Abstract: To observe the effects of reduced glutathione on promoting osseointegration of titanium implants in New Zealand rabbits with type I diabetes mellitus and to provide novel evidence for improving implants osseointegration. Diabetes mellitus models were established by injecting alloxan (80 mg/kg) into New Zealand rabbits. Success was verified by the blood glucose level. Titanium implants were then placed in the rabbits’ femoral epiphysis. Intravenous glutathione (GSH, 30 mg/kg) or saline, respectively, was started. The rabbits were divided into four groups: GSH-treated diabetes mellitus (GSH+DM), diabetes mellitus (DM), GSH-treated normal rabbits (GSH), normal control rabbits (NC). Four weeks after surgery, the rabbits were evaluated via general observation, micro-computed tomography (micro-CT), H&E staining and bone morphogenetic protein 2 (BMP-2) immunohistochemical staining. In results, general observation and micro-CT showed that wounds had healed, no implants had loosened, and there was good synostosis in all groups. Micro-CT also revealed that peri-implant threaded bone density was higher than that in peri-implant cancellous bone and was similar to that in femoral cortical bone. The implants tightly fused with surrounding bone tissue. HE staining revealed that large numbers of bone trabeculae, osteoblasts, and chondrocytes in the GSH+DM group and granulation tissue, mononuclear macrophages, and multinucleated giant cells in the DM group. New bone trabecular formation was rare. There was greater expression of BMP-2 in the GSH+DM group than in the GM group. The results suggest that GSH promotes new peri-implant bone formation and osseointegration in diabetic rabbits.

Key words: Glutathione, Diabetes mellitus, Titanium implants, Osseointegration, Bone tissue engineering

Introduction

Implants have become the ideal repair method for dentition defects and the edentulous jaw. Several systemic diseases, however, affect the process of implant osseointegration and greatly restrict the application of implant technology. The prevalence of diabetes mellitus (DM) is increasing sharply, with an estimated 380 million people (7.3 %) developing it by 2025 — up from 246 million cases (6 %) in 2007. An increasing number of DM edentulous patients are requiring dental implantation, but the success rate of dental implantation in diabetic patients has been significantly lower than that in the normal population (10-year survival rate 85.6–88.8 % vs. 98 %), which hinders further application of implant technology.

DM-induced chronic hyperglycemia affects various tissue structures and produces inflammatory cytokines, which stimulate bone resorption, inhibit osteoblast differentiation, alter the parathyroid hormone response, and influence bone metabolism, thereby reducing the formation of bone matrix and playing a crucial role in extracellular matrix adhesion, growth, and aggregation. Chronic hyperglycemia reduces peri-implant bone tissue formation and is associated with horizontal or vertical bone defects. After implantation, osseointegration is incomplete, the healing time is prolonged, new bone is immature, and no fiber-implant contact is formed. Diabetes mellitus has been consistently associated with deficient metabolism of the skeletal tissue, which, especially in T1DM, is primarily related to suppressed osteoblastic activity and reduced bone formation potential, irrespectively of the type of bone, the location and the mechanical loading characteristics.

Although the success and failure rates of implantation in DM patients have been studied extensively, only a few studies have focused on the effects of insulin treatment on implant osseointegration in these patients. Their results have suggested that insulin therapy could improve peri-implant bone formation and enhance new bone formation. Wang et al. found that local sustained application of recombinant rat insulin-like growth factor 1 was conductive to peri-implant bone remodeling. There was no improvement, however, in the jaw bone quality or quantity and no apparent effects on peri-implant osseointegration, which differs from the bone synostosis seen in non-DM patients.
Oxidation is highly involved in the antioxidant imbalance in DM patients. When implants are placed in alveolar bone, the body is being stressed and the intracellular level of reactive oxygen species rises, exceeding the scavenging capacity. A large amount of reactive oxygen species not only can directly attack cell proteins, lipids, nucleic acids, and other components, it activates a variety of intracellular signaling pathways that promote inflammation, accelerate extracellular matrix secretion, induce apoptosis, and accelerate synthesis of reactive oxygen species. Glutathione (GSH) is involved in the tricarboxylic acid cycle in vivo, activates various enzymes, and exerts antioxidation effects. As an important metabolism regulator and antioxidant, GSH scavenges oxygen free radicals, enhances antioxidant enzyme activity, and improves the body's defense capacity. It has therefore been widely applied in the drug treatment and adjuvant therapy of a variety of multi-system, multi-organ diseases, such as those involving liver or kidney damage as well as eye disease, cardiovascular disease, and Parkinson's disease. The present study aimed to explore the effects of antioxidant GSH intervention on implant osseointegration and provide an applicable solution to promote osseointegration efficiency for individuals in DM patients.

