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(72) Inventeurs/Inventors:
BRAIMAN-WIKSMAN, LIORA, IL;
SOLOMONIK, INESSA, IL

(73) Propriétaire/Owner:
HEALOR LTD., IL

(74) Agent: BORDEN LADNER GERVAIS LLP

(54) Titre : COMPOSITIONS PHARMACEUTIQUES ET METHODES PERMETTANT D'ACCELERER LA CICATRISATION DE PLAIES
(54) Title: PHARMACEUTICAL COMPOSITIONS AND METHODS FOR ACCELERATING WOUND HEALING

(57) Abrégé/Abstract:
A method and a pharmaceutical composition for inducing or accelerating a healing process of a skin wound are described. The method includes administering to the skin wound a therapeutically effective amount of an adipokine, an adipocyte modulator, an adipocyte, a cell capable of differentiating into an adipocyte, or a cell capable of secreting an adipokine, thereby inducing or accelerating the healing process of the skin wound.

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(71) Applicant (*for all designated States except US*): **HEALOR LTD.** [IL/IL]; Bar Ilan University, Life Science Building No. 212, P.O. Box 1550, 52 115 Ramat Gan (IL).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **BRAIMAN-WIKSMAN, Liora** [IL/IL]; 24 Stephan Weiss Street, 75 400 Rishon-LeZion (IL). **SOLOMONIK, Innesa** [IL/IL]; 3 Ein-Shemesh Street, 55 900 Ganei-Tikva (IL).

(74) Agent: **G.E. EHRLICH (1995) LTD.**; 11 Menachem Begin Street, 52 521 Ramat Gan (IL).

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**(54) Title:** PHARMACEUTICAL COMPOSITIONS AND METHODS FOR ACCELERATING WOUND HEALING

**(57) Abstract:** A method and a pharmaceutical composition for inducing or accelerating a healing process of a skin wound are described. The method includes administering to the skin wound a therapeutically effective amount of an adipokine, an adipocyte modulator, an adipocyte, a cell capable of differentiating into an adipocyte, or a cell capable of secreting an adipokine, thereby inducing or accelerating the healing process of the skin wound.

# PHARMACEUTICAL COMPOSITIONS AND METHODS FOR ACCELERATING WOUND HEALING

## FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to methods and pharmaceutical compositions for accelerating the healing process of wounds. In particular the present invention utilizes bioactive molecules which are secreted by adipocytes (adipokines), and bioactive molecules which regulate adipocytes differentiation, proliferation and/or activity, for inducing or accelerating the healing process of skin wounds.

The primary goal in the treatment of wounds is to achieve wound closure. Open cutaneous wounds represent one major category of wounds and include burn wounds, neuropathic ulcers, pressure sores, venous stasis ulcers, and diabetic ulcers.

Open cutaneous wounds routinely heal by a process which comprises six major components: (i) inflammation; (ii) fibroblast proliferation; (iii) blood vessel proliferation; (iv) connective tissue synthesis; (v) epithelialization; and (vi) wound contraction. Wound healing is impaired when these components, either individually or as a whole, do not function properly. Numerous factors can affect wound healing, including malnutrition, infection, pharmacological agents (e.g., actinomycin and steroids), advanced age and diabetes [see Hunt and Goodson in Current Surgical Diagnosis & Treatment (Way; Appleton & Lange), pp. 86-98 (1988)]. There is also a common problem of wound healing following surgical procedures in various parts of the body, the surgery succeeds but the opening wound fails to heal.

Skin is a stratified squamous epithelium in which cells undergoing growth and differentiation are strictly compartmentalized. In the physiologic state, proliferation is confined to the basal cells that adhere to the basement membrane. Differentiation is a spatial process where basal cells lose their adhesion to the basement membrane, cease DNA synthesis and undergo a series of morphological and biochemical changes. The ultimate maturation step is the production of the cornified layer forming the protective barrier of the skin (1, 2). The earliest changes observed when basal cells commit to differentiate is associated with the ability of the basal cells to detach and migrate away from the basement membrane (3). Similar changes are associated with the wound healing process where cells both migrate into the wound area and proliferative capacity is enhanced. These processes are mandatory for the restructuring of the skin layers and induction of proper differentiation of the epidermal layers.

The analysis of mechanisms regulating growth, differentiation and migration of epidermal cells has been greatly facilitated by the development of culture systems for mouse and human keratinocytes (2, 4). *In vitro*, keratinocytes can be maintained as basal proliferating cells with a high growth rate. Furthermore, differentiation can be induced *in vitro* following the maturation pattern in the epidermis *in vivo*. The early events include loss of hemidesmosome components (3, 5) and a selective loss of the α6β4 integrin and cell attachment to matrix proteins. This suggests that changes in integrin expression are early events in keratinocyte differentiation. The early loss of hemidesmosomal contact leads to suprabasal migration of keratinocytes and is linked to induction of Keratin 1 (K1) in cultured keratinocytes and in skin (1, 3, 6). Further differentiation to the granular layer phenotype is associated with down regulation of both β1 and β4 integrin expression, loss of adhesion potential to all matrix proteins and is followed by cornified envelope formation and cell death. Differentiating cells ultimately sloughs from the culture dish as mature squames (2, 7). This program of differentiation *in vitro* closely follows the maturation pattern of epidermis *in vivo*.

Wound healing may be induced *in vivo* by various bioactive agents which directly or indirectly promote growth, differentiation and/or migration of epidermal cells. Thus, U.S. Pat. Nos. 5,591,709 and 5,461,030 describe the use of non-steroidal anabolic hormone such as insulin, growth hormone, triiodothyronine and thyroxine for inducing wound closure. U.S. Pat. No. 5,145,679 describes the use of insulin and pancreatin for inducing wound closure. U.S. Pat. No. 6,541,447 describes the use of a mixture of growth factors and growth hormones for inducing wound closure, and WO02/09639 describes the use of PKC modulating agents for inducing wound closure. However, there is no teaching in the prior art for utilizing adipocytes, adipocyte modulators, or molecules secreted by adipocytes, for inducing or accelerating the processes associated with wound healing.

There is thus a widely recognized need for, and it would be highly advantageous to have, new approaches for promoting wound healing. The present invention provides a novel approach for treating wounds by utilizing adipocytes, cells capable of differentiating into adipocytes, products secreted by adipocytes and adipocyte modulators, for inducing or accelerating the processes associated with wound healing.

## SUMMARY OF THE INVENTION

While conducting experiments in wound healing research, the inventors of the present invention uncovered that adipocytes are closely associated with migrating keratinocytes at the wound gap during an early stage of the healing process, indicating that adipocytes, adipocyte modulators and adipokines are involved in, and hence may be used to influence, the wound healing process.

While reducing the present invention to practice, as is further delineated in the preferred embodiments and examples sections that follow, it was found that indeed administering to wounds an adipokine, or an adipocyte modulator, substantially and effectively promoted wound healing.

Hence, according to one aspect of the present invention, there is provided a method of inducing or accelerating a healing process of a skin wound, comprising administering to the skin wound a therapeutically effective amount of an adipokine, thereby inducing or accelerating the healing process of the skin wound.

According to another aspect of the present invention, there is provided a method of inducing or accelerating a healing process of a skin wound, comprising administering to the skin wound a therapeutically effective amount of an agent capable of modulating expression and/or secretion of an adipokine, thereby inducing or accelerating the healing process of the skin wound.

According to yet another aspect of the present invention there is provided a method of inducing or accelerating a healing process of a skin wound, comprising administering to the skin wound a therapeutically effective amount of an agent capable of modulating differentiation of adipocytes, thereby inducing or accelerating the healing process of the skin wound.

