

# Metformin accelerates wound healing in type 2 diabetic *db/db* mice

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**Abstract.** Wound healing impairment is increasingly recognized to be a consequence of hyperglycemia-induced dysfunction of endothelial precursor cells (EPCs) in type 2 diabetes mellitus (T2DM). Metformin exhibits potential for the improvement of endothelial function and the wound healing process. However, the underlying mechanisms for the observed beneficial effects of metformin application remain to be completely understood. The present study assessed whether metformin, a widely used therapeutic drug for T2DM, may accelerate wound closure in T2DM *db/db* mice. Genetically hyperglycemic *db/db* mice were used as the T2DM model. Metformin (250 mg/kg/day; intragastric) was administered for two weeks prior to EPC collection and wound model creation in *db/db* mice. Wound healing was evaluated by alterations in the wound area and the number of platelet endothelial cell adhesion molecule-positive cells. The function of the isolated bone marrow-derived EPCs (BM-EPCs) was assessed by a tube formation assay. The number of circulating EPCs, and the levels of intracellular nitric oxide (NO) and superoxide (O<sub>2</sub><sup>-</sup>) were detected by flow cytometry. Thrombospondin-1 (TSP-1) expression was determined by western blot analysis. It was observed that treatment with metformin accelerated wound healing, improved angiogenesis and increased the circulating EPC number in *db/db* mice. *In vitro*, treatment with metformin reversed the impaired BM-EPC function reflected by tube formation, and significantly increased NO production while decreasing O<sub>2</sub><sup>-</sup> levels in BM-EPCs from *db/db* mice. In addition, TSP-1 expression was markedly attenuated by treatment with

metformin in cultured BM-EPCs. Metformin contributed to wound healing and improved angiogenesis in T2DM mice, which was, in part, associated with stimulation of NO, and inhibition of O<sub>2</sub><sup>-</sup> and TSP-1 in EPCs from *db/db* mice.

## Introduction

The prevalence of diabetes mellitus is likely to exceed 591.9 million by 2035, and is becoming a serious international health crisis (1,2). The hallmark of diabetes mellitus is chronic hyperglycemia, resulting in vascular complications, including the impairment of angiogenesis, eventually resulting in disorders of wound healing and the development of refractory low extremity ulcerations (3,4).

A previous study demonstrated that circulating endothelial precursor cells (EPCs), a multifunctional population derived from bone marrow, are important for the promotion of angiogenesis and the maintenance of vascular homeostasis (5). An impairment in circulating EPCs may contribute to the pathogenesis of diabetic vasculopathy (6-8). Also, it has been observed that patients with type 1 and type 2 diabetes mellitus (T2DM) exhibited marked dysfunction in EPCs (9,10). In addition, a marked reduction in circulating EPCs was observed in diabetic patients (11-13). Preclinical experiments have demonstrated impaired tube formation in the EPCs of diabetic mice (14). These previous data indicated that the dysfunction of EPCs may be associated with a deterioration in wound healing in diabetes (15,16).

Metformin, an oral antihyperglycaemic agent, is the first-line drug in the clinic for patients with T2DM (17,18). Studies have demonstrated that metformin is able to increase the circulating EPC number in addition to improving the cellular function of EPCs in patients with T2DM (8,19). Thrombospondin-1 (TSP-1), a novel antiangiogenic adipokine, has been reported to be expressed in animal models susceptible to diabetes, including obesity and insulin resistance (20). Genetically augmented mRNA expression of TSP-1 has been observed in diabetes mellitus (21,22). A previous study demonstrated an apparent detrimental effect of TSP-1 on EPC function, which was reported to be negatively-correlated with nitric oxide (NO) regeneration in *in vitro* endothelial cells (23). Although metformin has been reported to be a regulatory factor for TSP-1 in patients with polycystic ovarian syndrome, little data are currently available in diabetes (24).

Xie *et al* (25) reported that the function of EPCs is associated with cellular oxidative stress. The study demonstrated that a

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**Abbreviations:** T2DM, type 2 diabetes mellitus; BM-EPCs, bone marrow-endothelial precursor cells; NO, nitric oxide; O<sub>2</sub><sup>-</sup>, superoxide; TSP-1, thrombospondin-1; CD31, platelet endothelial cell adhesion molecule; DAF-FM, 4-amino-5-methylamino-2',7'-difluorofluorescein; DHE, dihydroethidium; i.g., intragastric; Sca-1, ataxin-1; Flk-1, vascular endothelial growth factor receptor 2

**Key words:** T2DM, metformin, EPCs, wound healing, TSP-1

decrease in the NO level or excess generation of superoxide ( $O_2^-$ ) may result in a detrimental effect on EPCs, as indicated by impaired angiogenesis and tube formation. Little data is available concerning the role of metformin in improving impaired wound closure in T2DM. The present study hypothesized that metformin may be able to contribute to wound healing in T2DM mice, and that this protective effect may be partly attributed to an improvement in EPC function with the involvement of TSP-1 and cellular oxidative stress.

## Materials and methods

**Animals.** A total of 14 male C57BLKS/J *db/db* diabetic mice (age, 6 weeks; weight, 32–36 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and 7 male C57BL/6J non-diabetic mice (age, 6 weeks; weight, 16–18 g) were obtained from Sino-British SIPPR/BK Lab Animal Ltd. (Shanghai, China). Mice were housed in a well-ventilated holding room with a 12-h light-dark cycle at an ambient temperature of  $23\pm 2^\circ\text{C}$  and 70% humidity, with free access to water and food. All studies were in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Bethesda, MD, USA). The present study was approved by the Animal Care and Ethics Committee of Second Military Medical University (Shanghai, China).

**Experimental protocols.** Male *db/db* mice with obesity and hyperglycemia were used as a model of T2DM. Male age-matched C57BL/6J mice were used as control non-diabetic mice and received treatment with a vehicle. The *db/db* mice were randomly divided into 2 groups, either receiving the vehicle [0.5% carboxymethyl cellulose-Na; 10 ml/kg/day; intragastric (i.g.)] or metformin (250 mg/kg/day; i.g.; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 14 consecutive days. Whole blood samples from the tail veins of the mice were used for detecting blood glucose via a monitoring system (Maochang, Taipei, China). The mice were used for wound healing experiments, or anesthetized for the harvesting of bone marrow to isolate EPCs (BM-EPCs) (Fig. 1).