Materials and Methods

Materials and equipment

The study included 20 male clean-grade 5-month-old New Zealand white rabbits weighing 2.5–4.0 kg (provided by the Experimental Animal Center of Dalian University, Dalian, Liaoning Province, China).

Reagents and instruments used in this study include alloxan (Sigma, St. Louis, MO, USA), BMP-2 immunohistochemistry kit (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China), an electronic analytical balance (Shanghai Electronic Balance Factory, Shangai, China), electric low-speed mobile phone (NSK, Fukushima, Japan), type BX51 optical microscope (Olympus, Tokyo, Japan), type IX71/DP70 CCD image acquisition system (Olympus, Tokyo, Japan), ERM-3000 semi-automatic microtome (China Medical Device, Zhejiang, China), low-temperature high-speed centrifuge (Sigma, Tokyo, Japan), and glucometers and test strips (Sinocare Inc., Changsha, Hunan Province, China).

Model establishment and animal grouping

The involved rabbits were randomly given intravenous injections of GSH (30 mg/kg) or saline, respectively, and were divided into four groups: GSH-treated diabetes mellitus (GSH+DM), diabetes mellitus (DM), GSH-treated normal rabbits (GSH), normal control rabbits (NC).

After the rabbits were fed standard diet for 2 weeks, their blood glucose levels were determined prior to modeling. Animals were fasted for 8 h and fixed on the operation table, exposing the head and ears. The ear veins were disinfected with 75% alcohol and allowed to expand fully. Then, alloxan (50 mg/ml, 80 mg/kg per week for a total of three injections) was slowly injected into the ear veins. The control group was given injections of normal saline.

The blood glucose level was recorded at 2, 4, and 6 h after administration, and 10 mL of glucose injection and glucose solution (100 g/l) were injected within 24 h if needed to prevent hypoglycemia. During establishment of the model, blood glucose levels were monitored as following method every 24 h. When the fasting blood glucose level was higher than 19.4 mmol/l, the rabbits were given subcutaneous injections of insulin (1–4 U/kg). All the rabbits were fed standard diet throughout the trials. The success of creating a DM model was defined as accomplished by a fasting blood glucose level of >13.9 mmol/l. The fasting blood glucose levels reached their peak 2 weeks after injection of alloxan and were maintained at 14.3 mmol/l. They were significantly different from those in the control group (P<0.01). The license number of trial animal is SCXK (Liaoning) 2002-0002. The animal breeding and experiments in line with regulations of Dalian University, which is discussed and adopted by the Ethics Committee of Affiliated Zhongshan Hospital of Dalian University.

Dental implantation

Rabbits were anesthetized with intramuscular injection of sumianxin (0.1 ml/kg) and fixed in the supine position on the operating table. After the skin was disinfected and covered with a sterile towel, a 1.0-cm incision was cut on the left leg under local anesthesia of 0.5% lidocaine injection (2 mL containing epinephrine 4 ig/ml). The incision was into the right medial femoral epiphysis, exposing subcutaneous tissue and bone surface. A titanium implant (3.8 mm diameter, 5.0 mm length) was placed into the implant bed (2.0 mm diameter, 5.0 mm depth), which had been previously prepared at a speed of 1800 rps following continuous saline cooling. The titanium implant (SUNTEC Titanium Co., Ltd., Dalian, China) was in large particles with a blasting-etched surfaces. To the naked eye, SLA-treated Ti samples had a rough surface that exhibited no metallic luster and was dark gray in color. After mechanical polishing, there were uniform scratches on the surface of Ti, as observed under the scanning electron microscope. After SLA treatment, micro-level macropores with a pore size of 80-90 µm (grade A pores) and micropores with a pore size of 2-8 µm (grade B pores) were visible on the sample surface. Grade A pores were produced from strikes by corundum sands during sandblasting and were round or oval, and shallow and concave in shape; grade B pores were produced from etching and were round or oblong with sharp edges, but regular in size.
and shape, which is shown in Fig.1.

After implantation, the periosteum, subcutaneous tissue, and skin wound were sutured. The rabbits were housed in separate cages and allowed free activity. Intramuscular injection of penicillin (400,000 units) was given daily for 7 days. Blood glucose levels were monitored, and an appropriate amount of subcutaneous insulin (1–4 U/kg) was given if necessary to ensure a blood glucose level within the experimental range (higher than 19.4 mmol/l).

Figure 1. (a) mechanical polishing surface morphology of titanium implants surface, (b) SLA surface morphology of titanium implants surface, (c) SEM micrographs of SLA titanium implants, (d) SEM micrographs of SLA titanium implants.

