Published in final edited form as:

Ann Plast Surg. 2009 February ; 62(2): 180–186. doi:10.1097/SAP.0b013e31817fe47e.

Intracellular Adenosine Triphosphate Delivery Enhanced Skin Wound Healing in Rabbits

Jianpu Wang, MD, PhD^{*}, Qunwei Zhang, MD, PhD[†], Rong Wan, MD, PhD^{*,†}, Yiqun Mo, MD, PhD[†], Ming Li, BS^{*}, Michael T. Tseng, PhD[‡], and Sufan Chien, MD^{*}

^{*}Department of Surgery, University of Louisville School of Medicine, Louisville, KY

[†]Department of Environmental and Occupational Health Sciences, University of Louisville School of Medicine, Louisville, KY

[‡]Department of Anatomical Sciences and Neurobiology, University of Louisville School of Medicine, Louisville, KY

Abstract

Small unilamellar lipid vesicles were used to encapsulate adenosine triphosphate (ATP-vesicles) for intracellular energy delivery. This technique was tested in full-thickness skin wounds in 16 adult rabbits. One ear was rendered ischemic by using a minimally invasive surgery. The other ear served as a normal control. Four circular full-thickness wounds were created on the ventral side of each ear. ATP-vesicles or saline was used and the wounds were covered with Tegaderm (3M, St. Paul, MN). Dressing was changed and digital photos were taken daily until all the wounds were healed. The mean healing times of ATP-vesicles–treated wounds were significantly shorter than that of saline-treated wounds on ischemic and nonischemic ears. Histologic study indicated better-developed granular tissue and reepithelial-ization in the ATP-vesicles–treated wounds. The wounds treated by ATP-vesicles exhibited extremely fast granular tissue growth. More CD31 positive cells were seen in the ATP-vesicles–treated wounds. This preliminary study shows that direct intracellular delivery of ATP can accelerate the healing process of skin wounds on ischemic and nonischemic rabbit ears. The extremely fast granular tissue growth was something never seen or reported in the past.

Keywords

wound healing; ischemia; ATP; rabbit

Nonhealing wounds represent a significant cause of morbidity and mortality for a large portion of the population. At least 5 million Americans suffer from chronic wounds, and many of these stem from diabetes, poor functioning veins, or pressure sores.^{1,2}

Over the past 20 years, researchers have gained significant knowledge about the biochemical mechanisms underlying the process of wound repair but this knowledge has not resulted in

dramatic changes in wound closure or outcome. Despite thousands of remedies and dressings developed or advocated to treat chronic wounds, none has been proven effective.³

One of the major pathophysiologic events in slow-healing or nonhealing wounds is a deficient blood supply; the affected tissue exists in a partially ischemic state.⁴ This decreased blood and oxygen delivery to the wound cells results in significantly decreased cellular energy supply,⁵ which negatively impacts on nearly every aspect of the healing process from protein synthesis to cell migration and neovascularization.⁶

We hypothesized that by providing energy to the wound tissues, the healing process would be enhanced. This hypothesis has never been tested in the past. If proven correct, it would provide a new avenue for wound management. We have used a new intracellular ATP delivery technique in which highly fusogenic lipid vesicles (ATP-vesicles) are used to encapsulate magnesium-adenosine triphosphate (Mg-ATP). The diameter of the lipid vesicles ranged from 120 to 160 nm. When these lipid vesicles come into contact with the cell membrane, they fuse with the cells and deliver their contents into the cytosol. This intracellular ATP delivery technique has shown effective in cell culture and preservation.⁷ The direct intracellular delivery of ATP may circumvent tissue ischemic damage, and was shown effective in our skin wound treatment in nude mice.⁸ However, there are critical differences in the physiology and biochemistry among rodents, larger animals, and humans. The novel growth factor therapeutics reported as successful in animal experiments, failed miserably in human clinical trials; and this has been attributed, in large part, to the differences in pharmacodynamics between the rodent and the human.⁹ Furthermore, it is very difficult to create uniform ischemia in rodent wound models, most of the drugs are tested in normoxic wounds, which are very different from human chronic wounds. Since our final goal is to test the effectiveness of wound care drugs in humans, it is necessary to go beyond rodents. This article reports our results using the ATP-vesicles in the treatment of skin wounds in both nonischemic and ischemic wounds in rabbits.