**Analysis of wound closure.** A 6-mm circular wound was produced by punch biopsy, and digital images of the wound on the dorsum were captured every 2 days until the end of the experiment for all experimental mice. The wound areas were analyzed by tracing the wound margins and calculated using Image-Pro Plus software version 6.0 (Media Cybernetics, Rockville, MD, USA). The closure was expressed as a percentage area of the original wound area (26).

**Wound angiogenesis.** Wounds were harvested from mice on days 7 and 14 following the creation of the wound. Platelet endothelial cell adhesion molecule (CD31) staining was used to evaluate angiogenesis. Samples of skin at the wounded area and surrounding tissue (~1 cm in diameter, ~2 mm in thickness) were excised, bisected, and fixed in 10% formalin for 6 h at room temperature. The samples were subsequently embedded in paraffin. Following deparaffinization, rehydration with decreasing alcohol series, antigen retrieval (0.5 h at  $90^\circ\text{C}$  in 10 mM citrate buffer) and 5% serum blocking (3 h

at room temperature; Chemicon International, Inc., Temecula, CA, USA), the slides were incubated with an anti-CD31 antibody (2  $\mu\text{g}/\text{ml}$ ; cat. no. 550274; BD Biosciences, San Jose, CA, USA) for 1 h at room temperature and subsequently incubated with a biotinylated secondary antibody (1:500; cat. no. BA-9200; Vectastain Elite ABC kit; Vector Laboratories Ltd., Peterborough, UK) for 1 h at room temperature. The samples were counterstained with hematoxylin for 2 min at room temperature (27). CD31-positive tubular structures were considered to be capillaries and the capillary density in the wound healing area was quantified. One slide from each mouse was examined and, for each slide, two high-power fields (magnification,  $\times 200$ ) were examined using a light microscope. The capillaries were then counted.

**Quantification of circulating EPCs.** Circulating EPCs were determined according to a previously-described technique (28). Peripheral blood was acquired by removing the eyeballs from anesthetized mice. The samples were dissolved in PBS (1:1), following which gradient centrifugation liquid 1083 (Sigma-Aldrich; Merck KGaA) was used for the separation of peripheral blood mononuclear cells at  $400 \times g$  for 30 min. The mononuclear fraction was extracted and the erythrocytes were lysed with red blood cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Following washing, the samples were suspended for incubation (0.5 h at room temperature) using a buffer solution containing fluorescein isothiocyanate-ataxin-1 (Sca-1) (1:100; cat. no. 557405; BD Biosciences) and phycoerythrin-vascular endothelial growth factor receptor 2 (Flk-1) (1:100; cat. no. 555308; BD Biosciences) antibodies for flow cytometry detection and analyzed using FlowJo software version 7.6 (Tree Star Inc., Ashland, OR, USA). Sca-1/Flk-1 double-positive cells were defined as circulating EPCs.

**Isolation of the BM-EPCs.** The isolation and culturing of mouse BM-EPCs were in accordance with a previous technique (25). BM-EPCs were obtained from mouse tibias and femurs and seeded in 6-well plates coated with vitronectin (Sigma-Aldrich; Merck KGaA). Cells were cultured in endothelial growth medium-2 (Cambrex Corp., East Rutherford, NJ, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ . A total of 4 days subsequent to cultivation, the culture medium, including nonadherent cells, was changed for fresh medium and adherent cells were subjected to further culturing for 3 days. The supernatant was collected for western blot quantification and the cells were used for *in vitro* studies.

### *In vitro* cell assays

**Evaluation of the function of BM-EPCs.** A tube formation assay, a previously described method, was adopted to evaluate BM-EPC function (29). A total of 40,000 BM-EPCs were seeded in 96-well plates which were precoated with 50  $\mu\text{l}$ /well growth factor-induced Matrigel (BD Biosciences). Following 8 h of incubation at  $37^\circ\text{C}$ , images of tube morphology were captured using a computer-assisted microscope (Leica Microsystems GmbH, Wetzlar, Germany). Tube numbers were measured in five low power fields (magnification,  $\times 50$ ) for each sample at random.

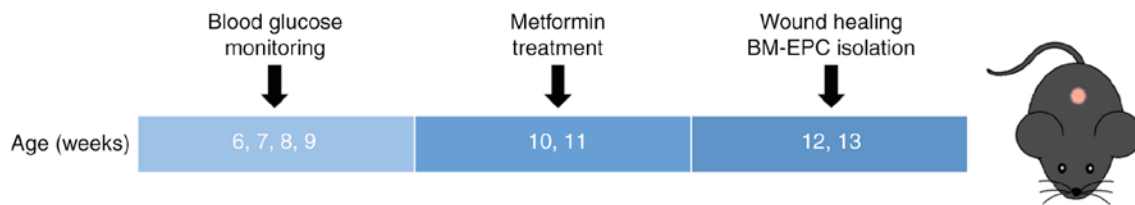


Figure 1. Illustration of experimental protocols. The blood glucose of *db/db* diabetic mice was monitored. Treatment with metformin (250 mg/kg/day; intragastric) was administered for 14 consecutive days. Wound healing and BM-EPC function were measured. BM-EPC, bone marrow-endothelial precursor cell.

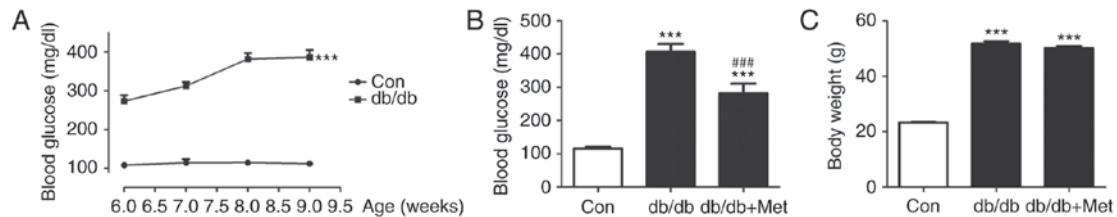


Figure 2. Alterations in blood glucose concentration and body weight of *db/db* mice. (A) In *db/db* mice, blood glucose was significantly elevated compared with the control.  $***P < 0.001$  vs. Con. Metformin (250 mg/kg/day for 14 days; intragastric) significantly decreased (B) blood glucose, although it did not alter (C) body weight in *db/db* mice.  $***P < 0.001$  vs. Con;  $###P < 0.001$  vs. *db/db*. Values are expressed as the mean  $\pm$  standard error of the mean ( $n = 7$  mice/group). Con, control; Met, metformin.

