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INTRACELLULAR DELIVERY OF ATP ENHANCED HEALING PROCESS IN FULL-THICKNESS SKIN WOUNDS IN DIABETIC RABBITS

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Abstract

Background—Small unilamellar lipid vesicles were used to encapsulate adenosine triphosphate (ATP-vesicles) for intracellular energy delivery and were tested for diabetic skin wounds in rabbits.

Methods—Diabetes was induced by alloxan. The mean peak blood glucose concentration was 505 mg/dl. One ear was created ischemic and eighty full-thickness wounds were created in 10 animals. ATP-vesicles or saline was used and their healing was compared.

Results—On the non-ischemic ears, mean closure time for ATP-vesicles-treated wounds was 13.7 days vs 16.4 days for saline-treated wounds ($p < 0.05$). On the ischemic ears, mean closure time for ATP-vesicles-treated wounds was 15.3 days vs 19.3 days for saline-treated wounds ($p < 0.01$). Histological study indicated better healing and re-epithelialization in the ATP-vesicles-treated wounds.

Conclusions—Intracellular delivery of ATP accelerated the healing process of diabetic skin wounds on ischemic and non-ischemic rabbit ears. The mechanisms deserve further study but may be related to improved cellular energy supplies.

Keywords

wound healing; diabetes; ATP; rabbit

INTRODUCTION

Diabetes affects approximately 246 million people worldwide [1] including 23.6 million in the USA [2] and these numbers are projected to double by 2030 [3]. Foot ulcers are estimated to occur in 15%-25% of all patients with diabetes in their life time and the overall cost of diabetic

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This is to certify that Sufan Chien is the partner of No vera, LLC in Louisville which plans to further develop and commercialize the ATP-vesicle for wound treatment.

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foot alone, including loss of work productivity, could be as high as \$20 billion per year [4]. Many foot ulcers ultimately need amputation, which is estimated at 60,000 cases per year in this country and represents the single largest group of non-traumatic amputation [5]. Over the past decades, researchers have gained significant knowledge about the biochemical and pathophysiological mechanisms underlying the process of diabetic wounds but this knowledge has not resulted in dramatic changes in wound closure or outcome [6]. The primary etiology of diabetic wounds includes neuropathy, ischemia and infection [7]. One of the major pathophysiologic events in non-healing diabetic wounds is a deficient blood supply. This decreased blood and oxygen delivery to the wound cells results in significantly decreased cellular energy supply [8] which negatively impacts on nearly every aspect of the healing process [9]. So far, no animal model is available to provide diabetic wounds which are also ischemic. The lack of suitable animal model has significantly limited research and development of drugs for diabetic wound care.

In the past, we reported that a direct intracellular delivery of highly fusogenic lipid vesicles encapsulated ATP (ATP-vesicles) could accelerate the full-thickness skin wound healing in non-diabetic rodents and rabbits [10-12]. We also reported that a rabbit ear wound model created by minimally invasive surgery was tolerable to diabetic animals [15]. Previous studies have shown that wound tissue relies heavily on the glycolytic pathway for energy production [8] and both ATP and PCr (phosphocreatine) were severely decreased in diabetic foot [13]. We hypothesized that wound hypoxia in diabetes results in low energy availability and this is the major cause of non-healing wounds. If we could deliver ATP into the cytosol of the wound tissue in diabetic subjects, the healing process would be greatly enhanced. This article reports our preliminary results of using ATP-vesicles in ischemic and non-ischemic wounds in diabetic rabbits.

METHODS

Preparation of ATP-vesicles

The ATP-vesicles were made by Avanti Polar Lipids Inc. (Alabaster, AL). The freeze dried ATP-vesicles were stored at -20 °C in freezer. They were mixed with a nonionic vanishing cream (Dermovan, San Anotio, TX) just before use. After reconstitution, the composition was: 100 mg/ml of Soy PC/DOTAP (50:1), Trehalose/Soy PC (2:1), 10 mM of KH_2PO_4 and 10 mM of Mg-ATP. The diameters of the lipid vesicles ranged from 120 to 160 nm which were measured by a DynaPro Particle Size Analyzer (Proterion Corporation NJ).

