal stromal cells were evaluated. Results showed that streptozotocin injection resulted in significant weight loss and increase in fasting blood glucose levels in the animals. Compared to the control mice, significantly greater bone surface/bone volume value was noted in the mandible and the femur. On the contrary, trabecular thickness was significantly decreased in the metaphysis area of the femur. Further, the bone volume/total volume significantly decreased in the diaphysis area of the femur and in the mandible. Bone volume density was significantly decreased in the femur but not the mandible. Bone marrow-derived mesenchymal stromal cells (BMSC) isolated from the streptozotocin-induced diabetic mice exhibited marked reduction in colony forming unit ability and cell proliferation. However, the proliferation ability of BMSC was rescued with the presence of basic fibroblast growth factor (bFGF). Together, the results suggest that streptozotocin-induced diabetic condition affects bone phenotype and BMSC’s behaviors. In addition, bFGF supplementation could be used to expand BMSC in vitro. Thus, streptozotocin-injected mice could be utilized as a model to evaluate bone regeneration and engineering in diabetic condition.

Keywords: bone, bone marrow-derived mesenchymal stromal cells, diabetic condition, streptozotocin

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Introduction

Diabetes mellitus is one of the major health problems worldwide. Uncontrolled blood glucose levels in diabetic patients result in increased risk of infection, cardiomyopathy, neuropathy, retinopathy, nephropathy, etc. (Ruggenenti and Remuzzi, 1998; Jongswaaij et al., 2013; Muhammad and Hashmi, 2013). In addition, the alteration of bone homeostasis and metabolism is noted in diabetic patients. In this regard, it was shown that diabetic patients had decreased serum osteocalcin and bone mineral density compared to normal patients (Chen et al., 2013). This impaired bone metabolism was more severe in patients affected with diabetic neuropathy, especially in male (Rasul et al., 2012; Chen et al., 2013). Growth factors related to bone formation/healing, vascular endothelial growth factor and bone morphogenetic protein-2, were reduced in cells obtained from diabetic patients (Ilic et al., 2012). Together, these results demonstrate the influence of hyperglycemic condition in bone homeostasis.

Several animal models have been established to mimic diabetic condition. Animal models are useful tools to investigate the science of diabetic physiopathology as well as mechanism. In addition, they could be utilized to examine novel potential treatment approach for reducing blood glucose levels, e.g. stem cell transplantation. Among the models, streptozotocin injection was widely used to induce diabetic-like conditions in several species, for example, mouse, rat, dog, pig and monkey (Bevilacqua et al., 1985; Dufrane et al., 2009; von Wilmowsky et al., 2010; Lee et al., 2014; Marangoni et al., 2014). Mouse is the standard species for in vivo study and is the least sentient mammalian species. Many molecular tools are also available for this particular species, making it a candidate species for physiopathological model for many diseases. However, the characterization of bone and bone marrow-derived mesenchymal stromal cell alteration in streptozotocin-induced diabetic mice model is yet lacking.

It was also noted that cell proliferation and osteogenic differentiation ability were reduced in streptozotocin-injected rats and mice (Jin et al., 2010; Stolzing et al., 2010; Zhao et al., 2013). Several growth factors were investigated to promote diabetic bone marrow stromal/stem cell proliferation as well as osteogenic differentiation, e.g. platelet-derived growth factor-BB (Zhao et al., 2014). Basic fibroblast growth factor (bFGF) is of interest to promote diabetic cell proliferation. The bFGF was employed as supplementation in culture medium of various stem cells, especially pluripotent stem cells (Quang et al., 2014). This molecule promoted stemness and proliferation of various cell types (Osathanon et al., 2011; Sukarawan et al., 2014).

Therefore, the present study aimed to investigate the effect of hyperglycemic condition in femoral and mandibular bone change in streptozotocin-induced diabetic mice. Further, the bone marrow-derived mesenchymal stromal cell behaviors from streptozotocin-induced diabetic mice and the influence of bFGF in these cells were examined.