According to still another aspect of the present invention there is provided a method of inducing or accelerating a healing process of a skin wound, comprising administering to the skin wound a therapeutically effective amount of an agent capable of attracting adipocytes to the skin wound, thereby inducing or accelerating the healing process of the skin wound.

According to an additional aspect of the present invention there is provided a method of inducing or accelerating a healing process of a skin wound, comprising administering to the skin wound a therapeutically effective amount of an agent capable of enhancing proliferation of adipocytes in the skin wound, thereby inducing or accelerating the healing process of the skin wound.

According to yet an additional aspect of the present invention there is provided a method of inducing or accelerating a healing process of a skin wound, comprising implanting into the skin wound a therapeutically effective amount of adipocytes, so as to induce or accelerate the healing process of the skin wound.

According to still an additional aspect of the present invention there is provided a method of inducing or accelerating a healing process of a skin wound, comprising implanting into the skin wound a therapeutically effective amount of preadipocytes, thereby inducing or accelerating the healing process of the skin wound.

According to yet an additional aspect of the present invention there is provided a method of inducing or accelerating a healing process of a skin wound, comprising implanting into the skin wound a therapeutically effective amount of stem cells, thereby inducing or accelerating the healing process of the skin wound.

According to a further aspect of the present invention there is provided a method of inducing or accelerating a healing process of a skin wound, comprising transforming cells of the skin wound to express and secrete an adipokine, thereby inducing or accelerating the healing process of the skin wound.

According to yet a further aspect of the present invention there is provided a pharmaceutical composition for inducing or accelerating a healing process of a skin wound, the pharmaceutical composition comprising, as an active ingredient, a therapeutically effective amount of an adipokine, and a pharmaceutically acceptable carrier being designed for topical application of the pharmaceutical composition.

According to still a further aspect of the present invention there is provided a pharmaceutical composition for inducing or accelerating a healing process of a skin wound, the pharmaceutical composition comprising, as an active ingredient, a therapeutically effective amount of an agent capable of modulating expression and/or secretion of an adipokine, and a pharmaceutically acceptable carrier being designed for topical application of the pharmaceutical composition.

According to yet a further aspect of the present invention there is provided a pharmaceutical composition for inducing or accelerating a healing process of a skin wound, the pharmaceutical composition comprising, as an active ingredient, a therapeutically effective amount of an agent capable of modulating differentiation of adipocytes, and a pharmaceutically acceptable carrier being designed for topical application of the pharmaceutical composition.

According to still a further aspect of the present invention there is provided a pharmaceutical composition for inducing or accelerating a healing process of a skin wound, the pharmaceutical composition comprising, as an active ingredient, a therapeutically effective amount of an agent capable of attracting adipocytes to the skin wound, and a pharmaceutically acceptable carrier being designed for topical application of the pharmaceutical composition.

According to yet a further aspect of the present invention there is provided a pharmaceutical composition for inducing or accelerating a healing process of a skin wound, the pharmaceutical composition comprising, as an active ingredient, a therapeutically effective amount of an agent capable of enhancing proliferation of adipocytes, and a pharmaceutically acceptable carrier being designed for topical application of the pharmaceutical composition.

According to still a further aspect of the present invention there is provided a method of determining a capacity of an adipokine or an adipocyte modulator to induce or accelerate a healing process of a wound, comprising administering the adipokine or the adipocyte modulator to the wound, and evaluating the wound for a keratinocytes migration and/or an epidermal closure, to thereby determine the capacity of the adipokine or the adipocyte modulator to induce or accelerate healing of wounds.

According to further features in preferred embodiments of the invention described below, the adipokine is selected from the group consisting of adipsin, adiponectin, resistin, leptin, lipoprotein lipase, angiotensinogen, angiotesin-like 4, 1-Butyrylglycerol, matrix metalloproteinase 2, matrix metalloproteinase 9, vascular endothelial growth factor, interleukin 6, and tumor necrosis factor $\alpha$. Preferably, the adipokine is adipsin, According to still further features in the described preferred embodiments the adipocytes modulating agent is a PPAR regulator, preferably a PPAR-$\gamma$ antagonist, more preferably GW9662.

According to still further features in the described preferred embodiments the adipocytes are human adipocytes. Preferably, autologous human adipocytes.

According to still further features in the described preferred embodiments the preadipocytes are human preadipocytes. Preferably, autologous human preadipocytes.

According to still further features in the described preferred embodiments the stem cells are human stem cells. Preferably, autologous human stem cells.

According to still further features in the described preferred embodiments the implanting further includes modulating expression and/or secretion of an adipokine.

According to still further features in the described preferred embodiments modulating is effected by differentiation.

According to still further features in the described preferred embodiments differentiation is effected by exposing the preadipocytes to a substance capable of enhancing differentiation of the preadipocytes into adipocytes.

According to still further features in the described preferred embodiments promoting differentiation is effected by exposing the stem cells to a substance capable of enhancing differentiation of the stem cells into adipocytes.

According to still further features in the described preferred embodiments the wound is selected from the group consisting of an ulcer, a burn, a laceration and a surgical incision.

According to still further features in the described preferred embodiments the pharmaceutical composition carrier is selected from the group consisting of an aqueous solution, a gel, a cream, a paste, a lotion, a spray, a suspension, a powder, a dispersion, a salve and an ointment.

According to still further features in the described preferred embodiments the pharmaceutical composition includes a solid support.

According to still further features in the described preferred embodiments the adipocytes modulator is an adipocyte differentiation modulator or an adipocyte activity modulator.

According to still further features in the described preferred the wound is an incision wound which is effected in an experimental animal.

According to still further features in the described preferred embodiments the administrating of an adipokine or an adipocyte modulator is effected in one or more concentrations.

According to still further features in the described preferred embodiments the administrating of an adipokine or an adipocyte modulator is effected in one or more applications.

The present invention provides novel pharmaceutical compositions and methods for treating wounds utilizing adipocytes, cells capable of differentiating into adipocytes, adipocytes modulators and molecules secreted by adipocytes, for inducing or accelerating wound healing.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

## BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1 illustrates the effect of insulin on adipocytes recruitment and epidermal cells migration at the wound area. Wounds were effected on the back of C57BL mice by incision. The wounds were treated daily with topical application of healing-inducing insulin (1 μM) for six days, then the mice were sacrificed and their wounds analyzed for epidermal cells migration and adipocytes recruitment. Epidermal cell migration was determined by K14 antibody staining and was considered positive if the wound was stained positive across the entire wound gap. Adipocytes recruitment was determined by H&E staining and was considered positive if adipocytes were detected inside the granulation tissue. The dark bars represent the insulin treatment and the light bars represent the buffer-treated control. The results are presented as percent of closed (positive) wounds and each bar represents the mean of six replications ± standard error.

FIGs. 2A-B are histochemical micrographs illustrating the association of adipocytes with the wound healing process. Wounds were effected on the back of C57BL mice by incision. The mice were sacrificed seven days after wounding, then sectioned and stained with K14 antibody to highlight migrating epidermal cells. The micrographs show that recruited adipocytes are present at the wound gap in abundance during an early stage of the wound healing process (Figure 2A, x20 magnification; Figure 2B, x10 magnification).

FIG. 3 illustrates the effect of a PPARγ antagonist (GW9662) on primary keratinocytes migration *in vitro*. Cultured keratinocytes were either untreated (control), or treated with 2 μM GW9662. The keratinocytes migration was observed under a light microscope. The upper panels show micrographs of pre-treated (Day 0) cultures, and the bottom left and panels show the resulting control and treated cultures, respectively (Day 2). The blue lines mark the edges of migrating keratinocytes and the arrows point out the enhanced migration of GW9662 treated cultures, as compared with the untreated control.