Animals and induction of diabetes

The study was approved by the Institutional Animal Care and Use Committee of the University of Louisville. A total of 10 young (8 -10 weeks and body weight of 1.7-2.0 kg) New Zealand white rabbits were used. Diabetes mellitus was generated by injection of alloxan (Sigma, St. Louis, MO) using a well-established technique [14]. Alloxan (100 mg/kg) was dissolved in normal saline and concentration was five percent (W/V), and immediately administered intravenously to the anesthetized rabbits (Ketamine hydrochloride 50 mg/kg and xylazine 5 mg/kg, IM) via the marginal ear vein over a period of 1 minute. A total of 10 ml of glucose (5% W/V) was administered subcutaneously 4, 8, and 12 hours after injection, and 20% glucose drink was provided for 1–2 days to offset transient hypoglycemia. Hyperglycemia normally developed after 48 hours and stabilized after 1 week (Fig.1). Because this dose of alloxan caused total destruction of pancreatic β cells, daily insulin injection was required. Blood glucose concentration was measured daily or twice a day using a blood glucose meter (LifeSpan, Inc Milpitas CA) for the first 4 weeks and once a week thereafter. If the blood glucose level was higher than 350 mg/dl, regular insulin (Novolin, Novo Nordisk Pharmaceuticals Inc. Princeton, NJ) 1-4U/kg was injected (SC) once or twice a day. Six animals

were kept for more than 9 months and their blood glucose levels and body weight were recorded every week.

Ischemic wound model

The ischemic ear model and wounds were created after hyperglycemia was stabilized for more than two weeks, using a minimally invasive technique reported before [15]. Briefly, the rabbits were anesthetized with ketamine hydrochloride (50 mg/kg) and xylazine (5 mg/kg, IM). One ear was rendered ischemic by using a minimally invasive surgical technique. The other ear served as a paired non-ischemic control. To create ischemic ear, three small vertical incisions were made on the vascular pedicles about 1 cm distal to the base of the ear. The central artery was ligated, divided and the accompanying nerve was cut as well. The cranial artery and vein were also cut, but the caudal artery and vein were preserved. A circumferential subcutaneous tunnel was made through the three incisions. All the subcutaneous tissues, muscles, nerves, and small vascular branches were discontinued. The skin incisions were closed with 5-0 prolene. Four circular full-thickness wounds were created on the ventral side of each ear with a 6-mm stainless steel punch. The perichondrium was also removed with the skin or separately. The base on which granulation and epithelization took place was the cartilage but the cartilage was not perforated.

Postoperative care

A Duragesic patch was attached to the back skin for releasing Fentanyl (25 µg/hour) for 2-3 days to reduce possible pain. On each ear, ATP-vesicles were used in one pair of wounds, and normal saline was used in the other two wounds. The wounds were covered with TegaDerm™ (3M, Minneapolis, MN). Dressing changes were made every day. The old dressings were removed and the wounds were cleaned with cotton swabs to remove any fluid, clots, fibrins, residual drugs, and any tissue debris. Digital photos were taken, new dressings were applied, and the wounds were covered again with TegaDerm™. Ear skin temperature was measured for comparison. This procedure was carried out until all wounds were healed.

Histological studies

In this group, 8 rabbits were used for wound healing comparison. Another 2 rabbits were sacrificed at days 2 and 17 for histological analysis before the wounds were healed. All the wound samples were taken for histological studies when the rabbits were finally sacrificed. The samples from the wounds were fixed in 4% buffered formaldehyde and embedded in paraffin. The paraffin blocks were cut in 6 µm and stained with hematoxylin and eosin for evolution of histological changes.

Statistical analysis

Results are reported as mean and standard deviation (SD). The saline and vesicles-ATP-treated wounds were compared by student t test and the changes of body weight were analyzed by one-way ANOVA with commercially available statistical software (GraphPad Software, Inc. San Diego, CA). A *p* value of < 0.05 was considered significant.