**General observation and micro-computed tomography scanning**

Rabbits were killed with an overdose of sumianxin (0.4 ml/kg) via intramuscular injection at 4 weeks after surgery. The rabbit femurs with the implants were dissected, and peri-implant bone tissue healing and growth were observed under micro-computed tomography (CT) (Siemens, Malvern, PA, USA).

**Decalcified bone tissue preparation**

The rabbits were killed 8 weeks after surgery. Their femurs were harvested, and the implants were removed and fixed in 4 % formalin. The fixed bone tissue was rinsed with saline and decalcified with 20 % EDTA solution in a microwave oven (50 °C). The decalcification process was intermittent, with each decalcification cycle consisting of 2 min of irradiation for a total of 30 cycles. Specimens were cooled at room temperature in a water bath for 2 min between cycles to ensure that the temperature of the decalcification solution was lower than 70 °C during irradiation. After bone decalcification, specimens were treated with a series of procedures, including conventional dehydration, transparency, soaking in wax, and embedding. They were then made into 5 μm thick slices, mounted, and dewaxed. The decalcification method in accordance with the HE staining instructions of Beijing Beijing-shirt Bridge Biotechnology Co. The slices were stained with HE and photographed under an optical microscope.

**Immunohistochemical staining of BMP-2 expression**

Paraffin sections were deparaffinized and incubated in 3 % H₂O₂ deionized water for 10 min to eliminate endogenous physical activity. They were then rinsed with distilled water and immersed in phosphate-buffered saline (PBS) for 5 min followed by high-temperature antigen retrieval. The sections were incubated with reagent A (blue liquid) at room temperature for 10 min, incubated with antibodies (1:100) at 4 °C overnight, and rinsed with PBS for 3 min three times, incubated with reagent B (yellow liquid) at room temperature for 10 min, and rinsed with PBS for 3 min three times. They were then incubated with reagent C (orange liquid) at room temperature for 10 min and rinsed with PBS for 3 min three
Table 1. Change in blood glucose levels (mmol/l) of rabbits before and after modeling in each group ($\bar{x} \pm S$).

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Baseline blood glucose</th>
<th>Blood glucose 1 week after modeling</th>
<th>Blood glucose 4 weeks after modeling</th>
</tr>
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<tr>
<td>Normal</td>
<td>18</td>
<td>4.92±0.78</td>
<td>5.23±0.65</td>
<td>5.17±0.82</td>
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<tr>
<td>Diabetes mellitus</td>
<td>18</td>
<td>5.07±0.83</td>
<td>20.69±2.33*</td>
<td>19.46±3.28*</td>
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</table>

*P<0.01, vs. before modeling.  #P<0.01, vs. control group.

Table 2. Change in body weight of rabbits before and after modeling in each group ($\bar{x} \pm S$).

<table>
<thead>
<tr>
<th>Group</th>
<th>No</th>
<th>Baseline body weight(g)</th>
<th>Body weight 1 week after modeling(g)</th>
<th>Body weight 4 weeks after modeling(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>18</td>
<td>2353±274</td>
<td>2410±248</td>
<td>2572±196</td>
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<tr>
<td>Diabetes mellitus</td>
<td>18</td>
<td>2289±239</td>
<td>2077±166*</td>
<td>1732±302*</td>
</tr>
</tbody>
</table>

*P<0.05, vs. before modeling.  #P<0.01, vs. control group.

Figure 2. Surgery of dental implant and General observation of the implant. (a) Drilling in the bone surface. (b) A titanium implant (3.8 mm diameter, 5.0 mm length) was placed into the implant bed. (c) The implant has been implanted in the bone. (d) the periosteum, subcutaneous tissue, and skin wound were sutured. (e) The general observation of the implant after implanted for 4 weeks. (f) The observation of the implant which had been removed.
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Figure 3. Micro-CT scans of the dental implant. (a) DM+GSH group. (b) DM group. (c) GSH group. (d) NC group.

Figures 4. HE staining of the implants. (a) DM+GSH group. The arrow points granulation tissue had grown into the fracture site (b) DM group. (c) GSH group. (d) NC group.
Specimens were developed with a chromogenic reagent (DAB), rinsed thoroughly with tap water, and counterstained. The cell nuclei were dehydrated and transparent. Specimens were mounted and observed under an optical microscope. Images were acquired.

Results

Establishment of alloxan-induced DM models

After intravenous injection of ALX 24-48h, rabbits appear apathetic, decreased activity, loss of appetite symptoms, significant weight loss occurred one week after. Blood glucose levels in rabbit ear venous blood were recorded at 1 and 4 weeks after injection. Results showed that blood glucose levels were >16.7 mmol/L, which was significantly higher than the baseline level and that for the normal control group (Table 1). It was evidence that indicated successful establishment of the DM models. Two rabbits died of convulsions after the alloxan injection. The 18 healthy rabbits that survived met the standard of modeling success. Body weights of the rabbits were significantly reduced after injection, with apparent emaciation. The body weights before and after the injection were significantly different ($P<0.01$) (Table 2).