Methods

Preparation of ATP-Vesicles

The ATP-vesicles were made by Avanti Polar Lipids Inc. (Alabaster, AL). The composition was 100 mg/mL of Soy PC/DOTAP (50:1), Trehalose/Soy PC (2:1), 10 mM KH_2PO_4 , and 10 mM 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, Piscataway, NJ). The lipid vesicles were freeze-dried and stored at -20°C until use. They were mixed with a nonionic vanishing cream (Dermovan, San Antonio, TX) immediately before use. The final concentration of Mg-ATP was 10 mM.

Animal and Wound Model

The study was approved by the Institutional Animal Care and Use Committee of the University of Louisville. A total of 16 young adult New Zealand white rabbits were used (less than 6 months old with a body weight of 1.5–2.5 kg). The ischemic ear model was created using a minimally invasive technique reported before.¹⁰ Briefly, the rabbits were

anesthetized with a mixture of ketamine hydrochloride 50 mg/kg and xylazine 5 mg/kg (IM). One ear was rendered ischemic and the other ear served as a paired normal (nonischemic) control. To create ischemic ear, 3 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 3 incisions. All the subcutaneous tissues, muscles, nerves, and small vascular branches were discontinued. The skin incisions were closed with 4-0 or 5-0 prolene. Four circular full-thickness wounds were created on the ventral side of each ear with a 6-mm stainless steel punch. Digital photos were taken and the wounds were treated with testing or control drugs. The rabbits were allowed to wake up after surgery and food and drinks were provided ad lib.

Postoperative Care

A Duragesic patch (Sandoz, Inc., Broomfield, CO) was attached to the back skin for releasing Fentanyl (25 $\mu\text{g/h}$) for 2 to 3 days to reduce possible pain. The ischemic ears were observed for blood circulation and the closed incisions were examined daily for signs of bleeding or infection. On each ear, ATP-vesicles were used in 1 pair of the wounds and normal saline was used in the other 2 wounds. The wounds were covered with Tegaderm (3M, Minneapolis, MN). Dressing changes were made and digital photos were taken everyday. Skin temperature was measured on each ear daily. In this group, 11 rabbits were used for wound healing comparison and they were kept until all wounds were healed. Another 5 rabbits were killed at days 1, 3, 6, and 13 for histologic analysis and immunohistochemical staining.

Histologic Studies

Immediately after the rabbits were euthanized, circular wounds were removed and immersed in 4% formaldehyde buffer overnight. The circular wound disks were bisected and one half was embedded in paraffin for light microscopic examination. The paraffin blocks were cut in 6- μm and the slides were used for hematoxylin and eosin staining or CD31 immunostaining. The other half of the tissue was submitted for transmission electron microscope processing, which was thinly sliced into 3-mm³ blocks for plastic embedding after postfixation in 2% osmium tetroxide and dehydration. The polymerized blocks were cut at 1-micron thickness, stained with toluidine blue for light microscopy and 800 Å thickness, and collected on copper grids, stained with lead citrate and urinal acetate, before they were examined in a CM10 Philips electron microscope (North American Philips, Co., Mahwah, NJ).