RESULTS

There was no death, bleeding or incisional infection in these animals. Among the 8 rabbits (64 wounds) that were followed until all wounds were healed, two wounds (one treated by ATP-vesicles and one treated by saline) on the ischemic ear in one rabbit were infected. These two wounds healed extremely slowly, and were excluded from wound healing time analysis. The remaining 62 wounds were used for healing comparison.

Blood glucose concentrations

Before alloxan injection, the average non-fasting blood glucose concentration was 104 ± 15 mg/dl. Two days after injection of alloxan, the peak blood glucose concentrations, normally taken immediately prior to insulin injection, increased to 407 to 600 mg/dl (Fig. 1). When insulin injection was given in the morning, blood glucose concentrations decreased gradually. About 1-5 hours after insulin injection, the mean blood glucose levels decreased to 150-350 mg/dl, and the concentrations rose again thereafter. In the rabbits with higher blood glucose concentrations, another dose of insulin was given again late in the afternoon.

Body weight

Before alloxan injection, the body weights ranged from 1.76 to 1.92 kg. Body weight increased gradually in all the diabetic rabbits. At the end of 9 months, their body weight ranged from 2.56 to 3.96 kg. The body weight increase was slower than that in normal rabbits, and there appeared to be a negative relationship between blood glucose level and body weight gain. There were significant differences of body weight gains among animals with different non-fasting blood glucose levels (Fig. 2).

The ischemic ear

Immediately after surgery, the ischemic ears became cool and cyanotic with a reduced sensation distal to the incision. The mean skin temperature differences was 5.0°C at the beginning, but decreased gradually to 0.6°C at the end of one month (Fig. 3). The most important ear artery, the central artery, had a strong pulse in the normal ear, but this pulse was not present in the ischemic ear. The ischemic ear movement was reduced but not totally eliminated because some muscles were still attached to the base of the ear.

Wound-closure times

The wound-closure times were determined by the wound management team, but were verified by a person who was blind to the treatment. Among the 62 wounds, the closure time was compared between the saline and ATP-vesicles treatments on non-ischemic ears (16 wounds in each group) and ischemic ears (15 wounds in each group because one infected pair were excluded from the comparison).

On the non-ischemic ears, wound closure times ranged from 12 to 22 days (mean 16.4 ± 3.4 days) for the saline-treated wounds. In the ATP-vesicles treated wounds, the healing times ranged from 9 to 19 days (mean 13.7 ± 4.9 days, $p < 0.05$). On the ischemic ear, the wound-closure times ranged from 16 to 27 days (mean 19.3 ± 4.2 days) for the saline-treated wounds. In the ATP-vesicles treated wounds, healing time ranged from 12 to 19 days (mean 15.3 ± 2.8 days, $p < 0.01$). There were significant differences in closure times between the two treatments on the ischemic or non-ischemic ears (Fig. 4). One example of healing comparison between the ATP-vesicles-treated and the saline-treated wounds on the non-ischemic and the ischemic ears is shown in Figure 5. More examples of healing comparisons are shown in Figure 6.

When the two saline-treated groups on the non-ischemic ear and ischemic ear were compared, the closure times were significant longer for the ischemic wounds than for the non-ischemic wounds ($p = 0.0172$). The closure times for the two ATP-vesicles-treated groups on the non-ischemic ear and ischemic ear were also different, but this difference did not reach statistical significance ($p = 0.1021$).

Extremely rapid granulation tissue growth by intracellular ATP delivery

One very special finding in this study was the extremely rapid granulation tissue growth in the wounds treated by intracellular ATP delivery. Granulation tissue growth started to appear as

early as one day after surgery on the non-ischemic ears. It lagged behind 1-3 days on the ischemic ears, but the wounds treated with ATP-vesicles also had faster granulation tissue growth than those treated by normal saline (Fig. 7).