Materials and Methods

Animal experiments: The animal experiment was approved by the Animal Care and Use Committee, Faculty of Dentistry, Chulalongkorn University (protocol number 123002). Five-week-old male C57BL/6Mac mice with body weight at baseline of approximately 20-30 grams were employed in the present study. The animals were kept in stainless steel, solid bottom, opened top cage in a conventional housing system with standard fluorescent light 12:12 hour-cycle. Temperature of the housing was also controlled. Standard diet and tap water were provided ad libitum. The mice were intraperitoneally injected with streptozotocin at concentration of 180 mg/kg for single dose. Body weight and fasting blood glucose levels were measured at 48 h, 1 week, 2 weeks, 4 weeks, 8 weeks, and 12 weeks after the streptozotocin injection. For blood glucose measurement, all animals were fasted for 12 h before measurement and the fasting blood glucose levels was measured using glucometer (Accu-Chek Performa, Roche, Mannheim Germany) using blood from tail tip. Mice with blood glucose level > 250 mg/dl were defined to have diabetic condition. The animals were maintained according to the condition approved by the Animal Care and Use Committee.

Micro-computerized tomography analyses: At 12 weeks after streptozotocin induction, the animals were sacrificed. The femurs and mandibles were dissected and fixed in 10% buffered formalin for 48 h. The specimens were evaluated using micro-computerized tomographic machine (μCT35, Scanco Medical, Switzerland) in PBS. The specimens were scanned with parameters as follows: 70 kVp, 114 μA, 8 watts, and voxel size 10 μm. The same threshold was assigned to all sample analyses in the study. The analyses were performed using 3D Analysis software from Scanco Medical. The distance transformation model was employed for all 3D analyses. Bone surface/bone volume was calculated based on the triangulation of surface model. Volume of interest (VOI) for mandible was set according to previous published protocol (Lee et al., 2010). Briefly, the furcation area of the first mandibular molar in bucco-lingual cross section slices was identified as the reference slice. Ten slices before and after the reference slice were included in the VOI. The tooth area in those VOI was omitted from the analysis. For femur, the identification of VOI was performed according to previous report (Lau et al., 2013). In this regard, the growth plate was identified as the reference slice. The metaphysis area was evaluated at 150-300 slices and the diaphysis area was evaluated at 600-700 slices apart from the growth plate.

Histological analyses: The specimens were decalcified using acid formalin solution (4% formalin/ 10% acetic acid solution) for 4 weeks in 4°C. Subsequently, the specimens were dehydrated through a graded series of ethanol and embedded in paraffin. The section was obtained at 5 μm in thickness and further stained with hematoxylin and eosin solution.
Cell isolation: At 12 weeks after streptozotocin administration, the mice were sacrificed and bone marrow-derived mesenchymal stromal cells were isolated. Briefly, femur bones were dissected and cleaned under sterile condition. Femoral marrow cavity was flushed with culture medium (Dulbecco’s Modified Eagle medium (Gibco, CA, USA) supplemented with 10% fetal bovine serum (Gibco, CA, USA) and 1% penicillin-streptomycin (Gibco, CA, USA)). Single cell suspension was obtained by pipetting up and down for several times. The cells were maintained in 60 mm tissue culture plates (Corning Incorporated, USA) and unattached cells were washed out using sterile PBS at 24 h after isolation. The culture medium was changed every three days for 2 weeks. These cells were considered as passage 0. Cells from passage 1-2 were employed in the study.

Colony forming unit assay: The cells were trypsinized and plated in 60 mm tissue culture plate at concentration of 500 cells/plate. The culture medium was changed every three days and the cells were maintained for 14 d. Thereafter, the cells were fixed with 10% buffered formalin (MERCK, Germany) and stained with coomassie blue. Under inverted light microscope observation, a clump of cells of approximately more than 50 cells was considered as colony.

