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In vitro assessment of the DNA damage and senility of CD117+ stem cells collected from diabetic mice

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Abstract. – OBJECTIVE: Angiogenesis impairment is a common feature of diabetes mellitus (DM), whereas CD117+ bone marrow cells (BMCs) injury might be responsible for such complication. In this study, we studied the effect of hyperglycemia on the DNA damage and senility of CD117+ bone marrow cells.

MATERIALS AND METHODS: We isolated CD117+ BMCs from the Streptozotocin (STZ) induced diabetes and healthy control mice. Oxidative stress was detected by flow cytometric analysis. γ-H2AX, which is the DNA damage mark, was detected by using Western blotting and immunofluorescence histochemistry. We also detected the expression of γ-H2AX and p16 by using Western blotting.

RESULTS: Compared with the control mice, the level of reactive oxygen species (ROS) was increased significantly in the CD117+ BMCs collected from the diabetic mice ($p<0.05$), and the percentage of γ-H2AX positive cells was higher significantly ($p<0.01$). The expression of γ-H2AX and p16 was increased significantly in the CD117+ BMCs from the diabetic mice.

CONCLUSIONS: Our experiments demonstrated the oxidative stress in CD117+ BMCs under DM conditions, while accelerating the DNA damage and senility in CD117+ BMCs as well.

Key Words:
Diabetes, CD117+ BMCs, DNA damage, Senility.

Introduction

DM is one of the most commonly diagnosed diseases, 285 million people are deeply troubled with diabetes, while it is estimated that there will be 439 million DM patients by 2030[1]. DM is often accompanied by multi-organ complications, including ischemic diseases[2,3]. Within the last years, impaired angiogenesis, which was closely associated with ischemic diseases, was identified in DM patients[4,5], whereas the exact mechanism remains unclear.

Previous studies[6-8] have proved that various types of stem cells in bone marrow can host the damaged area of tissues, then differentiating into endothelial cells and secrete some growth factors, thus exerting tissue repair activity. Decreased potency of BMCs for inducing angiogenesis can be found in patients and animals with DM[9-11]. It is well known that oxidative stress associates DM with cell damage[12]. Thus, we hypothesized that increased oxidative stress could induce DNA damage and cellular senility of CD117+ BMCs, which are the main cause for poor angiogenesis in DM.

Materials and Methods

Animals

Male, 6~8-week-old Balb/C mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). 12 mice were randomly divided into normal control and diabetes groups, 6 mice in each group. 60 mg/kg streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO, USA) administration by daily subcutaneous injection for 8 weeks were performed in diabetes group (n = 6). Citric acid administration by daily subcutaneous injection for 8 weeks in control group (n = 6) was performed. Animals were housed in controlled conditions of 12 h light, 12 h

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darkness and 22°C temperature. After 8 weeks of STZ injection we measured the body weight and blood glucose in all mice. The random blood glucose was higher than 15 mmol/L, indicating that the diabetes model was successfully prepared. All procedures performed in these studies involving mice were undertaken in accordance with the ethical standards of the institutional and/or national research committee. The approval was obtained from the Animal Care and Use Committee and Ethics Committee of Nanchang hospital affiliated Zhongshan University, Nanchang, China, before the commencement of the study (No: 17/NC/0153).

Blood Glucose Measurement

All mice were sacrificed by cervical dislocation after weighting, and blood was collected by eyeball extirpating. The blood glucose was measured by OneTouch Verio Flex Glucometer (Johnson & Johnson, Inc., New Brunswick, NJ, USA)

Separation of CD117+ BMCs

Bone marrow was collected from the femurs and tibiae of mice and was suspended in PBS. We isolated the bone marrow mononuclear cells by density gradient centrifugation and resuspended the cells in PBS. By using the antibody to the stem cell marker (CD117), $CD117^+$ cells were separated from the collected bone marrow mononuclear cells. Briefly, the cells were incubated with FITC-conjugated rat anti-mouse CD117 (c-Kit) monoclonal antibodies (Pharmingen, San Diego, CA, USA) for 60 mins at 4°C. After being washed, the cells were incubated with anti-FITC microbeads (Miltenyi Biotech, Bergis Gradbach, Germany) according to the manufacturer's instructions. $CD117^+$ cells and $CD117^-$ cells from the BMCs were separated by passing them through a magnetic-activated cell sorting system (MACS; Miltenyi Biotech, Bergis Gradbach, Germany).