No hypertrophic scar tissue formation in diabetic rabbits

Rabbits are known for their potential hypertrophic scar tissue formation in healed wounds, and rabbit ear wound model has been used for hypertrophic scar tissue formation study [16]. However, in our diabetic group which was intentionally kept for more than 9 months, no hypertrophic scar formation was found. Figure 8 is an example of healed wounds at three weeks, five months and eleven months after wounding. This finding is in sharp contrast to non-diabetic rabbits reported in the past [17] but this model fits the healing characteristics of human diabetic wounds.

Histology

Figure 9 is an example of histological comparison at day 2 post operation which showed the early process of wound healing. The ATP-vesicles treated wound is covered with granular tissue which contains numerous inflammatory cells such as neutrophils, lymphocytes and macrophages. The saline-treated wound shows almost no growth and is covered with small amount of blood clot and fibrins. Figure 10 is the example of the wound tissue taken from day 17 which compares the wound healing between different treatments. The ATP-vesicles treated wound shows full granular tissue coverage and complete re-epithelialization of the wound, while the saline-treated wound has less granular tissue and incomplete re-epithelialization. These findings indicate that the intracellular ATP-delivery caused faster and more complete healing than the wounds treated with normal saline.

DISCUSSION

The important findings of this experiment include: 1) Wound experiment can be performed successfully in rabbits with the combination of diabetes, ischemia, and nerve damage; 2) The delivery of ATP into the cell can accelerate the healing process of ischemic and non-ischemic skin wounds in diabetic rabbits; 3) The extremely rapid granulation tissue growth in the diabetic animals in this study is something never seen or reported in humans or any other land animals in the past; and 4) In rabbits with diabetes mellitus, no hypertrophic scar tissue was developed in the healed wounds. Diabetes mellitus is described as the global epidemic of the 21st century, and ulcers of the foot in diabetes take the greatest toll and are a source of major suffering and cost [1]. The pathogenesis of many diabetic complications remains incompletely understood, with multiple metabolic impairments playing complementary roles. According to the current knowledge, human diabetic foot ulcer is caused by a series of pathophysiologic changes induced by long-term hyperglycemia including neuropathy, ischemia and infection. First, in diabetes, nerve damage results from interacting metabolic abnormalities, worsened by disease of the vasa nervorum [18]. The damage affects peripheral sensation, innervation of the small muscles of the foot, and fine vasomotor control of the pedal circulation. In sensory neuropathy, loss of protective sensation leads to lack of awareness of incipient or actual ulceration. Next, ischemic necrosis of tissues beneath the callus leads to breakdown of skin and subcutaneous tissue, resulting in a neuropathic ulcer with a punched-out appearance. Also, foot tissues can become ischemic because of macrovascular disease (atherosclerosis), notably in the calf with relative sparing of proximal vessels. Ischemia also results from microvascular disease — both structural (capillary wall fragility, and thrombosis) and functional (vasomotor neuropathy with defective microcirculation and abnormal endothelial function) [19]. Although over 100 known physiologic factors may contribute to wound healing deficiencies in individuals with diabetes [20], the primary pathophysiology is associated with deficient blood supply in the non-healing wounds [21] which results in a significant decrease in cellular energy [8,22]. Energy is needed

for every phase of the wound healing process, and the decreased availability of ATP negatively impacts nearly every aspect of the healing process including protein and lipid biosynthesis, signal transduction, cell mitosis and migration, growth factor production, and maintenance of homeostasis [9]. This is because proteins built by linking its individual component amino acids with peptide bonds require chemical energy [23]. To compensate for the blood and oxygen supply, anaerobic glycolysis is increased in wound tissue [24]. However, this is inefficient for energy production, yielding only 1/16 the amount of ATP compared with aerobic energy production. Increasing oxygen supply may be one approach to improve energy production. The recent reviews showed hyperbaric oxygen therapy in diabetic wounds is still one of important methods in clinic [25,26]. However, the true effectiveness of increased oxygen tension on wound healing has not been completely established [27]. Oxygen has multiple functions in wound healing process and the effect of oxygen therapy in wound healing will be continuously explored in the future. However, oxygen alone is incapable of producing high-energy phosphates, and this may be one reason that such a therapy has not obtained a consistent result.