Wound Tissue Angiogenesis

Wound tissue angiogenesis was determined by CD31-positive cells using immunohistochemical staining. The biopsy samples were fixed in 4% formaldehyde buffer for 16 hours then placed in 70% ethanol before embedding. A mouse antihuman monoclonal antibody (Dako North America, Carpinteria, CA), which has been shown to have good cross-species reactivity in our experience and in the literature,¹¹⁻¹³ was used as the primary antibody. Most of the stain steps are performed in the Autostainer (Dako). All immunostained specimens were evaluated qualitatively under light microscopy. Following

assessment of the quality and distribution of the staining, a quantitative morphometry analysis was performed. In each microscopic field (a magnification of 400×), covering granular tissue either from the saline-treated group or from the ATP-vesicles-treated group in nonischemic wounds 6 days after surgery, the number of CD31-positive microvessels was counted as the total number of vessels per field. Five fields from each slide were counted and the finding was pooled for analysis.

Statistical Analysis

Results are reported as mean and standard deviation (SD). The saline and vesicles-ATP wounds were compared by either paired *t* test or Mann-Whitney test with commercially available statistical software (GraphPad Software, Inc. San Diego, CA). A *P* value of <0.05 was considered significant.

Results

Among the 16 rabbits, 5 were killed at different time periods for tissue biopsy study before the wounds were healed, and the remaining 11 rabbits were followed until all wounds were healed. There was no death or skin incision infection in these animals.

Overall Appearance

The ischemic ear became cool and cyanotic with a reduced sensation distal to the incision after surgery. The skin temperature differences between nonischemic ears and ischemic ears ranged from 3.2°C to 4.9°C at the beginning. It decreased gradually over time (Fig. 1). The most important ear artery, the central artery, had a strong pulse in the nonischemic ear, but this pulse was absent 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-Healing Times

Among the 11 rabbits, wound-healing time was compared between the ATP-vesicles and saline-treated wounds on the nonischemic ears and ischemic ears (22 wounds in each group). On the ischemic ears the healing times ranged from 19 to 30 days (mean 22.8 ± 4.1 days) for the saline-treated wounds versus 17 to 22 days (mean 18.0 ± 1.9 days) for the ATP-vesicles-treated wounds ($P = 0.0005$). On the nonischemic ears the healing times were 14 to 17 days (mean 15.5 ± 1.0 days) for the saline-treated wounds and 13 to 16 days (mean 13.9 ± 1.5 days) for the ATP-vesicles treated wounds ($P = 0.033$). There were significant differences in healing times between the 2 treatments on the ischemic and nonischemic ears (Fig. 2). Figure 3 is an example of the comparison of the healing process between the ATP-vesicles-treated and the saline-treated wounds on the ischemic ears. More examples of comparison of the 2 treatments on the nonischemic ears and the ischemic ears are shown in Figure 4.

Extremely Fast Granular Tissue Growth by Intracellular ATP Delivery

One very special finding in this study was the extremely fast granular tissue growth in the wounds treated by intracellular ATP delivery. Gross examination showed that the ATP-vesicles-treated wounds had granular tissue growth starting from only 1 day after surgery on the nonischemic ears. This granular tissue growth lagged behind about 2 to 3 days on the

ischemic ears, but the wounds treated with ATP-vesicles also had faster granular tissue growth than those treated by saline (Fig. 5).

Histology Study

Light Microscopy—Samples taken from the rabbit killed 3 days after surgery showed a significant granular tissue growth in the edge that extended toward the center in the ATP-vesicles-treated wounds on both the nonischemic ear and the ischemic ear. By contrast, the center of the wound treated by saline was without such changes and the wound edge was lined with normal epidermal cells infiltrated by a small amount of granulation tissue (Fig. 6). Figure 7 shows the details of histologic changes in the nonischemic ears at day 3 and day 6 postwounding. Three days after surgery, many more inflammatory cells such as neutrophils, macrophages, and lymphocytes were observed (Fig. 7B and D) in the ATP-vesicles-treated group than in the saline-treated group (Fig. 7A and C). Six days after surgery, a higher number of inflammatory cells appeared in both superficial and deep areas of the granulation tissue in the ATP-vesicles-treated wounds (Fig. 7F) than in the saline-treated wounds (Fig. 7E and G). The granulation tissue deposition increased with more fibroblasts development and capillary loops in the deeper layer of the wounds in the ATP-vesicles-treated group (Fig. 7H). Figure 8 shows the comparison at day 13 in the ischemic wound. The intracellular ATP-delivery caused faster and more complete healing, whereas the wound treated with saline remained unhealed.