Intracellular ROS Analysis

Isolated $CD117^+$ BMCs were incubated with 5 mmol/L 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA) (Invitrogen, Carlsbad, CA, USA) for 30 mins at 37°C. Flow cytometer (Becton Dickinson, Inc) was used to analyze fluorescence intensity in $CD117^+$ BMCs.

Immunofluorescent Staining of γ-H2AX

Isolated $CD117^+$ BMCs were plated onto the polylysine-coated 24-well chamber slides at 10^5 cells per well, and then, incubated at 37°C for 12 h. The cells were incubated with anti-γ-H2AX antibodies (Abcam, Cambridge, UK) for 1 hour at room temperature. After being washed for three times, the cells were stained with the FITC-combined secondary antibodies (Abcam, Cambridge, UK), and then, they were incubated with DAPI for 15 m and mounted in DABCO mounting medium (Leagene, Beijing, China).

Protein Extraction and Western Blot

Total protein was extracted from isolated $CD117^+$ BMCs through RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). Antibodies against γ-H2AX (1:5000, Abcam, Cambridge, UK), p16 (1:1000, Cell Signaling Technology, Boston, MA, USA), β-actin (1:5000, EnoGene Biotech Co., Nanjing, China) were used as primary antibodies, and a horseradish peroxidase-conjugated goat anti-rabbit antibody (1:10000, Beyotime Biotechnology, Shanghai, China) was used as a secondary antibody. β-actin was used as a loading control.

Statistical Analysis

All data were presented as mean ± SD. Statistical significance was determined by using the unpaired *t*-test for comparison between 2 means. $p < 0.05$ and $p < 0.01$ were considered as the significant and very significant results, respectively.

Results

STZ Treatment Induces DM in Balb/C Mice

Before STZ treatment, the body weight of diabetic and control mice was 20.21 ± 0.97 g and 19.91 ± 0.84 g respectively ($p>0.05$). The blood glucose of both groups was 7.60 ± 0.92 mmol/L and 7.68 ± 0.67 mmol/L, respectively ($p>0.05$). After STZ treatment, the body weight was 21.13 ± 0.79 g in diabetic mice and 28.14 ± 0.83 g in control mice, respectively. The blood glucose of both groups was 21.28 ± 1.46 mmol/L and 7.85 ± 0.66 mmol/L respectively. Compared with the control mice, the body weight of diabetic mice was significantly lower (Figure 1A), and the blood glucose levels of diabetic mice were significantly higher (Figure 1B). These results indicated that DM model was successfully constructed in STZ-treated mice.

DM Leads to ROS Imbalance in CD117+ BMCs

Oxidative stress plays a critical role in cell damage under DM conditions[12]; thus, we spec-

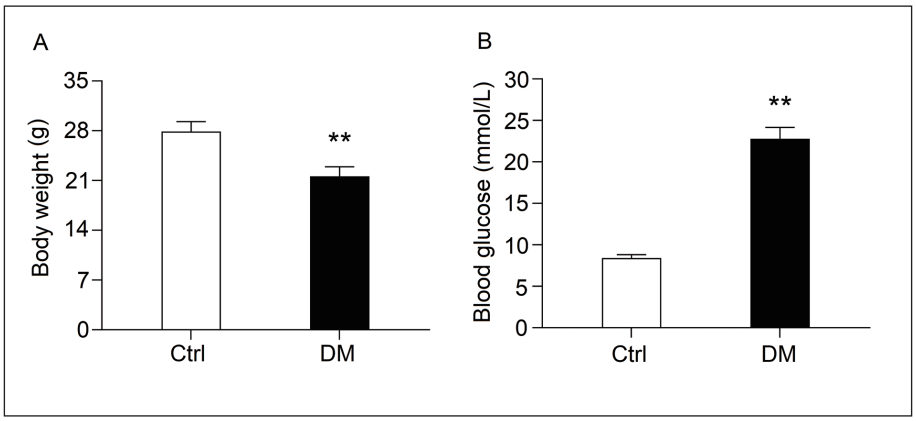

Figure 1. STZ treatment results in reduced body weight and high blood glucose. **A,** 8-week administration of STZ leads to reduced body weight; **B,** Higher blood glucose levels in STZ-treated mice. (Ctrl: Control mice, DM: Diabetic mice; n= 6 mice per group, $^{**}p < 0.01$).

ulated an unbalanced state in ROS generation and elimination might be observed in the CD117$^+$ BMCs from the diabetic mice. As shown in Figure 2, the levels of ROS in the CD117$^+$ BMCs were significantly higher in diabetic mice compared with the control mice, which suggested a disturbance of redox balance in CD117$^+$ BMCs under DM conditions.