Our group has demonstrated that intracellular delivery of ATP enhanced full-thickness skin wound healing process in nude mice and in non-diabetic rabbits [10,12]. In this study, we explored the possibility that such a treatment strategy might also be effective in diabetic wounds. Our preliminary data from biopsied wound tissues in diabetic patients indicated the existence of low energy status (unpublished data). The hypothesis is that, by supplementing energy supply, diabetic wound healing process may also be improved. The technique developed by our team is to use specially formulated and highly fusogenic small unilamellar lipid vesicles (with diameters of 120 - 160 nm) for intracellular Mg-ATP delivery. Because the composition of lipid vesicles is similar to that of the cell membrane, they readily fuse with the cells after contact. Our endothelial culture study showed that these vesicles fused with the cells and delivered water soluble carboxyfluorescein into the cytosol within 10 minutes and ATP-vesicles increased endothelial cell survival dramatically during hypoxia [28]. In skin penetration studies using rat and pig skin, it was found that lipid vesicles encapsulated Mg-ATP could penetrate the skin 10-20 fold faster than free Mg-ATP [11]. Our previous rodent study indicated that when ATP-vesicles were compared with its vehicle — lipid vesicles only, the vehicles did not show any enhanced healing effect [12]. In our pilot rabbit experiments, we used free Mg-ATP only, lipid vesicle only, and cream only, and these control drugs did not show any effect in enhancing the healing process in the ischemic or non-ischemic wounds. In the non-diabetic rabbit's study by our team, ATP-vesicles were compared with normal saline, and the latter did not show any effect [10]. Because at present time normal saline is still the standard wound dressing by most wound care specialists, the normal saline was used as the control treatment in this experiment. Our results indicated that the intracellular ATP delivery enhanced healing speed in diabetic rabbits in non-ischemic and ischemic wounds as compared to normal saline. The benefits appeared to be more prominent in the ischemic wounds than non-ischemic wounds. This is understandable because the ischemic wounds need extra energy for the cellular functions.

Diabetic wound healing is a complex process and numerous cell types, cytokines, growth factors, and enzymes are involved [29]. At the present time, the mechanisms by which intracellular ATP delivery enhances wound healing are not totally clear. Intracellular delivery of ATP into the wound cells can bypass the need for a fully intact blood circulation and still provide much-needed energy to all of the energy-starved cells to facilitate wound healing. We are not sure which cells will benefit most. However, the application of ATP-vesicles resulted in significantly decreased wound area, especially within the first 1-5 days in non-ischemic wounds and 2-7 days in ischemic wounds. This period correlates with skin constriction, macrophage stimulation, fibroblast proliferation and other fibroblast-dependant activities, such as collagen synthesis and extracellular matrix production [30]. ATP-vesicles appear to benefit all of these factors during the healing process. In the wound model we used, skin contraction

is minimal. While the outside skin of the rabbit ear has some mobility, the skin inside the rabbit ear is splinted by cartilage. As such, wound contraction is negligible [31]. Besides, the base on which granulation and epithelialization take place is the cartilage, this allows more accurate study of granulation and re-epithelialization process. The very early granulation tissue growth in the ATP-vesicles treated wounds indicated a rapid homing of many healing-related cells. Another possible mechanism is the upregulation of growth factors. Our previous nude mice study showed much higher VEGF upregulation of the wounds treated by ATP-vesicles than those treated by saline alone [12]. Although exogenous VEGF supplement was tried in the past, the value of such a supplement has not been established. This is because current advance in science has indicated that, to generate a functional vasculature, VEGF alone is not enough, all other vascular specific growth factors (VSGF), such as angioplatin family and ephrin family have to be involved too [32]. Supplement of cellular energy has the advantage of benefiting not only VEGF, but also other VSGF family members. Our previous rabbit non-diabetic wound study indicated that more CD31 positive cells appeared in the ATP-vesicles-treated wounds than those treated by normal saline [10]. The third mechanism is probably related to cytokine expressions. In a similar study using rabbit ear wound model, it was found that the expression of several pro-inflammatory cytokines (IL-1 β , MCP-1 and TNF- α) was significantly upregulated only 1 day after treatment [33]. This fast upregulation appeared to coincide with the time of the extremely rapid granulation tissue growth and may accelerate the healing process.