**Measurement of intracellular NO and  $O_2^-$ .** The intracellular NO level was determined using membrane-permeable 4-amino-5-methylamino-2', 7'-difluorofluorescein (DAF-FM) diacetate (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 7 days subsequent to BM-EPC culturing, the cells were harvested and incubated with DAF-FM diacetate ( $10^{-6}$  mol/l) for 30 min at  $37^\circ\text{C}$  and an additional 30 min at room temperature in dark. Following incubation, the DAF-FM fluorescence intensity in cells was measured by flow cytometry (25).

The intracellular  $O_2^-$  level was detected using the membrane-permeable dye dihydroethidium (DHE; Invitrogen; Thermo Fisher Scientific, Inc.), which is oxidized to ethidium bromide in the presence of  $O_2^-$ . Following 7 days of culturing, BM-EPCs were harvested and incubated with DHE ( $0.5 \times 10^{-6}$  mol/l) for 30 min at room temperature in dark. Following staining, the DHE fluorescence intensity in cells was examined by flow cytometry (25).

**Western blot analysis.** Western blotting was performed as previously described (30). TSP-1 secreted by BM-EPCs was obtained by condensing the BM-EPC culture media using a commercial filter device. The concentration of TSP-1 was determined using a bicinchoninic acid assay (Thermo Fisher Scientific, Inc.). For the western blotting experiments,  $\sim 30$ - $\mu\text{g}$  samples were loaded and run on an 8% SDS-PAGE gel. The proteins were electrophoretically transferred to nitrocellulose membranes. Subsequently, the membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature, washed and incubated with the primary antibody for TSP-1 (1:500; cat. no. ab85762; Abcam, Cambridge, UK) at  $4^\circ\text{C}$  overnight. IRDye 800-conjugated rabbit anti-mouse IgG was used as the secondary antibody (1:5,000; cat. no. 925-32212; LI-COR Biosciences, Lincoln, NE, USA) and incubated for 0.5 h at room temperature. The

bands were obtained using an Odyssey infrared imaging system (LI-COR Biosciences), and the expression levels of TSP-1 protein were quantified using Quantity One software version 4.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard error of the mean. Statistical significance was analyzed by one-way analysis of variance followed by the Newman-Keuls multiple comparison test, using GraphPad Prism Software version 5 (GraphPad Software, Inc., La Jolla, CA, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Effect of metformin on blood glucose and body weight in *db/db* mice.** A significant increase in blood glucose was observed in *db/db* mice compared with the control ( $338.4 \pm 27.6$  vs.  $112.1 \pm 1.5$  mg/dl;  $P < 0.001$ ; Fig. 2A). Pretreatment with metformin improved the blood glucose level (Fig. 2B), although it did not modify body weight in *db/db* mice (Fig. 2C).

**Metformin accelerates wound healing and angiogenesis in *db/db* mice.** In order to examine the effects of pretreatment with metformin on wound closure in *db/db* mice, alterations in the wounded skin were observed on alternate days until day 14. Fig. 3A exhibits the gross appearance of the wounds during the 14 days following injury. *Db/db* mice exhibited a marked delay in wound closure compared with the control. By contrast, wounds in *db/db* mice pretreated with metformin underwent gradual and progressive healing until reaching complete closure (Fig. 3A). Statistically, treatment with metformin significantly accelerated wound closure in *db/db* mice when compared with the untreated *db/db* mice ( $P < 0.05$ ; Fig. 3B).

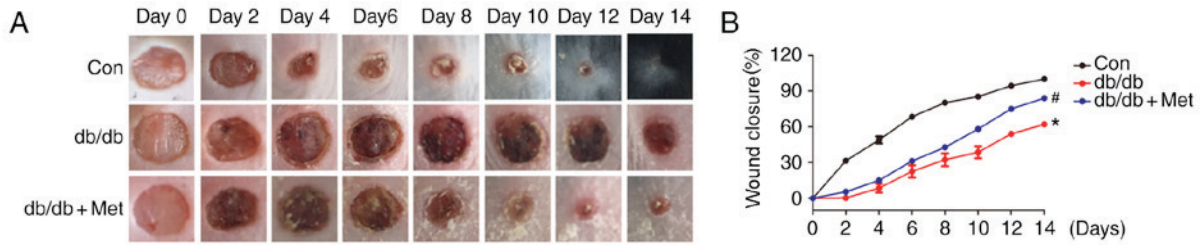


Figure 3. Metformin therapy accelerates wound closure in *db/db* mice. A dorsal skin wound was created via a 6-mm circular punch biopsy and digital images of the wound were captured every 2 days until day 14. (A) Representative images of wound healing. (B) Metformin therapy accelerated wound closure compared with control in *db/db* mice. \* $P < 0.05$  vs. Con; # $P < 0.05$  vs. *db/db*. Values are expressed as the mean  $\pm$  standard error of the mean ( $n = 5$  mice/group). Con, control; Met, metformin.