MTT assay: The cells were seeded in a 24-well plate at concentration of 12,500 cells/well. In some experiments, bFGF (Sigma, USA; 10 ng/mL) was added in the culture medium. The medium was changed every three days. At 1, 3, and 7 d after plating, the culture medium was removed and washed with sterile PBS. MTT solution (USB Corporation, USA) was added to the culture medium to make final concentration of 1 mg/mL. The cells were incubated with MTT solution for 10 min at 37°C. Formazan precipitation was dissolved with dimethyl sulfoxide and glycine buffer. Absorbance was measured by microplate reader at 570 nm.

Statistical analyses: Results were presented as mean±SD. Statistical significance was examined using independent sample t-test. P value below 0.05 was considered as statistical significance.

Results

Characterization of streptozotocin-induced diabetic mice: Results showed that streptozotocin-injected mice (n=8) exhibited a decrease in body weight compared to the control mice (n=4). The body weight of diabetic induced mice decreased through 12 weeks, while that of the control group slightly increased. A significant difference was noted at 2, 8, and 12 weeks (Fig 1 A). In addition, it was noted that the fasting blood glucose was significantly increased as early as 48 h after streptozotocin administration (Fig 1B). The fasting blood glucose increased significantly at all evaluated time-points compared to that of the control mice. The blood glucose levels were rapidly increased during the first 2 weeks and maintained its levels thereafter in the streptozotocin-injected groups. On the contrary, no significant change in the fasting blood glucose was noted in the control mice. These results demonstrated the diabetic-like condition in streptozotocin-induced mice as determined by weight loss and elevated blood glucose.

Characterization of femur bone from streptozotocin-induced diabetic mice: The representative radiographic images showed that the femur bones of streptozotocin-induced diabetic mice exhibited thinner cortical bone in diaphysis and metaphysis area compared to those of the control mice (Fig 2). Moreover, a slight increase in trabecular bones was observed in metaphysis area of femur bones from the streptozotocin-injected group.
Correspondingly, the quantitative μCT analyses demonstrated that femur bones of the streptozotocin-administration mice (n=7) had an increase in bone surface/bone volume and trabecular number in metaphysis area compared to the control mice (n=5) (Fig 3C and D). However, statistical significance was noted only for the bone surface/bone volume. The bone volume density, trabecular thickness, and trabecular separation in metaphysis area were significantly decreased in the streptozotocin-induced diabetic mice (Fig 3A, E, and F). In diaphysis area, the bone volume density and bone volume/total volume were significantly decreased, while the bone surface/bone volume was significantly increased in the streptozotocin-injected group (Fig 3G-I).

Histological evaluation presented that the cortical bone of metaphysis and diaphysis regions of femur bone from the streptozotocin-induced mice were thinner than those of the control mice (Fig 4). The femur of the diabetic-induced mice exhibited continuously trabecular bones, whereas the trabecular bones of the control femur bone were moderately interrupted (Fig 4B and E). The thickness of trabecular bone was relatively reduced in the streptozotocin-injected mice compared with the control mice (Fig 4).

Figure 3  Quantitative analyses from μCT measurement of femur bones from the control and streptozotocin-injected mice. The asterisks indicate statistically significant difference compared to the control mice at p<0.05. (Ctrl, control mice (n=5); DM, diabetic mice (n=7))

Figure 4  Representative histological images of femur bones from the control (A, B, C and G) and streptozotocin-injected mice (D, E, F and H). Difference in cortical and trabecular bone thickness was exhibited between the control and streptozotocin-injected femoral bone. Scale bar: 250 μm (A, B, D and E) and (C, F, G and H). (Ctrl, control mice; DM, diabetic mice)
Characterization of mandibular bone from streptozotocin-induced diabetic mice: The representative radiographic images of mandibular bone illustrated a decrease in cortex bone thickness and the trabecular bone area in the cross and horizontal section of the mandible (Fig 5). No obvious difference was noted in the longitudinal section of the mandibular bone. The radiographic quantitative measurement using μCT showed a significant decrease in bone volume/total volume in the streptozotocin-administered mice (n=6) compared to the control mice (n=3) (Fig 6). On the contrary, the bone surface/bone volume was significantly increased in the streptozotocin group (Fig 6C). Differences in other parameters were not statistically significant.