Electron Microscopy—A significant cell accumulation as early as day 1 was seen in the ATP-vesicles-treated wounds on the nonischemic rabbit ear. Scanning electron microscopy indicated numerous macrophages embedded in fibrin-like amorphous matrix. On the wound edge, the heaping cellular growth formed a small cellular mound over the epidermal keratinocytes as early as day 1. Daily application of ATP-vesicle seemed to sustain the newly arrived cells segregated by thin fibrin-like filamentous strands. At the level of ultrastructure granular tissue, 3 days after wounding on the nonischemic ear, a superficial layer dominated by cellular debris and degenerating cells is shown (Fig. 9A). The tissue interior consists of polymorphneutrophils (Fig. 9B), macrophages, fibrin, and red blood cells. Macrophages are identified by their large cell size, the tendency to engulf varying amounts of lipid droplets as well as the presence of cholesterol-like crystalline inclusions (Fig. 9C). Newly formed vessels in lamina propria are the conduit for the arriving inflammatory cells (Fig. 9D).

Morphometry Evaluation for CD31

Wound samples stained with mouse antihuman monoclonal antibodies shows more CD31 stained cells in the wounds treated with ATP-vesicles than in the saline-treated wounds (Fig. 10). A quantitative morphometry analysis confirmed that there was significant difference ($P = 0.0286$) between the ATP-vesicles-treated group (14.3 ± 2.7 capillaries/field) and the saline-treated group (5.5 ± 1.2 capillaries/field).

Discussion

Although the causes of nonhealing chronic wounds are multifactorial, the primary pathophysiology is associated with deficient blood supply,^{14,15} which results in a significant decrease in cellular energy.^{5,16,17} 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 (protein and lipid biosynthesis, signal transduction, cell mitosis and migration, growth factor production, and maintenance of homeostasis).⁶ This is because proteins built by linking its individual component amino acids with peptide bonds require chemical energy.¹⁸ Collagen is synthesized by fibroblasts,¹⁷ but fibroblasts are unable to replicate or synthesize collagen during ischemia and hypoxia.^{18,19} In the past, increasing wound oxygen supply has received substantial attention. The use of hyperbaric oxygen therapy is one example and has shown some favorable results,^{20–22} but the true effectiveness of increased oxygen tension on wound healing has never been established.^{18,23,24} To compensate for the reduced blood and oxygen supply, anaerobic glycolysis is increased in wound tissue.²⁵ However, this is inefficient for energy production, yielding only 1/16 the amount of ATP compared with aerobic energy production.²⁶

Our hypothesis is that if ATP can be delivered into the cytosol, the wound-healing process will be greatly enhanced. In the literature, there have been other 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.^{6,27} Using free Mg-ATP for intracellular delivery was reported in the past, but it is well known that a highly charged molecule like ATP does not cross the cell membrane in a quantity large enough to fulfill the metabolic requirement. Besides, the half-life of free ATP in the blood is less than 40 seconds, limiting its usefulness as an effective drug.