Although our study indicated the effectiveness of ATP-vesicles in wound healing, at this point, we are still not sure whether any of the individual components of the ATP-vesicles also played some supplemental roles in the healing process. Because the ATP-vesicles are not the simple physical mixture of these compounds, a complete comparison would have to include free-ATP only, lipid vesicles only, and free ATP plus lipid vesicles (without encapsulation). In the literature, there have been studies on extracellular ATP application for wound healing. These reports have indicated that extracellular ATP and ADP are mitogenic factors that enhance DNA synthesis and have synergism with growth factors during wound healing [34]. As indicated above, free Mg-ATP alone did not enhance the healing speed as compared to intracellular delivery in our pilot study. This does not exclude potential supportive effect. Because the phospholipids we used in the ATP-vesicles may also provide some nutrients to the wound tissue, they may also make some contributions. More work is under the way to delineate the role of each component in the contribution of healing mechanisms by which intracellular ATP delivery enhances wound healing.

The very rapid granulation tissues production in the ATP-vesicles group is something never seen or reported in the past in humans or any other land animals with any other drugs. Such a growth appeared to be very fast (only one day after application). The early growth was edematous and fragile, resembling some overgrowth of unhealthy granular tissue as seen very often in clinical wounds. It was easily mistaken as fibrin or clot accumulation. In our pilot study, we thought it might not be healthy and trimmed the excessive growth hoping that it would not hinder the re-epithelialization process. However, the growth always had a “root” connecting it to the wound edge. After extensive trimming, the growth stopped and the healing was delayed. In our healing study, we left the growth alone and found that it solidified during the process. The re-epithelialization did not climb up to cover the whole granular tissue. Instead, the epithelial cells made a tunnel through the growth and healed under the top of the growth. The cap became a crust and finally came off, revealing the beautiful totally healed wound underneath. We still do not know all the mechanisms related to this phenomenon other than discussed above. In a report using a similar rabbit wound model, it indicated that granulation tissue was barely seen 7 days after surgery [21]. This rapid granulation tissue growth in adult animals should be very attractive in clinical practice because, in many trauma or surgical cases, tissue defects need to be filled by granulation tissue before re-epithelialization takes place. The

important finding in this study is that it occurred equally rapidly in diabetic rabbits. This phenomenon is significant because impairment of leukocyte function and proliferation occurs in diabetic patients [35]. In the present study, many neutrophils, lymphocytes and macrophages were seen infiltrating the ATP-vesicles-treated wounds area only one day after surgery in the rabbits with diabetes mellitus even though they had severe hyperglycemia.

To our knowledge, this is the first report of using full-thickness skin wounds with ischemia and denervation in diabetic animals. Animal models play a key role in furthering our understanding of the underlying mechanisms involved in impaired diabetic wound healing and a critical role in the testing of new therapeutic strategies [36]. Diabetes mellitus is a very complex spectrum of diseases and so far no animal model has completely represented all human diabetic wounds. For the diabetic wound study, genetically or drug-induced diabetic animals without ischemia are usually used [37]. However, all models lack other important changes such as vasculopathy and neuropathy, characteristic in human diabetic ulcers, due to their short life span. We have created an ischemic and denervation model in the rabbit ear using the technique of minimally invasive surgery [15]. Due to its very small tissue disturbance, it can be safely used in diabetic rabbits. This model has following characteristics which are similar to diabetic wounds in human: (1) ischemia and nerve damage have been added to hyperglycemia; (2) wounds can be created in the animals with long-term hyperglycemia; (3) if infection is added to the wounds, it represents all pathophysiologic bases for human diabetic wounds. In this study, two wounds on the ischemic ear were infected and it took several weeks to heal. Although we could not draw any conclusion from only two wounds, this kind of wounds represent the most severe combination of diabetic wound pathophysiology that any animal model can attain: hyperglycemia, nerve damage, ischemia, and infection.