The longitudinal histological section of mandibular bone revealed no marked difference between the diabetic-induced mice and the control mice (Fig 7). However, the trabecular bones were slightly decreased in the mandible of the streptozotocin-injected mice (Fig 7A and D). The characters of alveolar bone and periodontal ligament were not histologically different in both groups.

Figure 5  Representative radiographic images of mandibular bones from the control (A, C and E) and streptozotocin-injected mice (B, D and F)

Figure 6  Quantitative analyses from μCT measurement of mandibular bones from the control and streptozotocin-injected mice. The asterisks indicate statistically significant difference compared to the control mice at p<0.05. (Ctrl, control mice (n=6); DM, diabetic mice (n=3))

Figure 7  Representative histological images of mandibular bones from the control (A-C) and streptozotocin-injected mice (D-F). These images show slight difference in trabecular and cortical bone of mandible between the two groups. Scale bar: 250 µm (A, B, D and E) and (C and F). (Ctrl, control mice; DM, diabetic mice)
Characterization of bone marrow-derived mesenchymal stromal cells from streptozotocin-induced diabetic mice: A marked reduction in the colony forming unit was noted in those cells isolated from the streptozotocin-induced diabetic mice (n=3) as compared to those of the control mice (n=3) (Fig 8A). Descriptively, the colony cell density was also decreased in the streptozotocin-injected mice compared to that of the control mice (Fig 8B and C). Cell proliferation was evaluated using MTT assay and the results illustrated that the bone marrow-derived mesenchymal stromal cell isolated from the streptozotocin-induced diabetic mice had cell proliferation impairment (Fig 9A). However, the cells from the control mice were able to proliferate as indicated by an increase in optical density of the formazan solution. These results may imply the cell proliferation impairment in bone marrow-derived mesenchymal stromal cell isolated from streptozotocin-induced diabetic mice. Interestingly, the bFGF supplementation was able to promote cell proliferation in the cells isolated from the streptozotocin-injected mice (Fig 9B).

Discussion

The present study described the characteristics of femoral and mandibular bone morphology as well as the bone marrow-derived mesenchymal stromal cell behaviors. As mentioned above, the examination of bone characters, especially mandible, in streptozotocin-induced diabetic mice is still limited. In this study, we noted the decrease in bone density, trabecular thickness, and bone volume in the femur, exhibiting osteoporotic-like features. Moreover, we demonstrated the thin discontinuous trabecular bone and reduction in cortical bone thickness in the streptozotocin-induced mice femur. Correspondingly, it was previously reviewed that both type I and II diabetes were associated with the increased risk of osteoporotic fracture (Antonopoulou et al., 2013). Moreover, it was reported that bone mineral density was reduced in type I diabetic patients (Abd El Dayem et al., 2011; Yan and Lee, 2013). This phenomenon correlated with low bone formation and high bone resorption markers in those patients (Abd El Dayem et al., 2011). Further, alloxan-induced diabetic rats exhibited an increase in trabecular distance but had a decrease in trabecular thickness and trabecular bone volume (Duarte et al., 2005). A spontaneous type 2 diabetic rat model demonstrated a decrease in trabecular bone volume, trabecular thickness and trabecular number (Zhang et al., 2009). Thus, the slight discrepancy of bone parameters in diabetic condition was noted between our study and other reports. This could be due to several reasons, e.g. the species difference, the diabetic induction technique, and the bone parameter measurement methods. Further, it was reported that age and diabetic duration influenced the bone mineral density in human (Hadjidakis et al., 2009). Thus, these streptozotocin-induced type 1 diabetic mice model should be further carefully characterized regarding bone metabolism and remodeling.