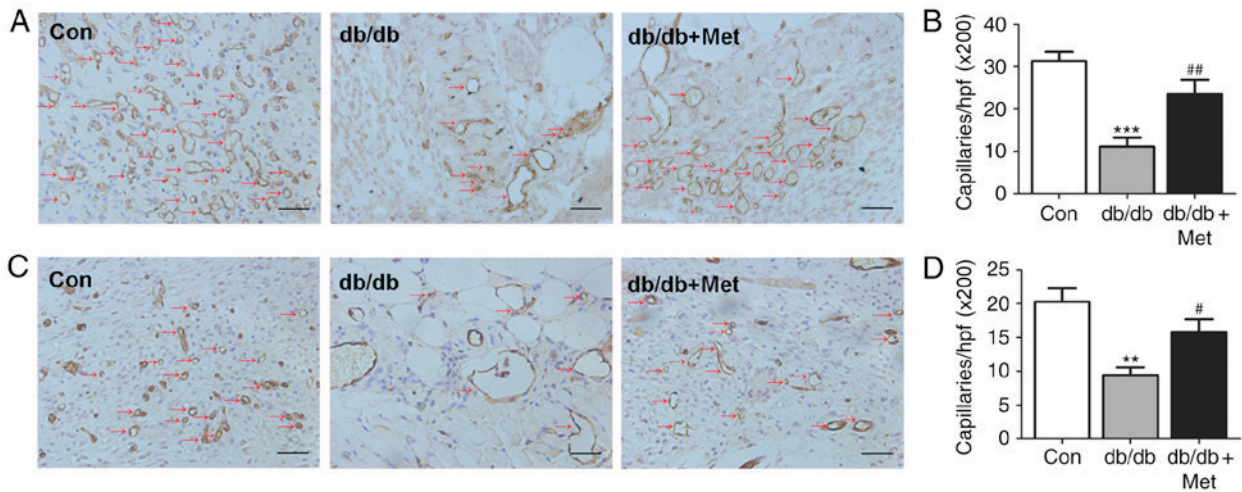


Figure 4. Metformin therapy enhances wound angiogenesis in *db/db* mice. A dorsal skin wound was created via a 6-mm circular punch biopsy and wound angiogenesis was measured on days 7 and 14. (A) Representative images of CD31 staining on day 7 and (B) quantitative analysis. (C) Representative images of CD31 staining on day 14 and (D) quantitative analysis. Red arrows indicate CD31-positive capillaries (magnification,  $\times 200$ ; scale bar,  $50 \mu\text{m}$ ). Quantitative analysis of capillaries in each field demonstrated that wound capillaries in metformin-treated *db/db* mice were increased on days 7 and 14 when compared with the untreated *db/db* mice. \*\*\* $P < 0.001$ , \*\* $P < 0.01$  vs. Con; ## $P < 0.01$ , # $P < 0.05$  vs. *db/db*. Values are expressed as the mean  $\pm$  standard error of the mean ( $n = 5$  mice/group). Con, control; Met, metformin; hpf, high-power field.

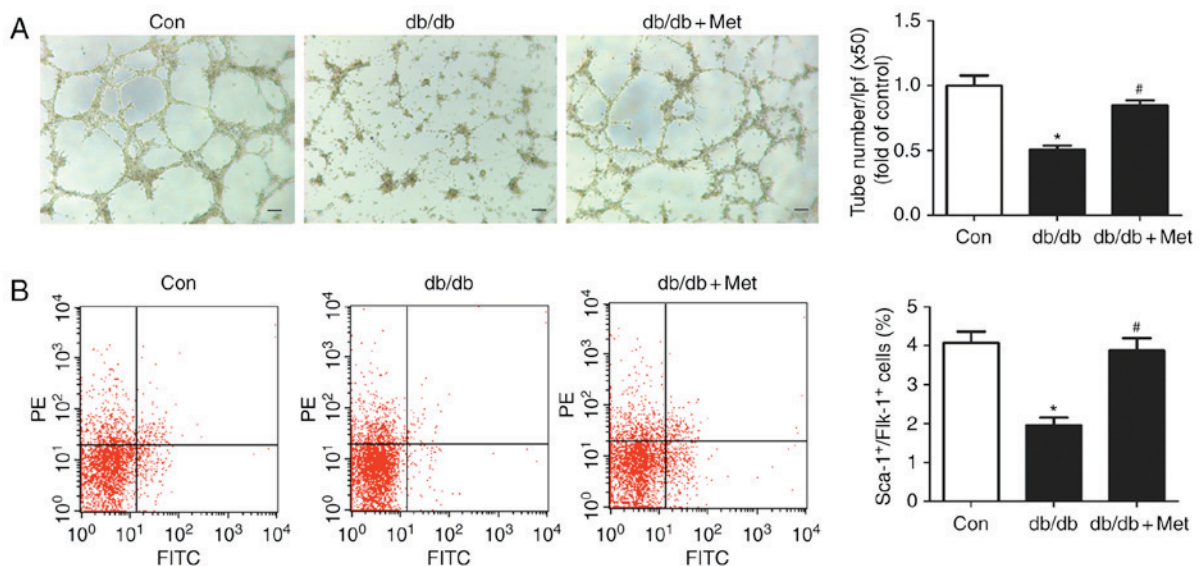


Figure 5. Metformin therapy ameliorates BM-EPC function in *db/db* mice. (A) Representative images of the tube formation assay of BM-EPCs. The number of tubes in each sample was calculated from 5 fields (magnification,  $\times 50$ ; scale bar,  $100 \mu\text{m}$ ) at random. Treatment with metformin ameliorated the tube formation of BM-EPCs. (B) EPC numbers were detected by flow cytometry and the percentage of Sca-1<sup>+</sup>/Flk-1<sup>+</sup> cells was calculated. Metformin elevated the circulating EPC number in *db/db* mice. \* $P < 0.05$  vs. Con; # $P < 0.05$  vs. *db/db*. Values are expressed as the mean  $\pm$  standard error of the mean ( $n = 7$  mice/group). Con, control; Met, metformin; BM-EPC, bone marrow-endothelial precursor cell; PE, phycoerythrin; FITC, fluorescein isothiocyanate; Sca-1, ataxin-1; Flk-1, vascular endothelial growth factor receptor 2; lpf, low-power field.