DM Causes DNA Damage in CD117$^+$ BMCs

To determine whether DM could induce DNA damage in the CD117$^+$ BMCs, DNA damage marker γ-H2AX, was examined by the Western blotting and immunofluorescent staining. As expected, the level of γ-H2AX protein (Figure 3A) and the percentage of γ-H2AX positive cells (Figure 3B and C) increased significantly in CD117$^+$ BMCs that derived from diabetic mice compared with control mice. These observations implied that diabetes might induce the DNA damage in CD117$^+$ BMCs.

DM Results in CD117+ BMCs Senility

Next, we investigated the senility of CD117$^+$ BMCs from diabetic and control groups, the cellular senescence marker p16 was examined by Western blotting. Our results indicated that p16 expression level (Figure 4) in CD117$^+$ BMCs was increased in diabetic mice compared with control mice. Therefore, DM might induce the CD117$^+$ BMCs senility.

Discussion

Recently, cell-based therapeutic angiogenesis has become the focus of attention for the treatment of ischemic diseases[13-15]. Bone marrow contains various kinds of primitive stem cells, including the endothelial progenitor cells (EPCs), which can differentiate into endothelial cells and secrete several growth factors[6,7]. However, in diabetic patients, this treatment did not improve clinical symptoms[9-11]. It was reported that the number of EPCs declined, and their functioning was impaired in DM patients, which induce poor angiogenesis during ischemic neovascularization and ultimately affect tissue repair[16,17]. In previous studies[18,19], increased oxidative stress has been shown in both type 1 and type 2 DM patients, even in patients without complications[18,19], and ROS have been implicated in DM and exerted a critical role in cellular DNA damage and senility[20,21].

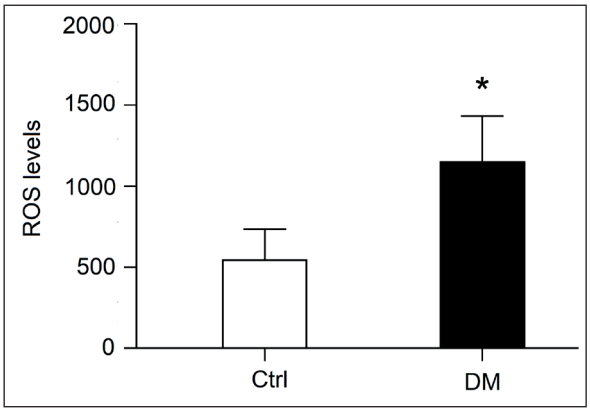

Figure 2. ROS production in DM mice. Flow cytometry analysis reveals higher levels of intracellular ROS in CD117$^+$ BMCs which derived from diabetic mice. (Ctrl: Control mice, DM: Diabetic mice; n = 3 independent experiments, $^{*}p < 0.05$).

$CD117^+$ BMCs is an important source of EPCs, while these cells can home to injured tissues and contribute to the formation of new blood vessels, which is highly involved in wound healing[22]. In present study, we found that the ROS generation in the $CD117^+$ BMCs from the diabetic mice was significantly higher than the control mice (Figure 2). It is known that ROS derived oxidative stress are responsible for the DNA damage[23], for instance, hydrogen peroxide can induce DNA single or double strand breaks[24]. As we showed, immunofluorescent staining and Western blotting revealed an upregulation of γ-H2AX in diabetic $CD117^+$ BMCs (Figure 3A and B). It is known that γ-H2AX is a variant of histone H2A in the mammalian cells, which is one of the earliest events involved in DNA damage response[25]. Therefore, upregulated-γ-H2AX in $CD117^+$ BMCs indicates DNA damage which induced by DM.

There are lots of stimuli has been proved to induce the cellular senescence, and these include telomere shortening, sustained mitogen stimulation, as well as DNA damage[26-28]. As mentioned above, ROS and DNA damage were presented in $CD117^+$ BMCs, while all of these are closely related to cellular senescence[29]. Thus, we speculated that DM is a primary cause of $CD117^+$ BMCs senility. The cycle arrest is an important feature of cellular senescence, as a key regulator of cell cycle, p16 has long been recognized as the mediator of senescence[30]. In present study, we found that the p16 was significantly increased in $CD117^+$ BMCs from the diabetic mice (Figure 4), which indicated that DM might result in the cellular senescence of $CD117^+$ BMCs.