Streptozotocin is a glucosamine-nitrosourea compound that is toxic to pancreatic beta cells (Graham et al., 2011). It was noted that mice receiving streptozotocin developed a diabetic condition that required insulin injection (Graham et al., 2011). Moreover, it has previously been reported that single-dose streptozotocin injection in mice resulted in damage of pancreatic islets and the insulin positive cells in those islets were markedly decreased (Deeds et al., 2011). We also noted the same phenomenon in the islets of streptozotocin-injected rats (data not shown). Corresponding with the present study, the high blood glucose levels and the loss of body weight were noted in streptozotocin-injected mice. However, it should be noted in the present study that the serum insulin levels as well as the beta cell viability were not characterized.
Thus, the interpretation of diabetic condition in the present study should be explicated with caution.

Diabetic condition influences the craniofacial bone structure. In this regard, it has been shown that diabetic infant rats had the reduction in craniofacial growth (Abbassy, et al., 2008). Particularly on mandible, the length and height were significantly lower in diabetic group (Giglio and Lama, 2001). In the present study, we did not focus on the development of the diabetic condition since the 5-week-old mice were employed. These streptozotocin-induced diabetic mice had thin buccal and lingual plates as well as thin mandibular cortex, including the reduction in bone volume/total volume. Interestingly, the mandibular bone density was not significantly different between the control and streptozotocin-induced mice. Corresponding with a previous study, the mandibular bone mineral density in type 2 diabetic patients slightly decreased compared to normal patients. However, no statistically significant difference was noted (Ay et al., 2005). The bone mineral density reduction in the femur but not in the mandible of the streptozotocin-induced diabetic mice might suggest that the different bones are preferentially affected by diabetic condition. In this respect, more susceptibility of the femoral bone to diabetic condition was observed in the streptozotocin-induced diabetic mice compared to the mandibular bone. Similarly, in a report on type II diabetic patients, bone mineral reduction was selectively found in distal radius but lumbar spine and femoral neck mineral change was not significantly different compared to the control patients (Majima et al., 2005).

The influence of diabetic condition on bone marrow-derived mesenchymal stromal cell behaviors was formerly reported in other species. In rat, the streptozotocin-induced diabetic condition resulted in the decrease in colony forming unit number and size of the isolated bone marrow mesenchymal stem cell (Stolzing et al., 2010). Moreover, the proliferation was markedly attenuated in bone marrow mesenchymal stem cell isolated from streptozotocin-injected rats (Jin et al., 2010). These cells were more susceptible to apoptosis when exposed to hypoxic condition, correlating with the reduction in vascular endothelial growth factor and insulin-like growth factor (Jin et al., 2010). Similarly, we noted the impaired cellular proliferation and colony forming unit ability in the bone marrow-derived mesenchymal stromal cell isolated from the streptozotocin-induced diabetic mice. Interestingly, the proliferation character of bone marrow-derived mesenchymal stromal cell-derived streptozotocin-induced diabetic mice was improved upon bFGF supplementation. It has been shown that bFGF was able to promote proliferation in several cell types, for example, murine compact bone-derived mesenchymal stem cells, rat bone marrow-derived endothelial progenitor cells, and human umbilical cord-derived mesenchymal stem cells (Guo et al., 2012; Ramasamy et al., 2012; Yamachika et al., 2012). Moreover, bFGF loaded biomaterials could enhance skin wound healing in diabetic rats (Yang et al., 2011).

In summary, the present study demonstrated that streptozotocin injection could lead to the hyperglycemic condition and body weight loss in mice. Further, this diabetic-like condition altered the morphological properties of femur and mandible as well as the bone marrow-derived mesenchymal stromal cell behaviors, suggesting the alternative diabetic model for investigation into bone metabolism and stem cell behaviors. Moreover, the results illustrated that bFGF supplementation promoted the proliferation of bone marrow-derived mesenchymal stromal cell isolated from streptozotocin-induced diabetic mice, implying the useful utilization of bFGF in \textit{ex vivo} expansion of bone marrow-derived mesenchymal stromal cell for further potential use.