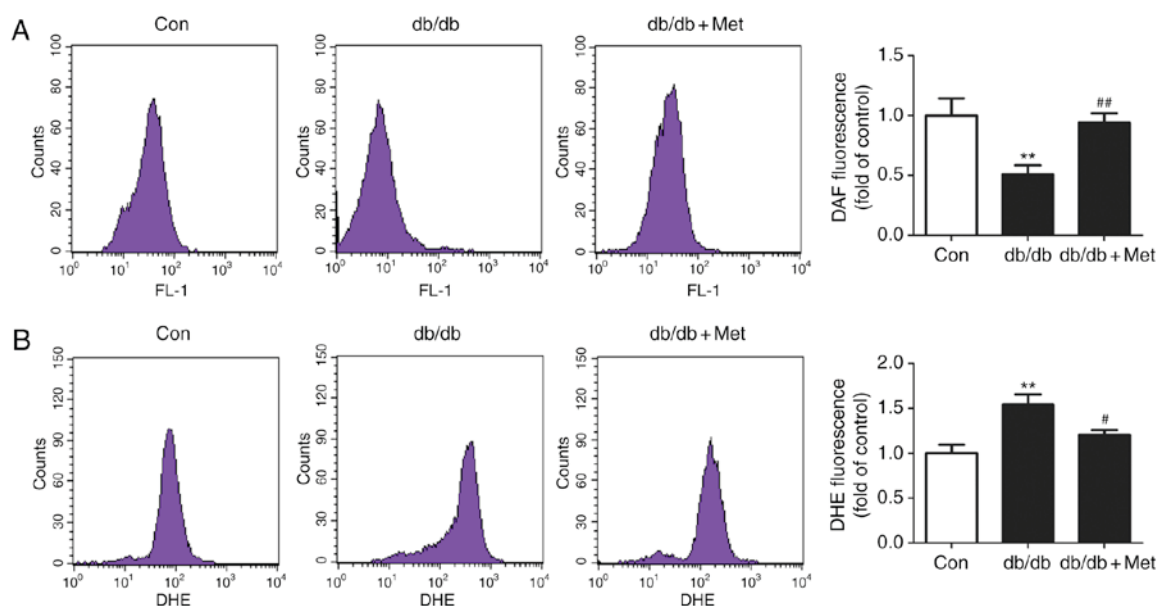


Figure 6. Metformin therapy enhances intracellular NO and suppresses intracellular  $O_2^-$  levels in BM-EPCs from *db/db* mice. (A) The intracellular NO level was determined by DAF-FM-staining and flow cytometry. Metformin increased the NO level in BM-EPCs from *db/db* mice. (B) DHE fluorescence intensity was determined by flow cytometry. Metformin decreased the intracellular  $O_2^-$  level in BM-EPCs from *db/db* mice. \*\* $P < 0.01$  vs. Con; # $P < 0.01$ , # $P < 0.05$  vs. *db/db*. Values are expressed as the mean  $\pm$  standard error of the mean (n=6-7 mice/group). Con, control; Met, metformin; BM-EPC, bone marrow-endothelial precursor cell; DAF-FM, 4-amino-5-methylamino-2', 7'-difluorofluorescein; DHE, dihydroethidium;  $O_2^-$ , superoxide; NO, nitric oxide.

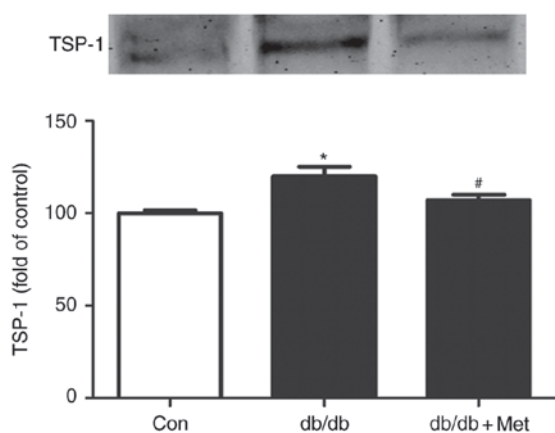


Figure 7. Metformin inhibits the secretion of TSP-1 in BM-EPC culture media from *db/db* mice. BM-EPCs were isolated and cultured from anesthetized mice. TSP-1 in BM-EPC culture media was subjected to western blotting, and metformin significantly decreased the expression of TSP-1 in BM-EPCs from *db/db* mice. \* $P < 0.05$  vs. Con; # $P < 0.05$  vs. *db/db*. Values are expressed as the mean  $\pm$  standard error of the mean (n=3 mice/group). Con, control; Met, metformin; TSP-1, thrombospondin-1.

In order to further examine the role of metformin treatment in skin neovascularization, the number of tubular structures, indicated by CD31 staining of the skin tissue harvested from the wound area, were calculated (Fig. 4). A significant decrease in capillary formation was observed in *db/db* mice on days 7 ( $P < 0.001$ ; Fig. 4B) and 14 ( $P < 0.01$ ; Fig. 4D) compared with the control. Capillary formation in *db/db* mice was significantly improved on days 7 ( $P < 0.01$ ; Fig. 4B) and 14 ( $P < 0.05$ ; Fig. 4D) following pretreatment with metformin.

*Metformin improves BM-EPC function in db/db mice.* In order to further examine the mechanism underlying the role of

metformin in accelerating wound closure, the tube formation capacity of EPCs from *db/db* mice was determined. Impaired EPCs were observed in *db/db* mice compared with the control. Pretreatment with metformin significantly improved tube formation capacity in *db/db* mice ( $0.70 \pm 0.04$  vs.  $0.54 \pm 0.04$ ;  $P < 0.05$ ; Fig. 5A). A decrease in the circulating EPC number was observed in *db/db* mice compared with the control, which was partially reversed by pretreatment with metformin ( $2.18 \pm 0.32\%$  vs.  $1.11 \pm 0.18\%$ ;  $P < 0.05$ ; Fig. 5B).

*Metformin increases intracellular NO production and decreases the intracellular  $O_2^-$  level in BM-EPCs from db/db mice.* There was significant decrease in intracellular NO in BM-EPCs from *db/db* mice compared with the control ( $P < 0.01$ ; Fig. 6A), and pretreatment with metformin significantly increased the intracellular NO level of BM-EPCs in *db/db* mice ( $P < 0.01$ ; Fig. 6A). By contrast, the intracellular  $O_2^-$  level in BM-EPCs from *db/db* mice was significantly elevated compared with the control ( $P < 0.01$ ; Fig. 6B). However, pretreatment with metformin significantly decreased the levels of intracellular  $O_2^-$  in BM-EPCs from *db/db* mice ( $P < 0.05$ ; Fig. 6B).

*Metformin inhibits TSP-1 secretion in BM-EPCs from db/db mice.* Elevated TSP-1 secretion was observed in BM-EPCs from *db/db* mice, as indicated by western blot analysis of the supernatant from the cell culture medium, when compared with the control ( $P < 0.05$ ; Fig. 7). By contrast, a significant decrease in TSP-1 was detected in the culture medium of BM-EPCs from metformin-pretreated *db/db* mice ( $P < 0.05$ ; Fig. 7).