As we known, stem/progenitor cells can divide asymmetrically. After division, one part of these cells still retains biological characteristics of stem cells, thus maintaining relative stability of stem/progenitor cells. Another part of these cells can differentiate into some specific kinds of cells to exert corresponding functions. DM-induced DNA damage might promote apoptosis of $CD117^+$ BMCs and lower their quantity in a whole, while

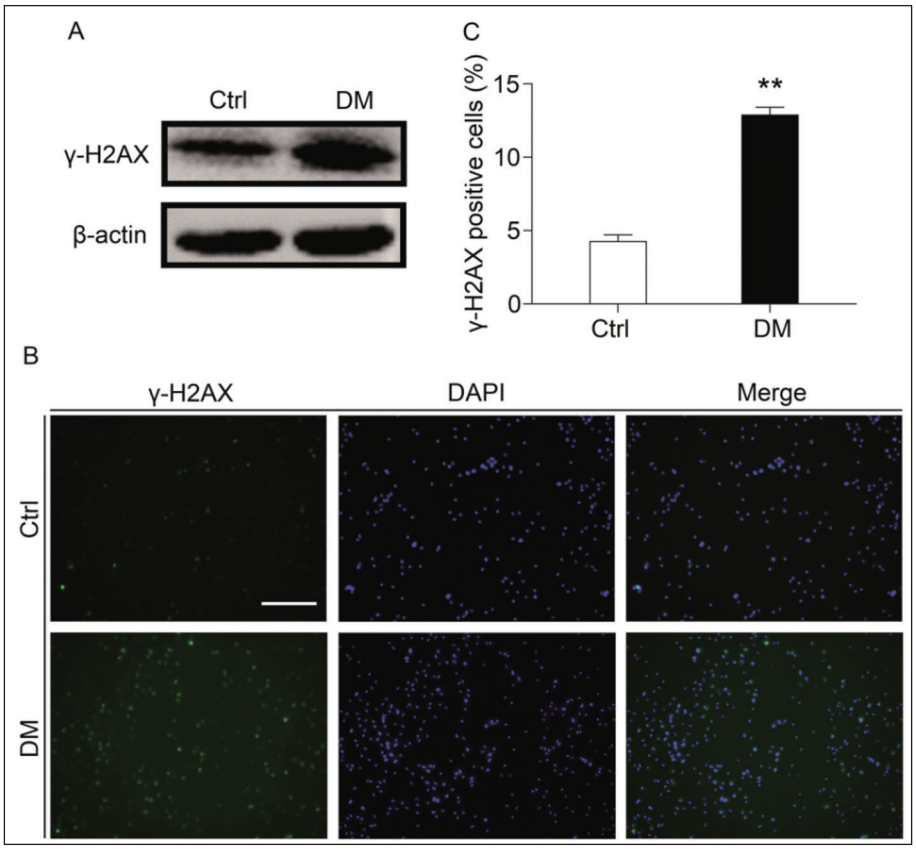

Figure 3. Diabetes causes DNA damage in $CD117^+$ BMCs. **A,** Western blot demonstrates upregulation of γ-H2AX in $CD117^+$ BMCs which derived from diabetic mice. **B-C,** Immunofluorescent staining reveals enhanced expression of γ-H2AX in $CD117^+$ BMCs which derived from diabetic mice. (Ctrl: Control mice, DM: Diabetic mice; β-actin is used as a loading control; Magnification: 400X; Scale bar: 50 nm; n = 3 independent experiments, ** $p < 0.01$).

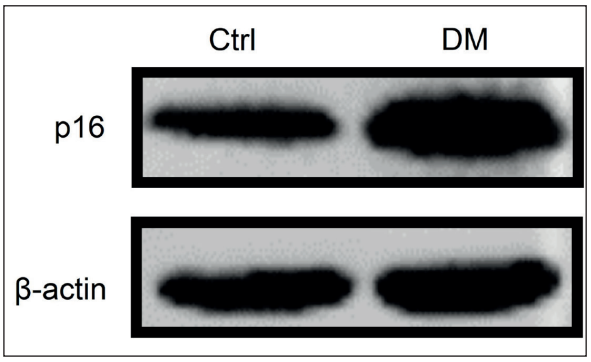

Figure 4. Diabetes upregulates the expression of p16 in CD117$^+$ BMCs. Western blot demonstrates upregulated expression of p16 in CD117$^+$ BMCs which derived from diabetic mice. (Ctrl: Control mice, DM: Diabetic mice; β-actin is used as a loading control).

DM-induced cellular senescence reduces the ability of asymmetric cell division in CD117$^+$ BMCs.

Thus, DM might affect CD117$^+$ BMCs in two ways, and ultimately reduces its ability of angiogenesis and tissue repair (Figure 5).