In this study, specially formulated and highly fusogenic small unilamellar lipid vesicles (with diameters of 120–160 nm) were used for intracellular Mg-ATP delivery. The technique was tested in various cell culture and preservation studies, and it showed protective effects during hypoxia. Although free ATP degrades quickly in the blood circulation, the encapsulation of drugs in the lipid vesicles can significantly extend its life.²⁸ Our freeze-dried ATP-vesicles can be kept for 6 to 12 months without degradation. In our previous rodent hind limb study, we found such encapsulation could maintain its *in vivo* effect for 4 to 6 hours. Our endothelial culture study showed that these vesicles fused with the cells and delivered water-soluble carboxyfluorescein into the cytosol within 10 minutes.^{7,29} In rat and pig skin penetration studies it was shown that lipid vesicles-encapsulated Mg-ATP could penetrate the skin 10- to 20-fold faster than free Mg-ATP.⁷ These characteristics make the technique suitable for wound care usage. In our pilot experiment, we used free Mg-ATP only, lipid vesicle only, saline only, or cream only, and these control drugs did not show any effect in enhancing wound healing. Our previous nude mice study showed promising results of such technique in full-thickness skin wound healing.⁸ Although rodent model has been popularly used for wound care studies, the differences in pathophysiology and pharmacokinetics, the ability of rodent to heal infected wounds quickly, and the ability of the loose skin to contract significantly in these animals, all have been blamed for unsuccessful

translation from bench to bedside of numerous wound care drugs.⁹ If a drug is expected to be successful, a testing model beyond rodent is required.

The results from this study indicated a promising effect of the intracellular ATP delivery technique. Not only did the wounds treated by the ATP-vesicles heal faster, but also the healing quality was better. The extremely fast granular tissues growth caused by intracellular ATP delivery is a very unique and very surprising finding. We have never seen such fast granular tissue growth in patients or any other land animals. To our knowledge, there has never been any report in the medical literature that shows such fast granular tissue growth by any other drugs. A report using a similar rabbit wound model indicated that granular tissue was barely seen 7 days after surgery.¹⁴ Although we still do not know all the mechanisms related to the enhanced wound healing and the extremely fast granular tissue growth in these animals at day 1 or 2, it should still be in the hemostasis and very early inflammation phase. The proliferation phase normally does not start until day 4 or 5.³⁰ As indicated above, ischemia is the major pathophysiologic event in nonhealing wounds. The reestablishment of blood circulation takes time. A direct intracellular delivery of ATP into the wound cells can bypass the need for fully intact blood circulation and still provide much-needed energy to all of the starved cells to facilitate wound healing. We are not sure which cells will benefit most. However, the application of ATP-vesicles resulted in a significantly decreased wound area, especially within the first 1 to 5 days in nonischemic wounds and 3 to 7 days in ischemic wounds. This period correlates with skin contraction, macrophage stimulation, fibroblast proliferation, and other fibroblast-dependant activities, such as collagen synthesis and extracellular matrix production.³¹ ATP-vesicles seem to benefit all of these factors during the healing process. The highly efficient granular tissue production in the ATP-vesicles group should also be very attractive in clinical practice because, in many surgical cases, tissue defects need to be filled by granular tissue before reepithelialization takes place.

Wound healing is a complex process and more than 100 physiologic factors are known to be involved in chronic diabetic wounds.³² Numerous cell types, cytokines, growth factors, and enzymes are involved in angiogenesis, collagen synthesis, and wound healing.³³⁻³⁵ Vascular endothelial growth factor (VEGF) was shown to be upregulated in our previous nude mice study in the wounds treated by ATP-vesicles compared with those treated by saline alone.⁸ CD31 staining is the most reliable and most popularly used technique for angiogenesis.³⁶ In the immunohistochemistry stained samples of the present study, more CD31-positive cells were present in the wound area in ATP-vesicles-treated wounds than in those treated by normal saline. The result seems to fit our hypothesis that providing energy to wound tissue causes functional neovascular generation. However, angiogenesis is induced by many factors and VEGF alone is not enough. All other vascular specific growth factors, such as angiopoietin family and eprin family, have to be involved too.³⁷ Unlike some other delivery techniques in which only a limited number of factors can be delivered, providing intracellular ATP has the possibility of supporting a coordinated upregulation of all growth factors. Another important factor is the involvement of pro- and anti-inflammatory cytokines.³⁸ In a similar study using a rabbit ear wound model, it was found that the expression of several proinflammatory cytokines (IL-1 β , MCP-1, and TNF- α) was significantly upregulated only 1 day after wounding (unpublished data). This fast

upregulation appeared to coincide with the time of the extremely fast granular tissue growth. More work is required in this area to fully understand the roles of these cytokines.