## Discussion

The principal findings of the present study were as follows: i) Metformin accelerated wound closure and improved

angiogenesis in *db/db* mice; ii) metformin improved BM-EPC function in *db/db* mice; iii) metformin augmented the decreased NO level and reduced the increased  $O_2^-$  production in the BM-EPCs of *db/db* mice; and iv) metformin decreased the serum TSP-1 level secreted from the BM-EPCs of *db/db* mice.

In the present study, *db/db* mice, with genetic hyperglycemia akin to adult-onset T2DM as a consequence of an inactive gene mutation affecting leptin receptors, were specifically selected as the T2DM model (31-33); this model has been widely recognized to be well-established animal model for investigating the association between hyperglycemia, EPC function and wound closure (34). Wound healing following tissue damage is a sophisticated pathophysiological process which requires comprehensive interactions between cells and a variety of signaling molecules (35). However, diabetes may cause disorders in the tissue microenvironment, in addition to impairments in cellular function and wound healing. Angiogenesis is important for promoting vessel formation which, in turn, provides regenerating tissue with the required oxygen and nutrients (35,36). CD31 is a typical marker for tracing vascular and endothelial cells (37). It was observed in the present study that healing capacity was decreased in genetically diabetic *db/db* mice, as indicated by staining of CD31, when compared with non-diabetic mice. These data were consistent with a previous report (38).

Currently, regarding the role of metformin on EPC function and wound healing, the published literature is subjected to a degree of controversy. For example, previous studies have indicated that metformin contributed to accelerated wound healing and an enhanced quantity of circulating EPCs and BM-EPC function in diabetes (39,40). Another study held the opposite opinion, that metformin treatment did not result in any alterations in the circulating EPC number, and even caused delayed wound healing in diabetes (41). The results of the present study demonstrated that treatment with metformin accelerated wound healing and improved angiogenesis.

EPCs, as precursors of endothelial cells, can mobilize from the bone marrow into the circulation and have been implicated in neovascularization following tissue injury (5,23,42). Reduced EPC numbers and impaired EPC function have been observed in diabetic patients (43). Metformin, a biguanide family member, is a commonly applied therapeutic drug for T2DM (44,45). Studies have demonstrated that metformin contributed substantially to increasing the circulating EPC number and maintaining endothelial cell function in patients with T2DM (19), while the associated mechanisms remain largely unclear. In the present study, *db/db* mice pretreated with metformin (250 mg/kg/day) for 2 weeks exhibited an increased EPC number and improved EPC function.

Gao *et al* (46) demonstrated that NO serves an important role in regulating tube formation in EPCs. Gallagher *et al* (16) demonstrated that induced expression of NO in bone marrow was highly correlated with increased mobilization of EPCs to the circulation. In the present study, metformin contributed to the elevation of NO levels in EPCs from *db/db* mice, in addition to a significantly increased circulating EPC number and improved EPC function. It has been demonstrated that a decreased intracellular  $O_2^-$  level may be accompanied by an improvement in EPC dysfunction and an enhanced NO level in EPCs (25,26). In the present study, it was observed that the

generation of  $O_2^-$  was increased, and treatment with metformin was able to inhibit the production of  $O_2^-$  in EPCs from *db/db* mice.

TSP-1, an endogenous anti-angiogenic mediator, has been demonstrated to be involved in vascular complications in diabetes (47). In a T2DM animal model, TSP-1 in the vessel walls was elevated at the mRNA and protein levels (22). In addition, preclinical studies suggested that the proangiogenic factor NO was a biological antagonist of TSP-1 (48,49). Ridnour *et al* (49) observed that the protective effect of NO on endothelial cells was accompanied by TSP-1 downregulation. Xu *et al* (50) reported that the TSP-1 level was upregulated in impaired retinal capillaries provoked by nitrate stress in diabetic rats. The results of the present study identified that TSP-1 was increased and NO was decreased in *db/db* mice, accompanied by wound healing delay, angiogenesis impairment and EPC dysfunction. Treatment with metformin was able to significantly alleviate disorders in wound healing, stimulate angiogenesis and improve EPC function, with TSP-1 levels inhibited and NO levels upregulated in BM-EPCs from *db/db* mice.

In conclusion, the results of the present study demonstrated that wound healing, angiogenesis and EPC function were impaired in *db/db* mice. Treatment with metformin was able to accelerate wound healing, which was possibly associated with an improvement in EPC function via a TSP-1/NO pathway.

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#### References

1. Chan JC, Cho NH, Tajima N and Shaw J: Diabetes in the Western Pacific Region-past, present and future. *Diabetes Res Clin Pract* 103: 244-255, 2014.
2. Thandavarayan RA, Garikipati VN, Joladarashi D, Suresh Babu S, Jeyabal P, Verma SK, Mackie AR, Khan M, Arumugam S, Watanabe K, *et al*: Sirtuin-6 deficiency exacerbates diabetes-induced impairment of wound healing. *Exp Dermatol* 24: 773-778, 2015.
3. Papanas N, Demetzos C, Pippa N, Maltezos E and Tentolouris N: Efficacy of a new heparan sulfate mimetic dressing in the healing of foot and lower extremity ulcers in type 2 diabetes: A case series. *Int J Low Extrem Wounds* 15: 63-67, 2016.
4. Zgheib C and Liechty KW: Shedding light on miR-26a: Another key regulator of angiogenesis in diabetic wound healing. *J Mol Cell Cardiol* 92: 203-205, 2016.
5. Li DW, Liu ZQ, Wei J, Liu Y and Hu LS: Contribution of endothelial progenitor cells to neovascularization (Review). *Int J Mol Med* 30: 1000-1006, 2012.
6. Fadini GP, Sartore S, Albiero M, Baesso I, Murphy E, Menegolo M, Grego F, Vigili de Kreutzenberg S, Tiengo A, Agostini C and Avogaro A: Number and function of endothelial progenitor cells as a marker of severity for diabetic vasculopathy. *Arterioscler Thromb Vasc Biol* 26: 2140-2146, 2006.
7. Kovacic JC, Moore J, Herbert A, Ma D, Boehm M and Graham RM: Endothelial progenitor cells, angioblasts, and angiogenesis-old terms reconsidered from a current perspective. *Trends Cardiovasc Med* 18: 45-51, 2008.