However, in this study, we did not reveal the mechanisms by which oxidative stress led to the DNA damage and senility in CD117$^+$ BMCs, while this should be achieved by analysis of DNA damage and senescence in CD117$^+$ BMCs after anti-oxidative treatment in diabetic mice.

Conclusions

In summary, our study has shown that the CD117$^+$ BMCs from diabetic mice have higher levels of ROS, which might induce DNA damage and senility of CD117$^+$ BMCs senility. Our results might disclose a potential mechanism of the poor angiogenic and tissue restorative potency in DM mice.

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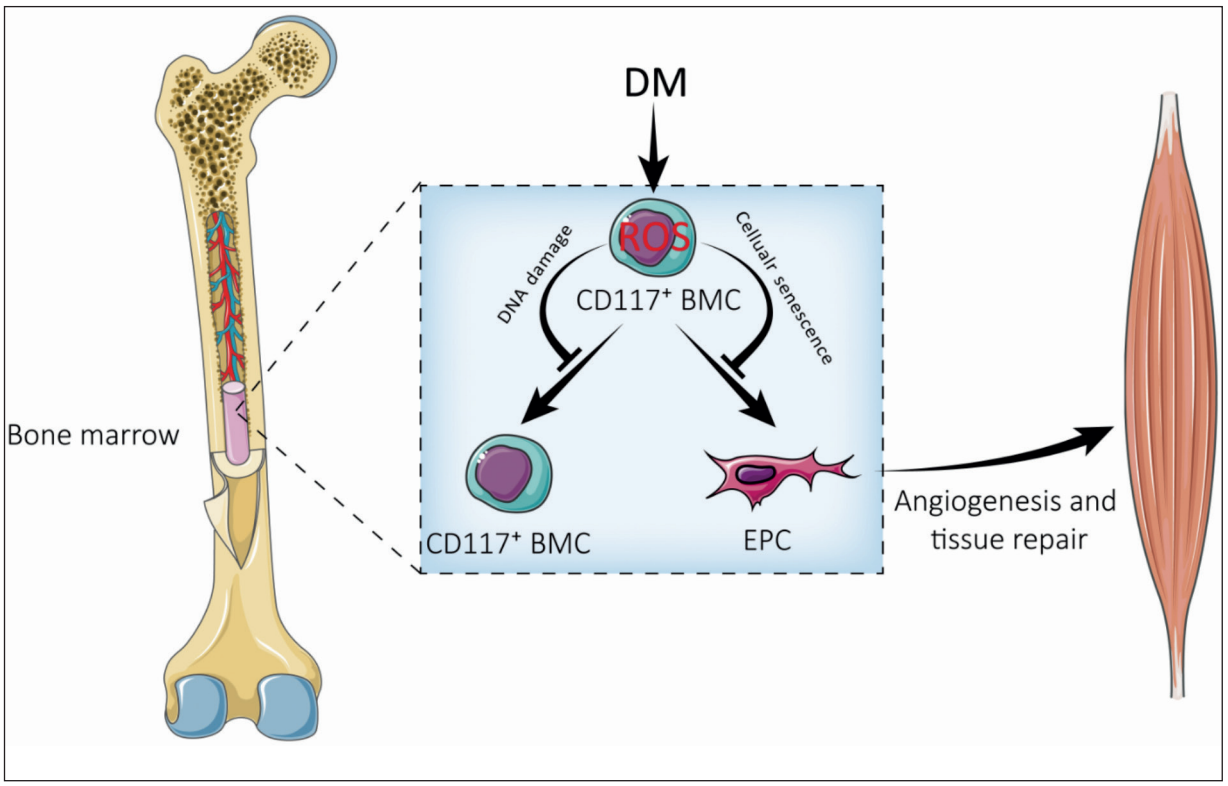

Figure 5. Schematic diagram of present study. DM induces ROS generation in CD117$^+$ BMCs, while this leads to DNA damage and cellular senescence in CD117$^+$ BMCs. DNA damage and cellular senescence might injure self-renew and differentiation of CD117$^+$ BMCs, which ultimately cause impairment of angiogenesis and tissue regeneration.

Ethical Approval

The study does not contain any studies with human subjects performed by the any of authors. All institutional and national guidelines for the care and use of laboratory animals were followed. The experimental protocol of this study was approved by the Animal Care and Use Committee and Ethics Committee, Nanchang hospital affiliated Zhongshan University.

Acknowledgements

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Conflicts of Interest

The authors declare no conflicts of interest.

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