FIG. 4 is a graph illustrating the effects of a PPARγ antagonist (GW9662) and adipsin on wound healing *in vivo*. Wounds were effected on the back of C57BL mice by incision and the wounds were then measured (Day 0). The wounds areas treated daily with topical application of PBS (control), adipsin, or GW9662 2 μM for six days. Mice were then sacrificed and their wound areas were measured (Day 6). The portion of the wound area contracted in the six days from the initial wound area was calculated (% wound contraction) for each treatment. The graph shows that both GW9662 and adipsin promoted a substantial wound contraction, as compared with the buffer-treated control.

FIG. 5 is a histochemical micrograph illustrating the effects of a PPARγ antagonist (GW9662) and adipsin on wound closure *in vivo*. Wounds were effected on the back of C57BL mice by incision and their area were then measured (Day 0). The wounds were treated daily, for six days, with topical application of PBS (control), adipsin 1 μM, or GW9662 2 μM. The mice were then sacrificed, their wounds were fixed with paraformaldehyde and observed under a binocular microscope at x5 magnification. The micrograph shows that the areas of wounds which were treated with GW9662, or with adipsin, are substantially smaller than the wound area of the buffer control.

FIG. 6 illustrates the effect of adipsin on epidermal cell migration and wound closure. Wounds were effected on the back of C57BL mice by incision. The wounds were treated daily with topical application of 1 μM adipsin for seven days, then sacrificed, sectioned and analyzed for epidermal closure and migration by K14 antibody staining. Epidermal closure was considered positive if the wound was stained positive through the entire wound gap. Epidermal migrating was considered positive if the wound was stained positive but not entirely across the wound gap. The bar graph shows that both epidermal closure and epidermal migration were markedly enhanced by adipsin. Each bar represents the mean of six replications.

FIG. 7 is a histochemical micrograph illustrating the effects of a PPARγ antagonist (GW9662) and adipsin on wound closure (contraction). Wounds were effected on the back of C57BL mice by incision and were treated daily, for 6 days, with adipsin (1μM), GW9662 (2μM), or untreated (control). The treated mice were sacrificed six days after wounding. Histochemical wound sections were performed, stained with H&E (upper panel) or with K14 antibody (lower panel), and observed under a light microscope at x5 magnification. The contraction was considered positive if both dermal wound sides (marked by black lines) could be observed in a single field. The opened wound area in the untreated control section (right) was too large to be contained in a single field (thus considered a negative dermal contraction), while the adipsin treated section (left) and the GW9662 treated section (center) show positive dermal contractions.

FIG. 8 illustrates the effect of a PPARγ agonist (troglitazone) on primary keratinocytes migration *in vitro*. Cultured keratinocytes were either untreated (control), or treated with 100 μM troglitazone, and their migration was observed under a light microscope. The upper panels (Time 0) show micrographs of pre-treated cultures, and the bottom left and right panels show the resulting control and treated cultures, respectively (within 48 hours). The lines mark the edges of cultured keratinocytes and indicate a substantially inhibited migration of cultured keratinocytes treated with troglitazone, as compared with the untreated control.

FIG. 9 is a histochemical micrograph illustrating the effects of insulin and a PPARγ agonist (troglitazone) on wound closure *in vivo*. Wounds were effected on the back of C57BL mice by incision and were treated daily, for 6 days, with topical application of PBS (control), insulin (10 nM), troglitazone (100 μM), or troglitazone (100 μM) + insulin (10 nM) combined. The mice were then sacrificed, their wounds were fixed with paraformaldehyde and observed under a binocular microscope at x5 magnification. The micrograph shows that the insulin-treated wound area is substantially smaller than the buffer control, while the troglitazone and the troglitazone + insulin treated wounds are substantially larger than the buffer control.

FIG. 10 illustrates the effect of insulin and a PPARγ agonist (troglitazone) on wound closure incidence. Wounds were effected on the back of C57BL mice by incision. The wounds were treated daily with topical application of PBS (control), insulin (10 nM), troglitazone (100 μM), or troglitazone (100 μM) + insulin (10 nM) for six days, then sacrificed, sectioned and analyzed for wound closure. The wound closure was determined by K14 and K1 antibody staining. Wound closure was considered positive if the wound was stained positive throughout the entire wound gap. Each bar represents the mean of six replications.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of methods and pharmaceutical compositions for accelerating the healing process of wounds. Specifically, the present invention utilizes adipocytes, cells capable of differentiating into adipocytes, bioactive molecules secreted by adipocytes (adipokines) and adipocyte modulators, for accelerating the healing process of skin wounds.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the examples section. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Adult skin includes two layers, a keratinized stratified epidermis and an underlying thick layer of collagen-rich dermal connective tissue providing support and nourishment. Skin serves as the protective barrier against the outside world. Therefore, any injury or break in the skin must be rapidly and efficiently mended. As described in the Background section hereinabove, the first stage of skin repair is achieved by formation of the clot that plugs the initial wound. Thereafter, inflammatory cells, fibroblasts and capillaries invade the clot to form a granulation tissue. The following stages involve re-epithelization of the wound, where basal keratinocytes have to lose their hemidesmosomal contacts and migrate into the granulation tissue to cover the wound. Following keratinocyte migration, keratinocytes enter a proliferative boost, which allows replacement of cells lost during wound formation. After the wound is covered by a monolayer of keratinocytes (i.e., epidermal closure) new stratified epidermis is formed and the new basement membrane is reestablished (8-11).

While conducting experiments in wound healing research, the inventors unexpectedly uncovered that adipocytes are closely associated with migrating keratinocytes at the wound area, during an early stage of the wound healing process. Accordingly, Example 1 of the Examples section that follows illustrates that the appearance of migrating keratinocytes at the wound gap was directly correlated with appearance of recruited adipocytes at the same area. Furthermore, insulin treated wounds recruited more adipocytes to the wound gap, and subsequently healed faster, than control, untreated wounds. This newly uncovered close association of adipocytes and migrating keratinocytes at healing wounds, coupled with the direct correlation observed between recruited adipocytes incidence and wound healing efficiency, indicate that adipocytes, adipocyte modulators and adipocyte products (adipokines) are involved in, and hence may be used to influence, the wound healing process.

Adipocytes secrete a number of bioactive molecules, known as adipokines, which play a role in the maintenance of energy homeostasis by regulating insulin secretion, insulin action, glucose and lipid metabolism, energy balance, inflammation, and reproduction.

However, the possible involvement of adipocytes-secreted bioactive molecules in wound healing has not been taught nor suggested by the prior art.

Based on the initial findings described above, and while further reducing the present invention to practice, the inventors of the present invention anticipated and thereafter uncovered that an exemplary adipokine, adipsin, which was chosen out of the list of known adipokones, substantially accelerated keratinocytes migration *in vitro* and effectively promoted healing of skin wounds *in vivo* (see in Example 3 of the Examples section which follows).

Thus, according to one aspect of the present invention there is provided a method of inducing or accelerating a healing process of a skin wound, comprising administering to the skin wound a therapeutically effective amount of an adipokine, thereby inducing or accelerating the healing process of the skin wound.

The term "wound" as used herein refers broadly to injuries to the skin and subcutaneous tissue initiated in any one of a variety of ways (e.g., pressure sores from extended bed rest, wounds induced by trauma, cuts, ulcers, burns, surgical incisions and the like) and with varying characteristics.