Implant healing

The wounds were well healed in all four groups. No ulceration was found, the implants fused with surrounding bone tissue without loosening, and the tapping sound was crisp (Fig. 2). Micro-CT scanning revealed that the peri-implant threaded bone density was higher than the peri-implant cancellous bone density and was similar to the femoral cortical bone density. The implants had tightly fused with the surrounding bone tissue (Fig. 3), with no significant differences among the four groups.
B signaling pathway. It plays an important role in the animal’s death that occurs with large doses of the drug. The rabbit’s weight (80 mg/kg). It effectively destroyed the rabbit’s pancreatic β cells and induced a diabetic state. This dose avoided the animal’s death that occurs with large doses of the drug. The standard for blood glucose levels in DM animals is inconsistent, mostly in the range of 11.1–16.7 mmol/L. Growing evidence has tended to define the fasting or nonfasting blood glucose levels at 11.1–16.7 mmol/L for successful creation of DM models. In this study, the successful DM model was defined based on a fasting blood glucose level >16.7 mmol/l.

During dental implantation, host tissue reactions include a series of cellular and molecular processes during tissue healing and fracture repair. Bone tissue molding and remodeling can be achieved through implant–bone surface fusion guided by osteoblasts and osteoclasts. Undifferentiated mesenchymal cells and progenitor cells migrate from the surrounding bone marrow, endosteum, and periosteum to the implant and proliferate. Under the action of local growth factors, these cells differentiate into osteoblasts or osteoclasts, which form osteoid tissue and, subsequently, mature bone. Histological findings have shown that active osteoclasts, thinning cortex, and thinning and sparse trabecular bone are present in the bone tissue of DM animals. Also, the number of osteoblasts are reduced, and the osteoblasts are flat or star-shaped. New bone formation is rarely seen. The reduced number of osteoblasts, inadequate osteoid formation, and slow bone deposition and mineralization contribute to bone remodeling and affect peri-implant bone density and osseointegration.

The mechanism of bone loss in DM remains unclear. Recent studies have shown that insulin deficiency, insulin-like growth factor reduction, inflammatory cytokine interleukins, tumor necrosis factor, and other factors may lead to decreased osteoblast activity and numbers, functional dysfunction, and collagen synthesis disorders, ultimately resulting in bone formation disorders. GSH is a good antioxidant synthesized naturally in human cells. It can be utilized and recycled via the glutamate recycling system. GSH, which consists of glutamic acid, cysteine, and glycine, can directly eliminate H₂O₂, eliminate toxic metabolites produced by reactive oxygen species, and repair oxidative damage. It can also directly protect –SH without biotransformation, serve as a hydrogen donor to promote peroxidase reduction reactions catalyzed by GPx, CAT, and SOD enzymes, directly scavenge free radicals, increase free radical scavenging activity, enhance the body’s antioxidant defense capacity, and attenuate oxidative damage. GSH is critically involved in the regulation of lipopolysaccharide-induced cytokine transcription and the I-KB/NF-κB signaling pathway. It plays an important role in cell signaling of reactive oxygen species under oxidative stress and prevents the oxidative stress response by adjusting the Ca²⁺ concentration in mitochondria. Reduced GSH can prevent Ca²⁺ overload in neurons and activate glutamate receptors in neurodegenerative diseases. At present, reduced GSH has been widely applied in the drug treatment and adjuvant therapy of a variety of multi-system, multi-organ diseases such as those
associated with liver and kidney damage, eye diseases, and cardiovascular diseases\(^9\). As an antioxidant, reduced GSH lowers the activity of redox-sensitive NF-κB, inhibits inflammatory cytokines (e.g., tumor necrosis factor-α, interleukin-6, interleukin-8), and attenuates oxidative stress injury\(^9\).

In this study, after diabetic rabbits were given intravenous injections of reduced GSH for 4 weeks, there was no significant difference between groups according to the results of general observation or micro-CT scanning. Peri-implant bone tissue grew well. Histological findings showed that intravenous injection of reduced GSH produced more osteoblasts and chondrocytes than did saline injection, new bone trabecular formation was faster, and trabecular bone began to mold and transform into lamellar bone. Also, there was greater expression of BMP-2 than in the saline-injected groups. This evidence indicates that the speed and degree of new bone formation were better after the GSH intervention than after saline injection. GSH is therefore regarded as a potential approach to improving implant osseointegration in DM patients. We believe that we have provided novel evidence that there is a way to increase the implantation success rate.

References