The wound model used in this study appeared to have many advantages. Unlike the wounds in rodents, which have high possibility of skin contraction, the rabbit ear skin is splinted by the cartilages, and this has eliminated skin contraction substantially. The technique for wound treatment reported here is easy to apply, has minimal expense, and has low toxicity. We have to point out that the use of small unilamellar vesicles is not our innovation, nor is the encapsulation of ATP into liposomes. Our innovations are (1) the use of small unilamellar vesicles that are highly fusogenic to encapsulate ATP and allow efficient cytosolic delivery of ATP and (2) the use of intracellular energy delivery for wound healing. If this approach can be translated to clinic patients, it will benefit millions of patients with diabetic wounds, pressure ulcers, and other types of acute and chronic wounds.

In conclusion, the results of this preliminary study show that intracellular delivery of ATP can accelerate the healing process in full-thickness skin wounds on ischemic and nonischemic rabbit ears. The extremely fast granular tissue growth in these wounds is a phenomenon never seen or reported before. If the full mechanism(s) is discovered by future research, it could have a major impact on medicine with great treatment potential in various human wounds.

Acknowledgments

The authors thank Sheron Lear in the Special Procedures Laboratory for her sample preparations and CD31 staining.

Supported in part by NIH grants DK74566 and AR52984.

References

1. Liechty KW, Sablich TJ, Adzick NS, et al. Recombinant adenoviral mediated gene transfer in ischemic impaired wound healing. *Wound Repair Regen.* 1999; 7:148–153. [PubMed: 10417750]
2. Strecker-McGraw MK, Jones TR, Baer DG. Soft tissue wounds and principles of healing. *Emerg Med Clin North Am.* 2007; 25:1–22. [PubMed: 17400070]
3. Page JC. Critiquing clinical research of new technologies for diabetic foot wound management. *J Foot Ankle Surg.* 2002; 41:251–259. [PubMed: 12194517]
4. Ehrlich HP, Grislis G, Hunt TK. Metabolic and circulatory contributions to oxygen gradients in wounds. *Surgery.* 1972; 72:578–583. [PubMed: 5057649]
5. Im MJ, Hoopes JE. Energy metabolism in healing skin wounds. *J Surg Res.* 1970; 10:459–464. [PubMed: 5476457]
6. Wang DJ, Huang NN, Heppel LA. Extracellular ATP shows synergistic enhancement of DNA synthesis when combined with agents that are active in wound healing or as neurotransmitters. *Biochem Biophys Res Commun.* 1990; 166:251–258. [PubMed: 1967937]
7. Chien S. Intracellular ATP delivery using highly fusogenic liposomes. *Meth Molecular Biol.* 2009 In press.
8. Chiang B, Essick E, Ehringer W, et al. Enhancing skin wound healing by direct delivery of intracellular adenosine triphosphate. *Am J Surg.* 2007; 193:213–218. [PubMed: 17236849]
9. Lindblad WJ. How should one study wound healing? *Wound Repair Regen.* 2006; 14:515. [PubMed: 17014661]
10. Chien S. Ischemic rabbit ear model created by minimally invasive surgery. *Wound Repair Regen.* 2007; 15:928–935. [PubMed: 18028143]