Wounds are typically classified into one of four grades depending on the depth of the wound: (i) Grade I: wounds limited to the epithelium; (ii) Grade II: wounds extending into the dermis; (iii) Grade III: wounds extending into the subcutaneous tissue; and (iv) Grade IV (or full-thickness wounds): wounds wherein bones are exposed (e.g., a bony pressure point such as the greater trochanter or the sacrum).

The term "partial thickness wound" used herein refers to wounds that encompass Grades I-III; examples of partial thickness wounds include burn wounds, pressure sores, venous stasis ulcers, and diabetic ulcers.

The term "deep wound" used herein is meant to include both Grade III and Grade IV wounds.

The term "chronic wound" used herein refers to a wound that has not healed within thirty days.

The term "healing" in respect to a wound refers to a process to repair a wound as by scar formation.

The present invention contemplates treating all wound types, including deep wounds and chronic wounds.

The term “adipokine” as used herein refers to any bioactive molecule which is secreted by adipocytes *in vivo* or *in vitro*, including, but not limited to, adipocyte-secreted enzymes, growth factors, cytokines and hormones. Preferably, the adipokine of the present invention is selected from the group consisting of complement factors D (adipsin), C3 and B; vascular endothelial growth factor (VGEF), Adiponectin (Acrp30); resistin; leptin; lipoprotein lipase (LPL); angiotensinogen; angiotesin like 4; 1-Butyrylglycerol (monobutyrin); matrix metalloproteinases 2 and 9; tumour neorosis factor α (TNFα), and interleukin 6. Preferably the adipokine is adipsin.

In addition to administering adipokines to wounds, the healing of wounds may be induced or accelerated according to certain embodiments of the present invention by an adipocytes modulator.

The phrase “adipocytes modulator” as used herein refers to any molecule capable of modulating expression and/or secretion of adipokines from adipocytes, adipocytes differentiation, adipocytes proliferation, adipocytes migration or attracting adipocytes to the wound gap.

Adipocytes are differentiated from preadipocytes in a process known as adipogenesis. In culture, adipogenesis is fully dependent on insulin, dexamethasone and isobuthylmethylxanthine, stressing the involvement of insulin, glucocorticoid and cAMP pathways.

While many signaling and biochemical pathways play an imperative role in this process, most of the known changes that occur during adipogenesis are at the gene transcription level. The key transcriptional factors involved in the adipogenic process include proteins belonging to the CCAAT/enhancer binding protein family, adipocyte determination and differentiation dependent factor 1 (also known as sterol regulatory element – binding protein 1), and peroxisome proliferators-activator receptor γ (Rangwala and Lazar, Ann. Rev. Nutt. 20:535-539, 2000).

The peroxisome proliferator-activated receptors (PPARs) comprise three types, PPARα, PPARß and PPARγ. They are ligand-inducible nuclear receptors which directly modulate gene activity by binding to defined nucleotide sequences in the promoter region of target genes. PPARγ plays a crucial role in the terminal differentiation by transactivation of adipocyte-specific genes. Recent results suggest a cross talk between PPARs and the cholesterol metabolism pathway in the epidermis. All PPAR isoforms are expressed in embryonic and mature skin. PPARγ expression dramatically increases at the late stages of fetal maturation. In postnatals, as well as in adult skin, the expression of PPARγ is decreased. An important role has been suggested for PPARβ and PPARα in keratinocyte differentiation during epidermis formation (Wabli W., Swiss Med. Wkly. 132: 83-91, 2002). It has been also demonstrated that PPARβ and PPARα are up-regulated at the edges of wounded skin and that null mice of these isoformes are wound healing impaired (Michalnik et al., J. Cell Biol. 154: 799-814, 2001). Yet, the involvement of PPARγ in the wound healing process has not been described nor suggested in the prior art.

U.S. Pat. No. 6,403,656 describes the use PPARγ activators for treating skin disorders related to an anomaly of the differentiation of epidermic cells. In addition, WO00/33724 describes the use of PPARγ activators, such as a prostaglandin J2 or D2, for treating obesity and diabetes. However, none of these disclosures teaches or suggests using PPARγ activators, or inhibitors, for use in healing wounds.

While further reducing the present invention to practice, the inventors of the present invention uncovered that PPARγ activity is inversely related to the wound healing process. Accordingly, Example 2 of the Examples section which follows illustrates that administering troglitazone, a PPARγ agonist, inhibited wound contraction. On the other hand, administering GW9662, which is a PPARγ antagonist, promoted wound contraction.

Thus, according to another aspect of the present invention there is provided a method of inducing or accelerating a healing process of a skin wound, comprising administering to the skin wound a therapeutically effective amount of an agent capable of modulating differentiation of adipocytes, thereby inducing or accelerating the healing process of the skin wound. The agent according to this aspect of the present invention may be any agonist or antagonist of any factor, such as a transcriptional factor which is involved in adipocytes differentiation, including, but not limited to, a protein belonging to the CCAAT/enhancer binding protein family, adipocyte determination and differentiation dependent factor 1, and PPAR$\gamma$. Preferably, the agent is a PPAR-$\gamma$ antagonist, more preferably GW9662.

In addition to adipocytes differentiation modulators, the wound healing process may be promoted by utilizing other adipocyte modulators. Thus, according to the teaching of the present invention, a healing process of a skin wound can be induced or accelerated by administering to the skin wound a therapeutically effective amount of an agent which is capable of: (i) modulating expression and/or secretion of an adipokine from adipocytes, (ii) enhancing adipocytes proliferation, (ii) enhancing adipocytes migration, or (iii) attracting adipocytes to the wound area.

An assay readily exercisable by one ordinarily skilled in the relevant art of determining whether a specific agent, e.g., an adipokine or an adipocyte modulator, is offered in context of the present invention to test whether any such specific agent is indeed an inducer or accelerator of a wound healing process.

Thus, the capacity of an adipokine or an adipocytes modulator to induce or accelerate a healing process of a skin wound can be determined by administering the adipokine or the adipocytes modulator in question to a skin wound and evaluating the treated wounds for keratinocytes migration and/or epidermal closure.

Preferably, the skin wound is effected on the backs of a C57BL mouse by incision and treated with one or more applications, each with one or more concentrations of the adipokine.

A desired time period post wounding, preferably about 6 days, the mouse is sacrificed and wound biopsies are sampled. The wound biopsies are then analyzed for keratinocytes migration to the wound gap and/or for epidermal closure of the wound gap, using methods known in the art, preferably using the procedures as described in the Examples section that follows.

A significant increase in the incidence of keratinocytes migration and/or epidermal closure, over an untreated control, would determine that a tested adipokine or adipocytes modulator is capable of inducing or accelerating a healing process of a skin wound.

According to another aspect of the present invention, adipocytes, preferably autologous adipocytes, are implanted into the wound, so as to induce or accelerate the healing process of the skin wound.

Adipocytes may be obtained from an adipose tissue of any animal source, preferably from a human donor, most preferably from an autologous human source. The adipose tissue may be sampled from a subcutaneous or perirenal site, preferably subcutaneous, using well recognized protocols such as surgical, suction, liposuction, penniculectomy, or via biopsy. The adipocyte cells are preferably separated from the adipose tissue sample by using enzymes which destroy physical cell contacts (e.g., collagenases), or by using mechanical agitation, sonic or ultrasonic energy and the like. The separated adipocytes can be cultured using suitable tissue culture techniques known in the art such as, for example, described in details in WO01/32129. The cultured adipocytes are allowed to grow until near-confluence is reached, then removed by gentle scrapping from the growth medium and implanted onto the wound.