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บทคัดย่อ

การศึกษากระดูกต้นขา กระดูกขากรรไกรล่างและเซลล์มีเซนไคม์จากไขกระดูกของหนูที่ได้รับการฉีดสารสเตรอยโซโทซิน

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การฉีดสารสเตรอยโซโทซินเป็นวิธีที่ง่ายในการกระตุ้นให้เกิดภาวะน้ำตาลในเลือดสูงในสัตว์ชนิดหนึ่ง การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาลักษณะของกระดูกต้นขาและกระดูกขากรรไกรล่างของหนูที่ได้รับการฉีดสารสเตรอยโซโทซิน นอกจากนี้ยังมีวัตถุประสงค์เพื่อศึกษาลักษณะของเซลล์มีเซนไคม์ที่แยกได้จากไขกระดูกของหนูด้วย ผลการศึกษาแสดงให้เห็นว่าการฉีดสารสเตรอยโซโทซินส่งผลให้หนูทดลองมีน้ำหนักลดลงและมีระดับของน้ำตาลในเลือดสูงอย่างมีนัยสัมพันธ์ทางสถิติ เมื่อเปรียบเทียบกับหนูในกลุ่มควบคุมพบว่า หนูที่ได้รับการฉีดสารสเตรอยโซโทซินมีค่าอัตราส่วนของพื้นที่ผิวกระดูกต่อปริมาตรกระดูกเพิ่มขึ้นอย่างมีนัยสัมพันธ์ทางสถิติทั้งในกระดูกต้นขาและกระดูกขากรรไกรล่าง แต่พบว่ามีความหนาของเส้นใยกระดูกยาวอย่างมีนัยสัมพันธ์ทางสถิติส่วนเมตาไฟซิสของกระดูกต้นขา นอกจากนี้ยังพบว่าอัตราส่วนของปริมาตรกระดูกต่อน้ำหนักของหนูมีค่าลดลงอย่างมีนัยสัมพันธ์ทางสถิติในกระดูกขากรรไกรล่างและส่วนโดยไขซิสของกระดูกต้นขา ค่าความหนาแน่นของกระดูกกระดูก(CON) ของเซลล์มีเซนไคม์ที่แยกได้จากไขกระดูกของหนูที่ได้รับการฉีดสารสเตรอยโซโทซินมีความสามารถในการสร้างโคโลนิและเพิ่มจำนวนของเซลล์ลดลงเมื่อเทียบกับกลุ่มควบคุม อย่างไรก็ตามผลการทดลองในจนบางที่กระดูกจะทำให้เพิ่มความสามารถในการเพิ่มจำนวนของเซลล์มีเซนไคม์ที่แยกได้จากไขกระดูกของหนูที่ได้รับการฉีดสารสเตรอยโซโทซิน ผลการศึกษาข้างต้นแสดงให้เห็นว่าการฉีดสารสเตรอยโซโทซินมีผลกระทบต่อลักษณะของกระดูกและคุณสมบัติของเซลล์มีเซนไคม์ที่แยกได้จากไขกระดูก ดังนั้นหนูที่ได้รับการฉีดสารสเตรอยโซโทซินอาจใช้เป็นต้นแบบในการศึกษาเกี่ยวกับการเจริญเติบโตและวิศวกรรมเนื้อเยื่อกระดูกในภาวะที่มีภาวะน้ำตาลในเลือดสูงได้

คำสำคัญ: กระดูก เซลล์มีเซนไคม์ที่แยกได้จากไขกระดูก ภาวะน้ำตาลในเลือดสูง สเตรอยโซโทซิน

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