11. Cartwright L, Farhat WA, Sherman C, et al. Dynamic contrast-enhanced MRI to quantify VEGF-enhanced tissue-engineered bladder graft neovascularization: pilot study. *J Biomed Mater Res.* 2006; 77:390–395.
12. Jones M, Clayton E, Noel C, et al. Immunohistochemical endothelial localisation-a novel method of vessel delineation in tendon tissue. *J Immunol Methods.* 2003; 280:175–181. [PubMed: 12972198]
13. Mall JW, Schwenk W, Philipp AW, et al. Intraperitoneal administration of the angiogenesis inhibitor thalidomide does not impair anastomotic healing following large bowel resection in a rabbit model. *World J Surg.* 2003; 27:1119–1123. [PubMed: 12947492]
14. Schaffer M, Witte M, Becker HD. Models to study ischemia in chronic wounds. *Int J Lower Extremity Wounds.* 2002; 1:104–111.
15. Silver, I. The physiology of wound healing. In: Hunt, T., editor. *Wound Healing and Wound Infection Theory and Surgical Practice.* New York: Appleton; 1980. p. 11-31.
16. Smith DG, Mills WJ, Steen RG, et al. Levels of high energy phosphate in the dorsal skin of the foot in normal and diabetic adults: the role of ³¹P magnetic resonance spectroscopy and direct quantification with high pressure liquid chromatography. *Foot Ankle Int.* 1999; 20:258–262. [PubMed: 10229283]
17. Fine, N.; Mustoe, T., editors. *Wound Healing.* Philadelphia: Lippincott; 2001.
18. Hunt TK, Pai MP. The effect of varying ambient oxygen tensions on wound metabolism and collagen synthesis. *Surg Gynecol Obstet.* 1972; 135:561–567. [PubMed: 5077722]
19. Stadelmann WK, Digenis AG, Tobin GR. Impediments to wound healing. *Am J Surg.* 1998; 176:39S–47S.
20. Gottrup F. Oxygen, wound healing and the development of infection. Present status. *Eur J Surg Acta Chirurgica.* 2002; 168:260–263. [PubMed: 12375606]
21. Pai MP, Hunt TK. Effect of varying oxygen tensions on healing of open wounds. *Surg Gynecol Obstet.* 1972; 135:756–758. [PubMed: 5084248]
22. Wang C, Schwaitzberg S, Berliner E, et al. Hyperbaric oxygen for treating wounds: a systematic review of the literature. *Arch Surg.* 2003; 138:272–279. discussion 280. [PubMed: 12611573]
23. Manning AM. Oxygen therapy and toxicity. *Vet Clinics North Am.* 2002; 32:1005–1020.
24. Niinikoski J. Hyperbaric oxygen therapy of diabetic foot ulcers, transcutaneous oxymetry in clinical decision making. *Wound Repair Regen.* 2003; 11:458–461. [PubMed: 14617286]
25. Almskog BA, Haljamae H, Hasselgren PO, et al. Local metabolic changes in skeletal muscle following high-energy missile injury. *J Trauma.* 1982; 22:382–387. [PubMed: 7077699]
26. Kristensen SR. A critical appraisal of the association between energy charge and cell damage. *Biochim Biophys Acta.* 1989; 1012:272–278. [PubMed: 2758039]
27. Taegtmeier H. Energy metabolism of the heart: from basic concepts to clinical applications. *Curr Probl Cardiol.* 1994; 19:59–113. [PubMed: 8174388]
28. Gabizon AA. Stealth liposomes and tumor targeting: one step further in the quest for the magic bullet. *Clin Cancer Res.* 2001; 7:223–225. [PubMed: 11234871]
29. Chien, S. Metabolic management. In: Toledo-Pereyra, L., editor. *Organ Procurement and Preservation for Transplantation.* 3rd. New York: Landes Bioscience: Springer; 2008. p. 1-42. Available at <http://eurekah.com/chaoter/3992>
30. Witte MB, Barbul A. General principles of wound healing. *Surg Clinics North Am.* 1997; 77:509–528.
31. Harding KG, Morris HL, Patel GK. Science, medicine and the future: healing chronic wounds. *BMJ.* 2002; 324:160–163. [PubMed: 11799036]
32. Brem H, Tomic-Canic M. Cellular and molecular basis of wound healing in diabetes. *J Clin Invest.* 2007; 117:1219–1222. [PubMed: 17476353]
33. Falanga V. Growth factors and wound healing. *Dermatol Clin.* 1993; 11:667–675. [PubMed: 8222350]
34. Falanga V. Growth factors and wound healing. *J Dermatol Surg Oncol.* 1993; 19:711–714. [PubMed: 8349910]

