The effect of B vitamin supplementation on wound healing in type 2 diabetic mice

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The aim of this study was to test the effects of B-group vitamin supplements on wound healing in diabetic mice. The mice in the experimental group were treated daily with 1 g/L B₆, 1.25 mg/L B₁₂, and 62.5 mg/L folic acid in their drinking water. Full-thickness excision wounds were created with 6-mm skin biopsy punches. Each wound closure was digitally photographed. Beginning on day 3 after wounding, the wound area in the diabetic mice was statistically larger than that of normal mice (p<0.05 vs diabetic mice). The diabetic mice treated with B vitamins displayed accelerated wound closure on day 3 (wound area 42.8 ± 11.3%, p<0.05). On day 9 after wounding, the wound area in the diabetic mice was also statistically larger than that of normal mice (p<0.05 vs diabetic mice). The diabetic mice treated with B vitamins displayed accelerated wound closure on day 3 (wound area 13.2 ± 16.8%, p<0.05). In addition, the high glucose level in the diabetic animals decreased significantly in response to B vitamin treatment. In conclusion, the results of this study indicate that B vitamin supplementation may improve wound healing in diabetic mice.

Key Words: B vitamin supplementation, wound healing, diabetic mice, glucose level, TNF-α

Diabetes mellitus (DM) is characterized by a host of complications that can affect many organs. Delayed wound healing is a major complication of diabetes that can enhance overall morbidity and mortality in diabetics.1,2 Impaired wound healing is characterized by delayed cellular infiltration and granulation tissue formation, decreased collagen organization, and reduced angiogenesis.3,4 The process of wound healing is a dynamic series of events involving inflammation, proliferation, maturation, and remodeling. In diabetes, there is a delayed influx of inflammatory cells into the wound site initially, but when inflammatory conditions become established, inflammatory cells prevent the deposition of matrix components and remodeling. It has been suggested that receptor for advanced glycation end-products (RAGE) is involved in this sustained inflammatory response. Indeed, there is enhanced expression of RAGE in slow-healing wounds in diabetic mice.5

Nutritional status has profound effects on immune responses.6,8 Strategies to prevent complications of diabetes, including nutritional and therapeutic support related to B vitamin supplementation, have emerged recently.7 Several studies have shown protective effects of B vitamins in diabetic patients.9 A previous study showed that the circulating and tissue concentrations of water-soluble vitamins were significantly decreased in diabetic subjects.10 In animal models, diabetes lowered the folate, vitamin B₆, and vitamin B₁₂ levels significantly in kidney, heart, liver, and muscle.11 These results indicate that diabetes causes depression of B vitamin in sufficiency, and combinational supplementation of B vitamins may be beneficent for wound healing in diabetes. The aim of this study was to test the effects of B-group vitamin supplements on wound healing in diabetic mice. Some studies showed the association of decreased serum levels of B vitamin and hyperhomocysteinemia. Hyperhomocysteinemia is associated with several microvascular diseases, such as coronary artery disease, cerebrovascular disease, peripheral arterial disease, and deep-vein thrombosis.11–13 Folic acid, vitamin B₁₂, and vitamin B₆ are cofactors for the enzymes involved in homocysteine metabolism. Folic acid, vitamin B₁₂, and vitamin B₆ deficiency and reduced enzyme activities inhibit the breakdown of homocysteine.14 Supplementation with these nutrients was shown to lower homocysteine levels in a number of clinical trials.15

Cystathionine β-synthase (CBS) catalyzes the first step of homocysteine transsulfuration as a rate-limiting enzyme. Genetically increased CBS expression would benefit from the protection due to the low homocysteine levels.16 We also examined CBS expression in liver.

Materials and Methods

Animals. Male 6-week-old KKAY (diabetic) and C57BL/6 (normal) mice were obtained from CLEA Japan (Tokyo, Japan). In this study, 15 and 17 mice of each strain were used. Type 2 diabetes is characterized by impaired insulin sensitivity and the resulting dysregulation of glucose and lipid metabolism. KKAY mice exhibit morbid obesity and metabolic abnormalities, including hyperglycemia, and are known to serve as an excellent model of type 2 diabetes mellitus.17 The mice were housed individually at a constant temperature (23 ± 2°C) and 55 ± 5% relative humidity, under a 12-h/12-h light/dark cycle (lights on at 07:00), and had free access to food and water. The body weight and blood glucose level of the mice were measured weekly. At the age of 10 weeks, the mice were used in the following experiments. The mice were separated randomly into four groups, as follows: control normal mice, experimental normal mice, control diabetic mice, and experimental diabetic mice. The mice in the experimental group were treated daily with 1 g/L B₆ (Sigma-Aldrich, St. Louis, MO), 1.25 mg/L B₁₂ (Sigma-Aldrich), and 62.5 mg/L folic acid (Sigma-Aldrich) in their drinking water and studied 4 weeks after exposure to the B vitamins to determine the effects on wound healing. Intake of B₆, B₁₂, and folic acid from food were 0.05 mg, 0.33 μg and 0.01 mg, respectively. Intake of B₆, B₁₂, and folic acid from supplemented drinking water were 8 mg, 0.01 mg and 0.5 mg, respectively. At 2 weeks after B vitamin exposure