Another important finding in this study is that there was no hypertrophic scar formation in the diabetic rabbit ear wounds. This is in sharp contrast to the previously reported phenomenon in non-diabetic rabbits [16]. This no-hypertrophic scar phenomenon is similar to human diabetic wounds. We still don't know all the mechanisms related to this phenomenon. The use of insulin might have contributed to this phenomenon. However, a complete research or discussion is beyond the scope of this article.

In summary, this study indicated that the intracellular ATP delivery technique is effective in enhancing healing process in full-thickness skin wounds in diabetic rabbits with a combination of hyperglycemia, ischemia, and nerve damage. To our knowledge, this is the first report using the combination of diabetes and ischemia in wounds in an experimental animal model. The extremely rapid granulation tissue growth in these adult animals is something never seen or reported before, and is very encouraging. Although we still do not know all the mechanisms by which intracellular ATP delivery enhances wound healing, the technology could have a major impact on our treatment of various human wounds such as diabetic wounds, pressure ulcers, and battlefield injuries if the results can be duplicated in humans.

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The peak blood glucose concentrations

Figure 1.

The peak blood glucose concentrations after injection of alloxan and these levels were maintained throughout the experimental period. The lowest blood glucose concentrations ranged from 94 to 225 mg/dl, normally 2 to 3 hours after insulin injection.

Figure 2.

Change of body weight in the 6 diabetic rabbits which were kept for more than 9 months. There were significant differences among animals with different blood glucose levels (500-600 mg/dl vs. 300-450 mg/dl or 350-550 mg/dl, $p < 0.01$).

Ear temperature differences

Figure 3.
Comparison of mean skin temperature differences between the ischemic and non-ischemic ears during the first month after surgery.

Comparison of wound closure times

Figure 4.

Comparison of wound closure times between the saline and ATP-vesicles treated groups on the non-ischemic and ischemic ears.

* $p < 0.05$, ** $p < 0.01$ ATP-vesicles vs. Saline; # $p < 0.05$ between non-ischemic and ischemic wounds.

Figure 5.

An example of wound healing comparison between the ATP-vesicles treated wounds and saline treated wounds on the non-ischemic ear and ischemic ear.

Figure 6. More examples of wound healing comparisons between the ATP-vesicles-treated wounds and saline-treated ones on the non-ischemic ears and ischemic ears.

Figure 7.

An example of the extremely rapid granulation tissue growth in the wounds treated by the ATP-vesicles. On the non-ischemic ear (left), granulation tissue started to appear only one day after surgery. In the saline-treated wounds very little granulation tissue was found after 2-3 days. On the ischemic ear (right), a similar phenomenon in ATP-vesicles treated wounds occurred but with 1-3 days delay. Almost no granulation tissue was found in the saline-treated wounds at 7 days.

Figure 8.
An example of healed wounds on one diabetic rabbit ear. A-3 weeks after surgery; B-5 months after surgery; and C-11 months after surgery. D is an enlarged view of one wound in C. No hypertrophic scar formation is seen.

Figure 9.

Histologic comparison of two wounds on non-ischemic ear at day 2. The ATP-vesicles treated wound area is covered by numerous inflammatory cells such as neutrophils, lymphocytes and macrophages. The saline-treated wound has almost no cell infiltration. No bacterial colonization is seen in these wounds.

Figure 10.

A comparison of two wounds at 17 days after surgery. The intracellular ATP-delivery caused faster and more complete healing and re-epithelialization while the wound treated with saline remained unhealed.