Adipocytes can also be generated from cultured preadipocytes. The term "preadipocyte" as used herein refers to any cell which is capable of differentiating into an adipocyte. Preferably, preadipocytes are human adipocytes, more preferably autologous adipocytes isolated from the patient's own adipose or other tissue. The adipose tissue may be sampled from subcutaneous or perirenal sites using well recognized protocols such as surgical, suction liposuction, penniculectomy, or via biopsy. The preadipocyte cells may be isolated from the sampled tissue by using methods such as described by Rodbell *et al.* (Meth. Enzymol. 31:103-114, 1974). Isolated preadipocytes can be grown, expanded and differentiated into adipocytes *in vitro*, using methods and procedures such are described by Hauner *et al.* (Journal Clin. Invest., 34:1663-1670, 1989), Digby *et al.* (Diabetes 5:138-141, 1998), and WO00/44882. The differentiated adipocytes can be harvested from the culture medium using harvesting methods such as described by Freshney (Culture of Animal Cells pp. 310-312, 3rd Ed., 1994) and implanted onto the wound preferably via a grafting chamber.

The grafting chamber can be removed from the wound after at least 1 day, preferably after at least 1 week subsequent to the implantation of the adipocytes. Optionally, the implanted adipocytes are exposed to an adipocyte modulator, such as, without limitation, a PPAR-$\gamma$ antagonist, preferably GW9662.

According to another aspect of the present invention, preadipocytes are implanted into the wound, so as to induce or accelerate the healing process of the skin wound. The preadipocytes can be isolated, grown and expanded *in vitro*, using the methods and procedures such as described for adipocytes hereinabove, but omitting the differentiation step. The non-differentiated preadipocytes are harvested from the culture medium and implanted onto the wound using procedures such as described for adipocytes hereinabove. Optionally, the implanted preadipocytes are exposed to an adipocyte modulator, such as, without limitation, a PPAR-$\gamma$ antagonist, preferably GW9662.

Adipocytes and/or preadipocytes can also be generated from cultured stem cells. The phrase "stem cells" as used herein refers to embryonic or adult cells which are not terminally differentiated, which can divide without limit, and divides to yield cells that are either stem cells or which irreversibly differentiate to yield a new type of cell such as a preadipocytes or an adipocyte.

Isolation and *ex vivo* expansion of stem cells can be performed using methods well known in the art. For example, Van Epps *et al.* (Blood Cells 20:411, 1994) and Emerson S. G. (Blood 87:3082, 1996) describe procedures for isolation and human hematopoietic stem cells from bone marrow, peripheral blood or a neonatal umbilical cord blood, and their expansion in culture. Human embryonic stem cells (hESC), can be prepared from human blastocyst cells, obtained from human in vivo preimplantation embryos, or *in vitro* fertilized embryos, using methods such as described in U.S. Pat. No. 5,843,780, and by Reubinoff *et al.* (Nature Biotech. 18:399, 2000). Human mesenchymal stem cells (hMSC) can be isolated and expanded using methods such as described in U.S. Pat. Nos. 5,197985, 5,486,359 and 6,214,369. The hMSC are found in bone marrow, blood, dermis and periosteum which are capable of differentiating into any of the specific types of mesenchymal tissues, such as an adipose tissue.

Stem cells can be administered directly onto the skin wound and allowed for differentiation into adipocytes *in vivo* with or without the co-administration of factors facilitating such differentiation. Alternatively, the stem cells can be differentiated into preadipocytes or adipocytes *ex vivo* and then implanted onto the wound.

Cultured hMSC can be induced for adipogenic differentiation, using methods such as described in U.S Pat. No. 6,322,784. Accordingly, adipocytes can be generated from primary hMSC by exposing the cells to a glucocorticoid and a compound capable of upregulating cAMP production, or by inhibiting degradation of cAMP, such as a phosphodiesterase inhibitor. The adipocytes generated from stem cells are subsequently harvested and implanted onto wounds using procedures such as described above, so as to induce or accelerate the healing process of the skin wound.

In an alternative embodiment of the present invention, wound cells are transformed to express and secrete an adipokine thereby inducing or accelerating the healing process of the skin wound.

The wound cells may be of any cell type which is involved in the wound healing process, such as keratinocytes, adipocytes or preadipocytes. The cells can be transformed by a polynucleotide encoding an adipokine such as adipsin, adiponectin, resistin, leptin, lipo protein lipase, angiotensinogen, angiotesin-like 4, 1-Butyrylglycerol, matrix metalloproteinase 2, matrix metalloproteinase 9, and tumour neorosis factor α. Alternatively, the cells can be transformed by a polynucleotide encoding a polypeptide capable of an adipokine activity, such as the polynucleotide encoding adipsin/complement D activity which is described in U.S. Patent 5,223,425.

The suitable polynucleotide can be introduced into cells by any one of a variety of known methods within the art. Such methods can be found generally described in Sambrook *et al.*, [Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992)]; Ausubel *et al.*, [Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Maryland (1989)]; Chang *et al.*, [Somatic Gene Therapy, CRC Press, Ann Arbor, MI (1995)]; Vega *et al.*, [Gene Targeting, CRC Press, Ann Arbor MI (1995)]; Vectors [A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston MA (1988)] and Gilboa *et al.* [Biotechniques 4 (6): 504-512 (1986)] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see United States patent 4,866,042 for vectors involving the central nervous system and also United States patents 5,464,764 and 5,487,992 for positive-negative selection methods for inducing homologous recombination.

A preferred approach for introducing a polynucleotide encoding an adipokine into wound cells is by using a viral vector. Viral vectors offer several advantages including higher efficiency of transformation, and targeting to, and propagation in, specific cell types. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through specific cell receptors, such as cancer cell receptors.

Retroviral vectors represent one class of vectors suitable for use with the present invention. Defective retroviruses are routinely used in transfer of genes into mammalian cells [for review see Miller, A.D., Blood 76: 271 (1990)]. A recombinant retrovirus including an adipokine encoding polynucleotide can be constructed using well known molecular techniques. Portions of the retroviral genome can be removed to render the retrovirus replication defective and the replication defective retrovirus can then packaged into virions, which can be used to infect target cells through the use of a helper virus and while employing standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in, for example, Ausubul *et al.*, [eds, Current Protocols in Molecular Biology, Greene Publishing Associates, (1989)]. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells endothelial cells, lymphocytes, myoblasts, hepatocytes and bone marrow cells.

Another suitable expression vector may be an adenovirus vector. The adenovirus is an extensively studied and routinely used gene transfer vector. Key advantages of an adenovirus vector include relatively high transduction efficiency of dividing and quiescent cells, natural tropism to a wide range of epithelial tissues and easy production of high titers [Russel, W.C. [J. Gen. Virol. 81: 57-63 (2000)]. The adenovirus DNA is transported to the nucleus, but does not integrate thereinto. Thus the risk of mutagenesis with adeno viral vectors is minimized, while short term expression is particularly suitable for treating cancer cells, such as multidrug resistant cancer cells. Adenoviral vectors used in experimental cancer treatments are described by Seth *et al.* [Adenoviral vectors for cancer gene therapy. In: P. Seth (ed.)

Adenoviruses: Basic biology to Gene Therapy, Landes, Austin, TX , (1999) pp. 103-120].

Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and regulatory elements that are specific for the desired cell type. The viral vector may also include a nucleotide sequence encoding a signal for secretion of the antibody fragment to the outside of the cell. Secretion signals generally contain a short sequence (7-20 residues) of hydrophobic amino acids. Secretion signals suitable for use in this invention are widely available and are well known in the art, see, for example by von Heijne [J. Mol. Biol. 184:99-105 (1985)] and by Lej *et al.*, [J. Bacteriol. 169: 4379 (1987)].