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(at the age of 12 weeks), the mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (Somnopentyl; 50 mg/kg body weight; Schering-Plough, Munich, Germany). An intraperitoneal injection of 0.1 ml of lidocaine (Xylocaine, 1:8 dilution; Astra Zeneca, Osaka, Japan) was administered to control bleeding and provide additional anesthesia. The animal’s dorsal hair was shaved and two full-thickness excision wounds were created with 6-mm skin biopsy punches (Kai Medical, Solingen, Germany). Each wound closure was digitally photographed at the indicated time points (0, 1, 3, 7, 9, 11 and 14 days). The wound areas were quantified using Adobe Photoshop Elements 2.0. All procedures were approved by the Animal Experimentation Committee at Nihon University School of Dentistry, Tokyo, Japan.

Body weight and blood glucose. We observed the general condition of the mice and measured their body weights and blood glucose levels. Peripheral venous blood was taken from the tail of each mouse every week after 12 h of fasting. The fasting plasma glucose level was measured with a glucose measuring device (Bayer Medical, Tuttlingen, Germany). Each wound closure was digitally photographed at the indicated time points (0, 1, 3, 7, 9, 11 and 14 days). The wound areas were quantified using Adobe Photoshop Elements 2.0. All procedures were approved by the Animal Experimentation Committee at Nihon University School of Dentistry, Tokyo, Japan.

Enzyme-linked immunosorbent assay (ELISA). The levels of TNF-α and IL-6 in the serum of the mice were determined by ELISAs using commercially available kits (BioSource International, Inc., Camarillo, CA), according to the manufacturer’s instructions.

Real-time PCR. For RNA isolation, liver was fixed immediately in RNA stabilization reagent (RNALater; Qiagen, Valencia, CA), and the samples were homogenized. Total RNA was extracted using an RNeasy Mini kit (Qiagen). Complementary DNA was synthesized using a Ready-To-Go T-Primed First-Strand kit (Amersham Biosciences, Tokyo, Japan). The primer and probe sets for TNF-α, IL-6 and cystathionine β-synthase (CBS) were from Applied Biosystems (Foster City, CA). Real-time PCR was performed on an ABI PRISM 7700 Sequence Detector (Applied Biosystems) using the following parameters: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and primer extension at 60°C for 1 min. The expression level of each gene was first normalized to that of the glyceraldehyde-3-phosphate dehydrogenase gene in the same sample. The data are shown as means ± SD.

Statistical analysis. The data were analyzed using SPSS software (ver. 16.0 for Windows; SPSS Inc., Chicago, IL). Statistical analyses were performed using Tukey-Kramer test.

Fig. 1. Effect of B vitamin supplementation on blood glucose level in C57BL/6 and KKAy/Ta mice. Peripheral blood was taken from the tail vein and the fasting blood glucose level was measured on every weeks. *p<0.05 (Tukey-Kramer test), C57BL/6 control, control C57BL/6 mice (n = 7); KKAy/Ta control, control KKAy/Ta mice (n = 8); C57BL/6 test, C57BL/6 mice treated with B-vitamins (n = 8); KKAy/Ta test, KKAy/Ta mice treated with B-vitamins (n = 9).

Fig. 2. Effect of B vitamin supplementation on body weight in C57BL/6 and KKAy/Ta mice. Body weight was measured on every weeks. C57BL/6 control, control C57BL/6 mice (n = 7); KKAy/Ta control, control KKAy/Ta mice (n = 8); C57BL/6 test, C57BL/6 mice treated with B-vitamins (n = 8); KKAy/Ta test, KKAy/Ta mice treated with B-vitamins (n = 9).
Results

Fig. 1 shows the effects of B vitamins on glucose levels. The high glucose level in the diabetic animals decreased significantly in response to B vitamin treatment. B vitamin therapy showed no apparent effect on body weight in normal or diabetic mice (Fig. 2). We examined serum TNF-α and IL-6 levels using ELISAs on day 14. However, no significant change in serum TNF-α or IL-6 levels was observed in the B vitamin-treated diabetic mice (Fig. 3). Next, real-time PCR was used to measure the levels of TNF-α and IL-6 mRNA expression in liver. A significant reduction in the expression of these genes was observed in the B vitamin-treated diabetic mice (Fig. 4A and B). The levels of cystathionine β-synthase (CBS) mRNA expression in liver significantly increased in the B vitamin-treated diabetic mice compared with control diabetic mice (Fig. 4C).