8. Liao YF, Chen LL, Zeng TS, Li YM, Fan Yu, Hu LJ and Ling Yue: Number of circulating endothelial progenitor cells as a marker of vascular endothelial function for type 2 diabetes. *Vasc Med* 15: 279-285, 2010.
9. Tepper OM: Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation* 106: 2781-2786, 2002.
10. Wang CH, Ting MK, Verma S, Kuo LT, Yang NI, Hsieh IC, Wang SY, Hung A and Cherng WJ: Pioglitazone increases the numbers and improves the functional capacity of endothelial progenitor cells in patients with diabetes mellitus. *Am Heart J* 152: 1051.e1-8, 2006.
11. Fadini GP, Miorin M, Facco M, Bonamico S, Baesso I, Grego F, Menegolo M, de Kreutzenberg SV, Tiengo A, Agostini C and Avogaro A: Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. *J Am Coll Cardiol* 45: 1449-1457, 2005.
12. Nakamura K, Oe H, Kihara H, Shimada K, Fukuda S, Watanabe K, Takagi T, Yunoki K, Miyoshi T, Hirata K, *et al*: DPP-4 inhibitor and alpha-glucosidase inhibitor equally improve endothelial function in patients with type 2 diabetes: EDGE study. *Cardiovasc Diabetol* 13: 110, 2014.
13. Yue WS, Lau KK, Siu CW, Wang M, Yan GH, Yiu KH and Tse HF: Impact of glycemic control on circulating endothelial progenitor cells and arterial stiffness in patients with type 2 diabetes mellitus. *Cardiovasc Diabetol* 10: 113, 2011.
14. Badr G, Hozzein WN, Badr BM, Al Ghamdi A, Saad Eldien HM and Garraud O: Bee venom accelerates wound healing in diabetic mice by suppressing activating transcription factor-3 (ATF-3) and inducible nitric oxide synthase (iNOS)-mediated oxidative stress and recruiting bone marrow-derived endothelial progenitor cells. *J Cell Physiol* 231: 2159-2171, 2016.
15. Gallagher KA, Goldstein LJ, Thom SR and Velazquez OC: Hyperbaric oxygen and bone marrow-derived endothelial progenitor cells in diabetic wound healing. *Vascular* 14: 328-337, 2006.
16. Gallagher KA, Liu ZJ, Xiao M, Chen H, Goldstein LJ, Buerk DG, Nedeau A, Thom SR and Velazquez OC: Diabetic impairments in NO-mediated endothelial progenitor cell mobilization and homing are reversed by hyperoxia and SDF-1 alpha. *J Clin Invest* 117: 1249-1259, 2007.
17. Hattori Y, Hattori K and Hayashi T: Pleiotropic benefits of metformin: Macrophage targeting its anti-inflammatory mechanisms. *Diabetes* 64: 1907-1909, 2015.
18. Li DJ, Huang F, Lu WJ, Jiang GJ, Deng YP and Shen FM: Metformin promotes irisin release from murine skeletal muscle independently of AMP-activated protein kinase activation. *Acta Physiol (Oxf)* 213: 711-721, 2015.
19. Chen LL, Liao YF, Zeng TS, Yu F, Li HQ and Feng Y: Effects of metformin plus gliclazide compared with metformin alone on circulating endothelial progenitor cell in type 2 diabetic patients. *Endocrine* 38: 266-275, 2010.
20. Varma V, Yao-Borengasser A, Bodles AM, Rasouli N, Phanavanh B, Nolen GT, Kern EM, Nagarajan R, Spencer HJ III, Lee MJ, *et al*: Thrombospondin-1 is an adipokine associated with obesity, adipose inflammation, and insulin resistance. *Diabetes* 57: 432-439, 2008.
21. Dabir P, Marinic TE, Krukovets I and Stenina OI: Aryl hydrocarbon receptor is activated by glucose and regulates the thrombospondin-1 gene promoter in endothelial cells. *Circ Res* 102: 1558-1565, 2008.
22. Stenina OI, Krukovets I, Wang K, Zhou Z, Forudi F, Penn MS, Topol EJ and Plow EF: Increased expression of thrombospondin-1 in vessel wall of diabetic Zucker rat. *Circulation* 107: 3209-3215, 2003.
23. Tie L, Chen LY, Chen DD, Xie HH, Channon KM and Chen AF: GTP cyclohydrolase I prevents diabetic-impaired endothelial progenitor cells and wound healing by suppressing oxidative stress/thrombospondin-1. *Am J Physiol Endocrinol Metab* 306: E1120-E1131, 2014.
24. Tan BK, Adya R, Chen J, Farhatullah S, Heutling D, Mitchell D, Lehnert H and Randeve HS: Metformin decreases angiogenesis via NF-kappaB and Erk1/2/Erk5 pathways by increasing the antiangiogenic thrombospondin-1. *Cardiovasc Res* 83: 566-574, 2009.
25. Xie HH, Zhou S, Chen DD, Channon KM, Su DF and Chen AF: GTP cyclohydrolase I/BH4 pathway protects EPCs via suppressing oxidative stress and thrombospondin-1 in salt-sensitive hypertension. *Hypertension* 56: 1137-1144, 2010.
26. Li ZP, Xin RJ, Yang H, Jiang GJ, Deng YP, Li DJ and Shen FM: Diazoxide accelerates wound healing by improving EPC function. *Front Biosci (Landmark Ed)* 21: 1039-1051, 2016.
27. Cai J, Lu S, Yao Z, Deng YP, Zhang LD, Yu JW, Ren GF, Shen FM and Jiang GJ: Glibenclamide attenuates myocardial injury by lipopolysaccharides in streptozotocin-induced diabetic mice. *Cardiovasc Diabetol* 13: 106, 2014.
28. Marrotte EJ, Chen DD, Hakim JS and Chen AF: Manganese superoxide dismutase expression in endothelial progenitor cells accelerates wound healing in diabetic mice. *J Clin Invest* 120: 4207-4219, 2010.
29. Chen JK, Deng YP, Jiang GJ, Liu YZ, Zhao T and Shen FM: Establishment of tube formation assay of bone marrow-derived endothelial progenitor cells. *CNS Neurosci Ther* 19: 533-535, 2013.
30. Lee MH, Choi EN, Jeon YJ and Jung SC: Possible role of transforming growth factor-beta1 and vascular endothelial growth factor in Fabry disease nephropathy. *Int J Mol Med* 30: 1275-1280, 2012.
31. Aoyama H, Daitoku H and Fukamizu A: Nutrient control of phosphorylation and translocation of Foxo1 in C57BL/6 and db/db mice. *Int J Mol Med* 18: 433-439, 2006.
32. Bao Q, Shen X, Qian L, Gong C, Nie M and Dong Y: Anti-diabetic activities of catalpol in db/db mice. *Korean J Physiol Pharmacol* 20: 153-160, 2016.
33. Tamura Y, Murayama T, Minami M, Yokode M and Arai H: Differential effect of statins on diabetic nephropathy in db/db mice. *Int J Mol Med* 28: 683-687, 2011.
34. Galiano RD, Tepper OM, Pelo CR, Bhatt KA, Callaghan M, Bastidas N, Bunting S, Steinmetz HG and Gurtner GC: Topical vascular endothelial growth factor accelerates diabetic wound healing through increased angiogenesis and by mobilizing and recruiting bone marrow-derived cells. *Am J Pathol* 164: 1935-1947, 2004.
35. Galeano M, Altavilla D, Cucinotta D, Russo GT, Calò M, Bitto A, Marini H, Marini R, Adamo EB, Seminara P, *et al*: Recombinant human erythropoietin stimulates angiogenesis and wound healing in the genetically diabetic mouse. *Diabetes* 53: 2509-2517, 2004.
36. Zhang XN, Ma ZJ, Wang Y, Li YZ, Sun B, Guo X, Pan CQ and Chen LM: The four-herb Chinese medicine formula Tuo-Li-Xiao-Du-San accelerates cutaneous wound healing in streptozotocin-induced diabetic rats through reducing inflammation and increasing angiogenesis. *J Diabetes Res* 2016: 5639129, 2016.
37. Caligiuri G, Groyer E, Khallouf-Laschet J, Al Haj Zen A, Sainz J, Urbain D, Gaston AT, Lemitre M, Nicoletti A and Lafont A: Reduced immunoregulatory CD31+ T cells in the blood of atherosclerotic mice with plaque thrombosis. *Arterioscler Thromb Vasc Biol* 25: 1659-1664, 2005.
38. Tellechea A, Kafanas A, Leal EC, Tecilazich F, Kuchibhotla S, Auster ME, Kontoes I, Paolino J, Carvalho E, Nabzdyk LP and Veves A: Increased skin inflammation and blood vessel density in human and experimental diabetes. *Int J Low Extrem Wounds* 12: 4-11, 2013.
39. Desouza CV: Does drug therapy reverse endothelial progenitor cell dysfunction in diabetes? *J Diabetes Complications* 27: 519-525, 2013.
40. Lin JT, Chen HM, Chiu CH and Liang YJ: AMP-activated protein kinase activators in diabetic ulcers: From animal studies to Phase II drugs under investigation. *Expert Opin Investig Drugs* 23: 1253-1265, 2014.
41. Ochoa-Gonzalez F, Cervantes-Villagrana AR, Fernandez-Ruiz JC, Nava-Ramirez HS, Hernandez-Correa AC, Enciso-Moreno JA and Castañeda-Delgado JE: Metformin induces cell cycle arrest, reduced proliferation, wound healing impairment in vivo and is associated to clinical outcomes in diabetic foot ulcer patients. *PLoS One* 11: e0150900, 2016.
42. Mao L, Huang M, Chen SC, Li YN, Xia YP, He QW, Wang MD, Huang Y, Zheng L and Hu B: Endogenous endothelial progenitor cells participate in neovascularization via CXCR4/SDF-1 axis and improve outcome after stroke. *CNS Neurosci Ther* 20: 460-468, 2014.
43. Kim KA, Shin YJ, Kim JH, Lee H, Noh SY, Jang SH and Bae ON: Dysfunction of endothelial progenitor cells under diabetic conditions and its underlying mechanisms. *Arch Pharm Res* 35: 223-234, 2012.
44. Ghosh S, Lakshmanan AP, Hwang MJ, Kubba H, Mushannen A, Triggle CR and Ding H: Metformin improves endothelial function in aortic tissue and microvascular endothelial cells subjected to diabetic hyperglycaemic conditions. *Biochem Pharmacol* 98: 412-421, 2015.