35. Nanney LB, Caldwell RL, Pollins AC, et al. Novel approaches for understanding the mechanisms of wound repair. *J Investig Dermatol Symp Proc.* 2006; 11:132–139.
36. Sauer H, Bekhite MM, Hescheler J, et al. Redox control of angiogenic factors and CD31-positive vessel-like structures in mouse embryonic stem cells after direct current electrical field stimulation. *Exp Cell Res.* 2005; 304:380–390. [PubMed: 15748885]
37. Yancopoulos GD, Davis S, Gale NW, et al. Vascular-specific growth factors and blood vessel formation. *Nature.* 2000; 407:242–248. [PubMed: 11001067]
38. Nwomeh BC, Yager DR, Cohen IK. Physiology of the chronic wound. *Clin Plast Surg.* 1998; 25:341–356. [PubMed: 9696897]

Figure 1.
The mean skin temperature differences between the ischemic and nonischemic ear ranged from 4.7°C to 1.5°C, decreasing with time.

Figure 2. Comparison of wound healing times between the saline and ATP-vesicles treated wounds on normal and ischemic ears.

Figure 3.

An example of wound healing process on an ischemic ear. The healing time of ATP-vesicles-treated wound is 18 days and the saline-treated wound is 25 days.

Figure 4. More examples of wound healing comparisons between ATP-vesicles-treated wounds and saline-treated ones on the nonischemic ears and the ischemic ears.

Figure 5.

An example of the extremely rapid granular tissue growth in the wounds treated by ATP-vesicles on rabbit ear. On the nonischemic ear (left), granular tissue starts to appear only 1 day after surgery. In the saline-treated wounds very little granular tissue is found after 2 to 3 days. On the ischemic ear (right), a similar phenomenon in ATP-vesicles– treated wounds occurs but with 2 to 3 days delay. Almost no granular tissue is found in the saline-treated wounds at 7 days.

Figure 6.
An example of wound histology 3 days after surgery. The wound treated by ATP-vesicles shows granular tissue growth on both nonischemic and ischemic ears. The wounds treated by saline have almost no granular tissue. Arrows indicate the wound edge.

Figure 7.

The detailed histologic changes 3 (A–D) and 6 (E–H) days after surgery on the nonischemic ears. Three days after surgery, many inflammatory cells such as neutrophils, macrophages, and lymphocytes are observed (B and D, indicated by →) in the ATP-vesicles–treated group than those in the saline-treated group (A and C). Six days after surgery, a larger number of inflammatory cells appeared in both superficial and deep areas of the granulation tissue (F, indicated by arrowheads) in the ATP-vesicles–treated wounds than those in the saline-treated wounds (E and G), and granulation tissue deposition improved, and more fibroblasts development (H, indicated by *) and capillary loops (H, indicated by →) in the deeper layer of the wounds in the ATP-vesicles–treated group.

Figure 8. Histologic view of the wound at 13 days after surgery. The wound treated with ATP-vesicle shows complete reepithelialization whereas the saline-treated one is still open with much less granulation.

Figure 9.

Ultrastructural feature of the wound healing under the influence of ATP on the nonischemic ear 3 days after surgery. A, Interface between the granular tissue and the epidermal keratinocytes. Note the presence of red blood cells and 2 polymorphonuclear leukocytes (PMNs) in the field rich in fibrin (F). B and C, The presence of PMNs and macrophages. The former is identified by the presence of secretory granules and the mutilobed nuclei, whereas the latter contains numerous lipid droplets (L) and numerous needle-like cholesterol cleft (arrows). D, A common feature found in lamina propria where newly formed vessels are surrounded by the migrating PMNs.

Figure 10. CD31 staining of wound tissue on nonischemic ear 6 days after surgery. There are many more CD31-positive cells in the ATP-vesicles-treated wound (A) than in the saline-treated wound (B).