The recombinant vector can be administered in several ways. If viral vectors are used the procedure can take advantage of their target specificity and consequently, such vectors do not have to be administered locally at the tumor site. However, local administration can provide a quicker and more effective treatment. Administration of viral vectors can also be performed by, for example, intravenous or subcutaneous injection into the subject. Following injection, the viral vectors will circulate until they recognize host cells with appropriate target specificity for infection.

According to the present invention an adipokine or an adipocytes modulator can be used in therapy *per se* or as an active ingredient of a pharmaceutical composition.

As used herein the phrase "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of an administered active ingredient. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping and/or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which can be used pharmaceutically.

According to the present invention, the pharmaceutically acceptable carrier is suitable for topical application and can be, for example, but is not limited to, a gel, a cream, a paste, a lotion, a spray, a suspension, a powder, a dispersion, a salve and an ointment, as is further detailed hereinunder. Solid supports can also be used for prolonged release of the active ingredient into the wound.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients effective to induce or accelerate wound healing.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein, the assay disclosed herein and the Examples section that follows.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from a skin wound assay using experimental animals as described hereinabove. Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals. The data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See, e.g., Fingl, *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to levels of the active ingredient which are sufficient to, for example, induce wound healing (minimal effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from *in vitro* data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration.

Depending on the severity and responsiveness of the wound to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until diminution of wound is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredients. The pack may, for example, comprise metal or plastic foil formed into a tube for dispensing formulations for topical administration. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as if further detailed above.

It will be appreciated that the preferred mode of administration of the active ingredients of the present invention is topical - local administration, yet systemic administration, via acceptable administration routes, such as oral, intramuscular, intravenous, subcutaneous, transdermal, peritoneal, and the like using suitable formulations, as is well known in the art are not excluded.

Thus, the present invention provides novel methods and compositions for use in treatment of wounds by utilizing adipocytes, cells which can differentiate into adipocytes, adipocyte modulators and molecules secreted by adipocytes, for inducing or accelerating healing of wounds safely and effectively.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

## *EXAMPLES*

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A Laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531;

5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells -A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization -A Laboratory Course Manual" CSHL Press (1996).

Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader.

## *MATERIALS AND EXPERIMENTAL METHODS*

***Materials:*** All standard chemicals were from (Sigma-Aldrich, St. Louis, USA). Paraplast Embedding Medium was also purchased from Sigma. Anti keratin 14 antibody and anti keratin 1 polyclonal antibody were purchase from Bacto-Covance (Richmond, CA USA). Biotinylated goat anti-rabbit antibody and streptavidin-horseradish peroxidase (HRP) were purchased from ZYMED Laboratories Inc. (San Francisco, CA USA). GW9662 was purchased from Cayman Chemicals (Ann Arbor Michigan USA). Adipsin (complementing factor D) was purchased from Calibiochem

(San Diego CA USA). Hematoxylin was purchased from DAKO corp. (Carpinteria CA USA) eosin was purchased from ICN Biomedicals Inc. (Aurora Ohaio USA) and entellan was purchased from MERCK (Darmstadt Germany).

***Isolation and culture of murine keratinocytes:*** Primary keratinocytes were isolated from newborn skin as described in reference 18. Keratinocytes were cultured in Eagle's Minimal Essential Medium (EMEM) containing 8 % Chelex (Chelex-100, BioRad) treated fetal bovine serum. To maintain a proliferative basal cell phenotype, the final $Ca^{2+}$ concentration was adjusted to 0.05 mM. Experiments were performed five to seven days after plating.

***Keratinocytes migration assay:*** Primary mouse keratinocytes were untreated or treated with, PPAR$\gamma$ antagonist GW9662 ($\mu$M) or PPAR$\gamma$ agonist troglitazone ($\mu$M). Wound Scratch Assay was performed 24 hours following treatment, and representative fields were photographed immediately after the wounding (Day 0) and 48 hours later (Day 2). The average of wound closure represented as percentage in comparison to the initial width of the wound scratch (% of wound closure).

***Wound healing assay:*** Wounds were effected on backs of C57BL mice by 20 mm incision and were treated daily, for 6 days, with various agents. The mice were sacrificed six days after wounding. Wound biopsies were sampled, processed and analyzed morphologically and/or histochemically for various wound healing parameters, i.e., wound contraction, adipocytes migration and differentiation, epidermal migration, and epidermal closure.

***Wound contraction analysis:*** The wound area was measured before and after treatment and the percent reduction of the wound area was calculated.

***Preparation of paraffin embedded wound sections:*** Wound biopsies were fixed in 4 % paraformaldehyde then dehydrated in increasing concentrations of ethanol (50 – 100 %). The dehydrated preparations were immersed first in paraffin 50 % and xylene 50 % solution, then in pure paraffin. The paraffin blocks were then sectioned by a microtome and the sections were mounted on Super Frost$^{+TM}$ slides.

***H&E staining:*** Parafin embedded wound-section slides were incubated at 60 °C for 60 minutes and were the de-parafinated by washing the slides twice with toluene (100%) for 10 minutes, once with ethanol 100 % for 15 minutes and once with ethanol 100 % for 10 minutes. The de-parafinated slides were stained with hematoxylin (ready to use solution) for 10 minutes, rinsed with water, stained with eosin (0.5% in DDW) for 5 minutes, then washed with 70 % ethanol for 1 minute. Thereafter, the slides were dehydrated by washing once with 95 % ethanol for 5 minutes, twice with 100 % ethanol for 5 minutes, and twice with xylene (100%) for 10 minutes, then sealed using entellan (MERCK Darmstadt Germany).

***Keratin 4 and Keratin 14 staining:*** Parafin embedded wound-section slides were de-parafinated as described for H&E staining above and incubated in blocking solution (5 % BSA and 5 % Tween 20™ in PBS) for 1 hour. The slides were then incubated with either anti-keratin 1, or with anti-keratin 14 antibody (Babco-Covance) in (1:1000) blocking solution (5 % BSA and 5 % Tween 20™ in PBS) at 4 °C overnight. Thereafter, the slides were washed five times with washing buffer (5 % Tween 20™ in PBS) followed by incubation with biotinilated goat anti-rabbit antibody (ZYMED Laboratories Inc.) suspended (1:200) in blocking solution (5 % BSA and 5 % Tween 20™ in PBS) for 1 hour. The slides were then washed three times with washing buffer followed by incubation with a secondary biotinilated streptavidin antibody in blocking solution (1: 300) for 1 hour at room temperature. Thereafter, the slides were washed twice with washing buffer for 5 minutes, once with PBS for 5 minutes and once with TRIS buffer (0.05 M in PBS), followed by incubation in DAB reagent (2 tablets 1 gold 1 silver dissolved in DDW) for color development. The reaction was terminated by immersing the slides in water followed by counterstaining with eosin (ICN, 0.5% in DDW).

## *EXPERIMENTAL RESULTS*

## *EXAMPLE 1*

### *Association of adipocytes with migrating keratinocytes during the wound healing process*

Migrating epidermal cells (keratinocytes) and recruited adipocytes were observed in seven day old wound tissue (Figures 2A-B). The observed recruited adipocytes appeared essentially free of stored fat (i.e., early adipocytes). As can be seen in Figure 1, migrating keratinocytes were observed across the entire wound gap in about 60 % of the untreated wounds, while recruited adipocytes were also present in about 60 % of the untreated wounds. Figure 1 also shows that migrating keratinocytes and recruited adipocytes were observed in about 90 % and 80 % of insulin-treated wounds, respectively.