45. Shi Y, He Z, Jia Z and Xu C: Inhibitory effect of metformin combined with gemcitabine on pancreatic cancer cells *in vitro* and *in vivo*. *Mol Med Rep* 14: 2921-2928, 2016.
46. Gao L, Li P, Zhang J, Hagiwara M, Shen B, Bledsoe G, Chang E, Chao L and Chao J: Novel role of kallistatin in vascular repair by promoting mobility, viability, and function of endothelial progenitor cells. *J Am Heart Assoc* 3: e001194, 2014.
47. Bhattacharyya S, Marinic TE, Krukovets I, Hoppe G and Stenina OI: Cell type-specific post-transcriptional regulation of production of the potent antiangiogenic and proatherogenic protein thrombospondin-1 by high glucose. *J Biol Chem* 283: 5699-5707, 2008.
48. Isenberg JS, Wink DA and Roberts DD: Thrombospondin-1 antagonizes nitric oxide-stimulated vascular smooth muscle cell responses. *Cardiovasc Res* 71: 785-793, 2006.
49. Ridnour LA, Isenberg JS, Espey MG, Thomas DD, Roberts DD and Wink DA: Nitric oxide regulates angiogenesis through a functional switch involving thrombospondin-1. *Proc Natl Acad Sci USA* 102: 13147-13152, 2005.
50. Xu L, Xun G, Yao Z, Liu Y, Qiu Y, Liu K, Zhu D, Gu Q, Xu X and Ho PC: Effects of generated trans-arachidonic acids on retinal capillary during nitrate stress in diabetic rats. *Ophthalmologica* 222: 37-41, 2008.