Full-thickness punch biopsy wounds (6 mm) were created on two sites of the shaved back of normal and diabetic mice, and the wounded areas were monitored serially (Fig. 5). Beginning on day 3 after wounding, the wound area in the diabetic mice was $63.4 \pm 21.4\%$, compared with $30.2 \pm 12.9\%$ in the normal mice ($p < 0.05$). The diabetic mice treated with B vitamins displayed accelerated wound closure on day 3 (wound area $42.8 \pm 11.3\%$, $p < 0.05$). On day 9 after wounding, the wound area in the diabetic mice treated with B vitamins was $19.2 \pm 7.8\%$, which was significantly lower than the untreated diabetic mice ($p < 0.05$).
mice was 24.2 ± 15.9%, compared with 2.1 ± 2.4% in the normal mice (p<0.05). The diabetic mice treated with B vitamins displayed accelerated wound closure on day 3 (wound area 13.2 ± 16.8%, p<0.05). The area under the curves (AUC) of control normal mice, experimental normal mice, control diabetic mice and experimental diabetic mice were 20.6 ± 7.7%, 18.7 ± 5.3%, 38.3 ± 11.9% and 27.0 ± 12.3%, respectively. It was found that the AUC of experimental diabetic mice was significantly lower than that of control diabetic mice (p<0.05).

Discussion

This study investigated the effects of B vitamins on wound healing in diabetic mice. The concentration of B vitamins in the drinking water has been described previously. Some studies found that the combinatorial supplementation of B vitamins may be more effective. Several studies have shown the potential role of vitamin B₆ supplementation against the effects of oxidative stress. Increased plasma and tissue lipid oxidation has also been reported in rats receiving a B₆-deficient diet. Vitamin B₆ inhibits the formation of advanced glycation end products (AGEs). Vitamin B₆ appears to act by a mechanism analogous to that of AGE breakers (i.e., by reaction with dicarbonyl intermediates in AGE formation). These observations indicate that vitamin B₆ may be beneficial for wound healing in diabetics.

In patients with diabetes, elevated homocysteine (Hcy) levels have been reported to be associated with endothelial dysfunction. Elevated Hcy levels can decrease the endothelium-derived signaling molecule nitric oxide, which, in turn, regulates vascular function. It has been reported that hyperhomocysteinemia is associated with microvascular failure. Hcy can be methylated to form methionine, catalyzed by the enzyme methionine synthase and folic acid and vitamin B₁₂ cofactors. The conversion of Hcy to cystathionine is catalyzed by cystathionine β-synthase in the liver. Our results also showed that cystathionine β-synthase mRNA expression in liver of diabetic mice was increased by B vitamin supplementation. One possible explanation is that B vitamin supplementation may improve microcirculation in the wound healing process via Hcy reduction.

In this study, blood glucose levels were reduced significantly by B vitamin supplementation at weeks 2 and 3. No significant difference was observed in body weight. A previous human study also showed that B vitamin supplementation for 8 weeks in adults with metabolic syndrome resulted in improved fasting blood glucose and insulin levels. Although the mechanisms by which B vitamins decrease blood glucose are not clearly understood, several hypotheses have been suggested. One possible mechanism is that Hcy may inhibit insulin-stimulated tyrosine phosphorylation of the insulin receptor subunit. Another possibility is that B vitamins lower oxidative stress, which, in turn, reduces inflammatory activity such as TNF-α production in the liver. In this study, a significant reduction in the expression of TNF-α mRNA was observed in the B vitamin-treated diabetic mice liver. However, no significant change in serum TNF-α level was observed. This indicates that B vitamin supplementation has limited effectiveness against inflammatory cytokine expression. In this study, the high glucose level in the diabetic animals decreased significantly in response to B vitamin treatment. However, wound closure in the B vitamin-treated diabetic mice was delayed compared to normal mice. Hyperglycemia could directly contribute to poor wound healing in diabetics as well as indirectly by glycation.

In conclusion, the results of this study indicate that B vitamin supplementation may improve wound healing in diabetic mice. B vitamins may also stimulate glucose utilization. Furthermore, B vitamins may reduce oxidative stress and result in TNF-α expression in liver. Various questions remain about the connection between B vitamins and diabetic complications. In addition, dose of supplementation of this study was extremely higher than reference nutrient intake. Further study is needed to determine the mechanisms underlying such effects and appropriate dose of supplementation.

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Abbreviations

CBS  cystathionine β-synthase
DNA  deoxyribonucleic acid
GAPDH  glyceraldehyde 3-phosphate dehydrogenase
Hcy  homocysteine
IL  interleukin

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Conflict of Interest

No potential conflicts of interest were disclosed.