These results reveal that migration of keratinocytes to the wound gap area is closely associated with recruitment of adipocytes to the same area during an early stage of the wound healing process. The results thus indicate that migrating adipocytes, which are not fully differentiated into fat accumulating cells, are involved in the wound healing process.

## *EXAMPLE 2*

### *The effect of PPARγ modulators on keratinocytes migration and wound contraction*

The effect of inhibiting or enhancing the activity of peroxisome proliferators-activated receptor gamma (PPARγ) on keratinocytes migration was evaluated *in vitro*. As can be seen in Figure 3, the treatment of cultured primary mouse keratinocytes with the PPARγ antagonist GW9662 promoted keratinocytes migration. On the other hand, the treatment of cultured keratinocytes with the PPARγ agonist troglitazone inhibited keratinocytes migration (Figure 8).

The effect of inhibiting or enhancing the activity of PPARγ using PPARγ antogonist and agonist, respectively, on wound healing was also evaluated *in vivo*. Accordingly, incision wounds were effected on the back of C57BL mice and were treated daily, for 6 days, with PBS buffer (control), or with various agents. The mice were sacrificed six days after wounding and the wounds were then analyzed. As can be seen in Figures 4, 5 and 6, the treatment with GW9662 (a PPARγ antagonist) promoted wound contraction, as compared with the control. On the other hand, a similar treatment with troglitazone (a PPARγ agonist) inhibited wound contraction (Figure 9). Furthermore, troglitazone impaired insulin-induced wound healing (Figures 9 and 10).

Hence, the results clearly demonstrate that PPARγ activity hinders the healing of wounds and that PPARγ inhibition can effectively promote the wound healing process. Accordingly, the results indicate that PPARγ antagonists, such as GW9662 can be used to effectively accelerate wound healing.

## *EXAMPLE 3*

### *The effect of adipsin on keratinocytes migration and wound contraction*

The effect of adipsin (complementing Factor D, which is secreted from adipocytes) on wound healing was evaluated *in vivo*. Accordingly, incision wounds were effected on the back of C57BL mice and were treated daily, for 6 days, with PBS buffer (control), or with 1 μM adipsin. The mice were sacrificed six days after wounding and the wounds were then analyzed. As illustrated in Figures 4, 5 and 7, adipsin substantially promoted wound contraction (Figures 4, 5 and 7). In addition, adipsin increased epidermal closure from about 15 % to about 30 %, and increased keratinocytes migration from about 30 % to about 65 %, as compared with the buffer control (Figure 6).

These results demonstrate that an adipokine, such as adipsin, can effectively induce or accelerate wound healing.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the broad scope of the appended claims. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

REFERENCES CITED BY NUMERALS
(Additional references are cited in the text)

1. Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S.H. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell, *19*: 245-254, 1980.

2. Yuspa, S.H., Kilkenny, A.E., Steinert, P.M., and Roop, D.R. Expression of murine epidermal differentiation markers is tightly regulated by restricted extracellular calcium concentrations in vitro. J.Cell Biol., *109*: 1207-1217, 1989.

3. Fuchs, E. Epidermal differentiation: the bare essentials. J.Cell Biol., *111*: 2807-2814, 1990.

4. Yuspa, S.H. The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis--Thirty-third G.H.A. Clowes Memorial Award Lecture. Cancer Res., *54*: 1178-1189, 1994.

5. Hennings, H. and Holbrook, K.A. Calcium regulation of cell-cell contact and differentiation of epidermal cells in culture. An ultrastructural study. Exp.Cell Res., *143*: 127-142, 1983.

6. Tennenbaum, T., Li, L., Belanger, A.J., De Luca, L.M., and Yuspa, S.H. Selective changes in laminin adhesion and $\alpha 6\beta 4$ integrin regulation are associated with the initial steps in keratinocyte maturation. Cell Growth Differ., *7*: 615-628, 1996.

7. Tennenbaum, T., Belanger, A.J., Quaranta, V., and Yuspa, S.H. Differential regulation of integrins and extracellular matrix binding in epidermal differentiation and squamous tumor progression. J.Invest.Dermatol., *1*: 157-161, 1996.

8. Weinstein, M.L. Update on wound healing: a review of the literature. Mil.Med., *163*: 620-624, 1998.

9. Singer, A.J. and Clark, R.A. Cutaneous wound healing. N.Engl.J.Med., *341*: 738-746, 1999.

10. Whitby, D.J. and Ferguson, M.W. Immunohistochemical localization of growth factors in fetal wound healing. Dev.Biol., *147*: 207-215, 1991.

11. Kiritsy, C.P., Lynch, .B., and Lynch, S.E. Role of growth factors in cutaneous wound healing: a review. Crit.Rev.Oral Biol.Med., *4*: 729-760, 1993.

**CLAIMS:**

1. Use of adipsin for the preparation of a pharmaceutical composition adapted for topical application for inducing or accelerating a healing process of a skin wound.

2. The use of claim 1, wherein said pharmaceutical composition comprises adipsin.

3. The use of claim 1 or 2, wherein said skin wound is a pressure sore from extended bed rest, a wound induced by trauma, a cut, an ulcer, a burn or a surgical incision.

4. The use of any one of claims 1 to 3, wherein said pharmaceutical composition is an aqueous solution, a gel, a cream, a paste, a lotion, a spray, a suspension, a powder, a dispersion, a salve or an ointment.

5. The use of claim 4, wherein said pharmaceutical composition includes a solid support.

6. Use of a viral vector comprising a polynucleotide coding for adipsin for the preparation of a pharmaceutical composition for transforming cells of a skin wound to express and secrete said adipsin, thereby inducing or accelerating a healing process of the skin wound.

7. The use of claim 6, wherein said viral vector is an adenoviral vector.

8. The use of claim 6, wherein said pharmaceutical composition is adapted for local administration.

9. The use of claim 6, wherein said pharmaceutical composition is adapted for subcutaneous administration.

10. A pharmaceutical composition for topical application for inducing or accelerating a healing process of a skin wound comprising adipsin in admixture with a pharmaceutically acceptable diluent or carrier.

11. The pharmaceutical composition of claim 10, wherein said pharmaceutical composition is an aqueous solution, a gel, a cream, a paste, a lotion, a spray, a suspension, a powder, a dispersion, a salve or an ointment.

12. The pharmaceutical composition of claim 11, wherein said pharmaceutical composition includes a solid support.

13. A pharmaceutical composition for transforming cells of a skin wound to express and secrete adipsin, thus inducing or accelerating a healing process of a skin wound, comprising a therapeutically effective amount of a viral vector comprising a polynucleotide coding for said adipsin in admixture with a pharmaceutically acceptable diluent or carrier.

14. The pharmaceutical composition of claim 13, wherein said viral vector is an adenoviral vector.

15. The pharmaceutical composition of claim 13 or claim 14 in locally administrable form.

16. The pharmaceutical composition of claim 13 or claim 14 in subcutaneously administrable form.

17. A commercial package comprising a pharmaceutically effective amount of adipsin together with instructions for topical application for inducing or accelerating a healing process of a skin wound.

18. A commercial package comprising a pharmaceutically effective amount of a viral vector comprising a polynucleotide coding for adipsin together with instructions for transforming cells of a skin wound to express and secrete said adipsin, thus inducing or accelerating a healing process of said skin wound.

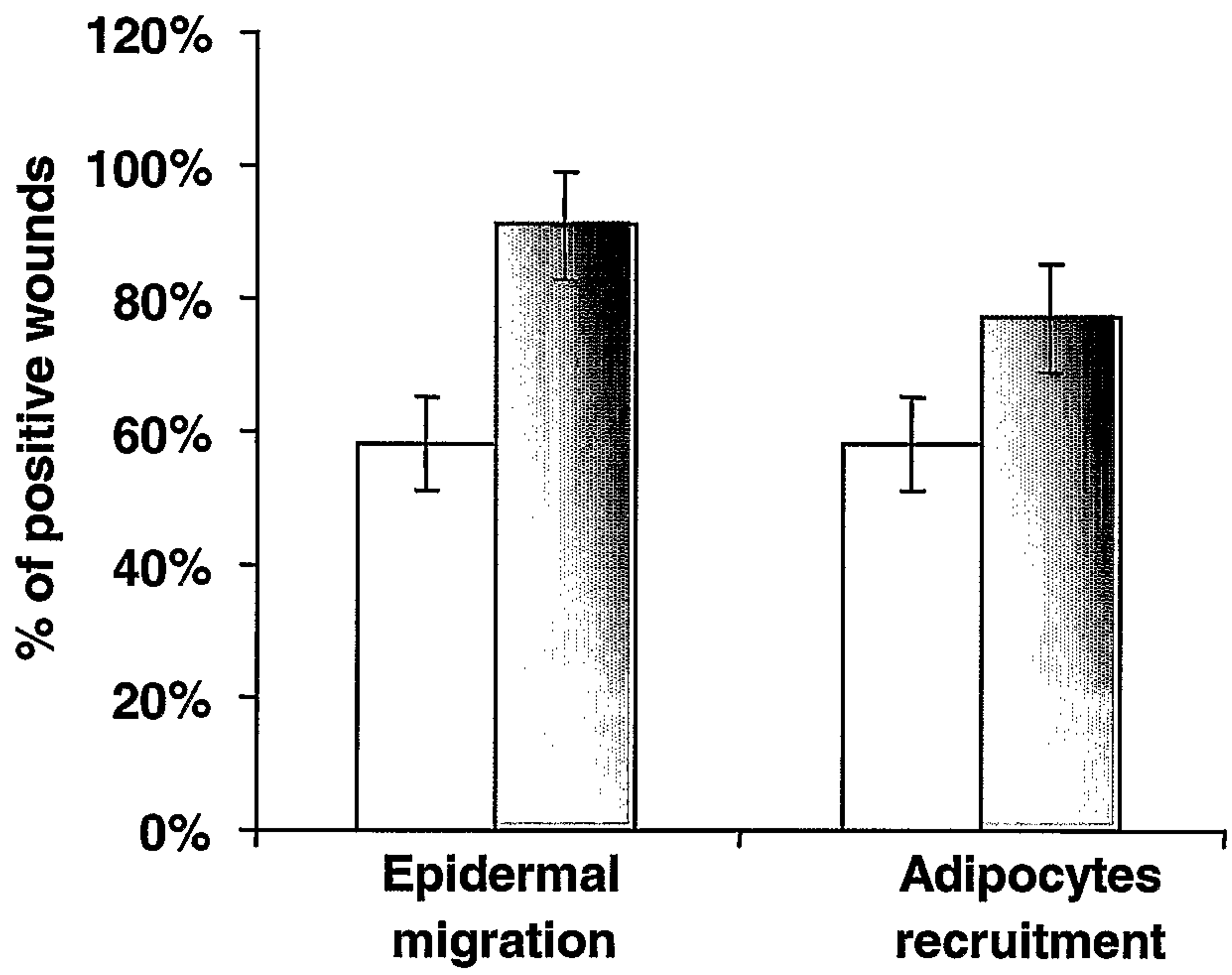
120%
100%
80%
60%
40%
20%
0%
% of positive wounds
Epidermal migration
Adipocytes recruitment

**Fig. 1**

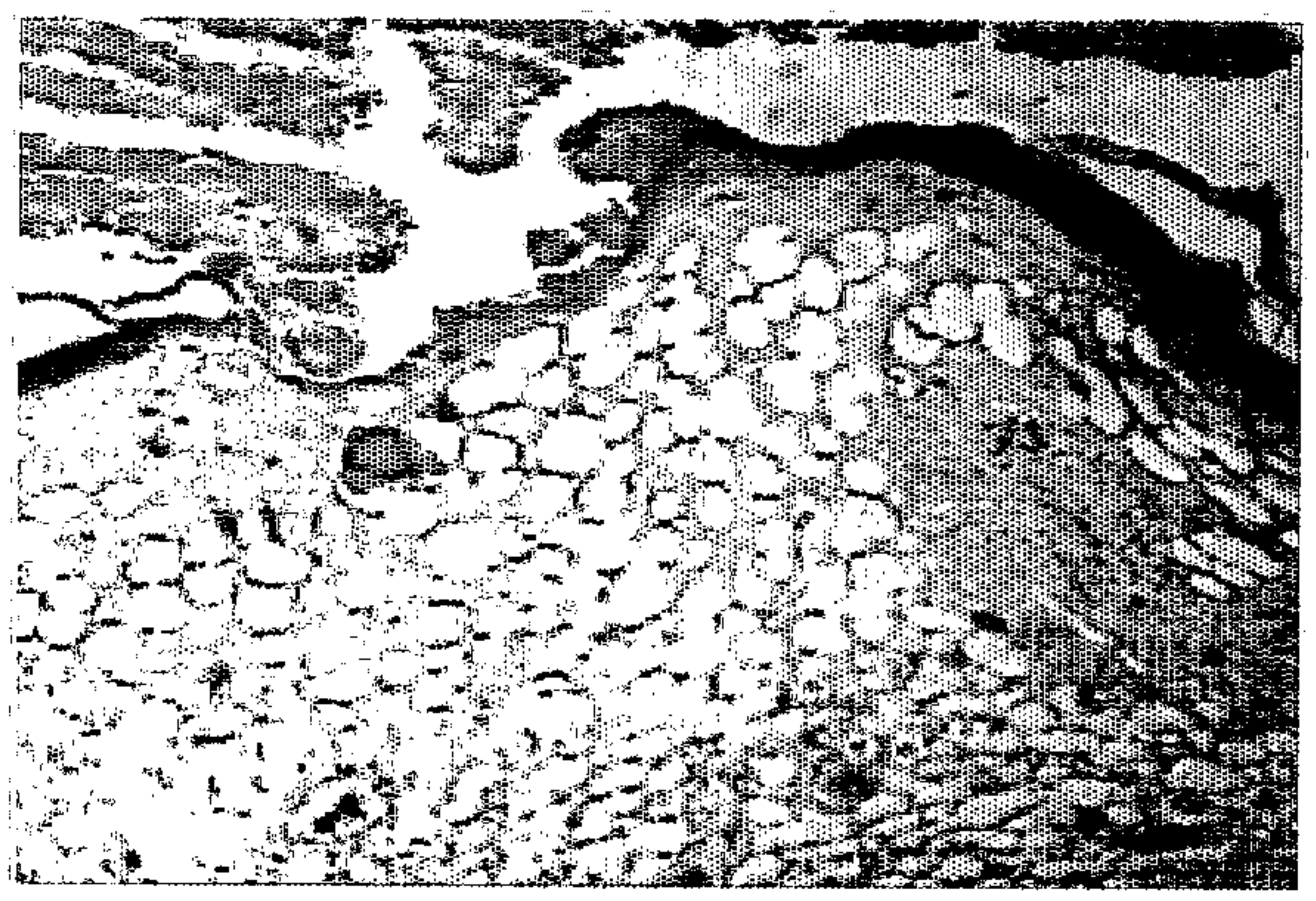

Fig. 2A

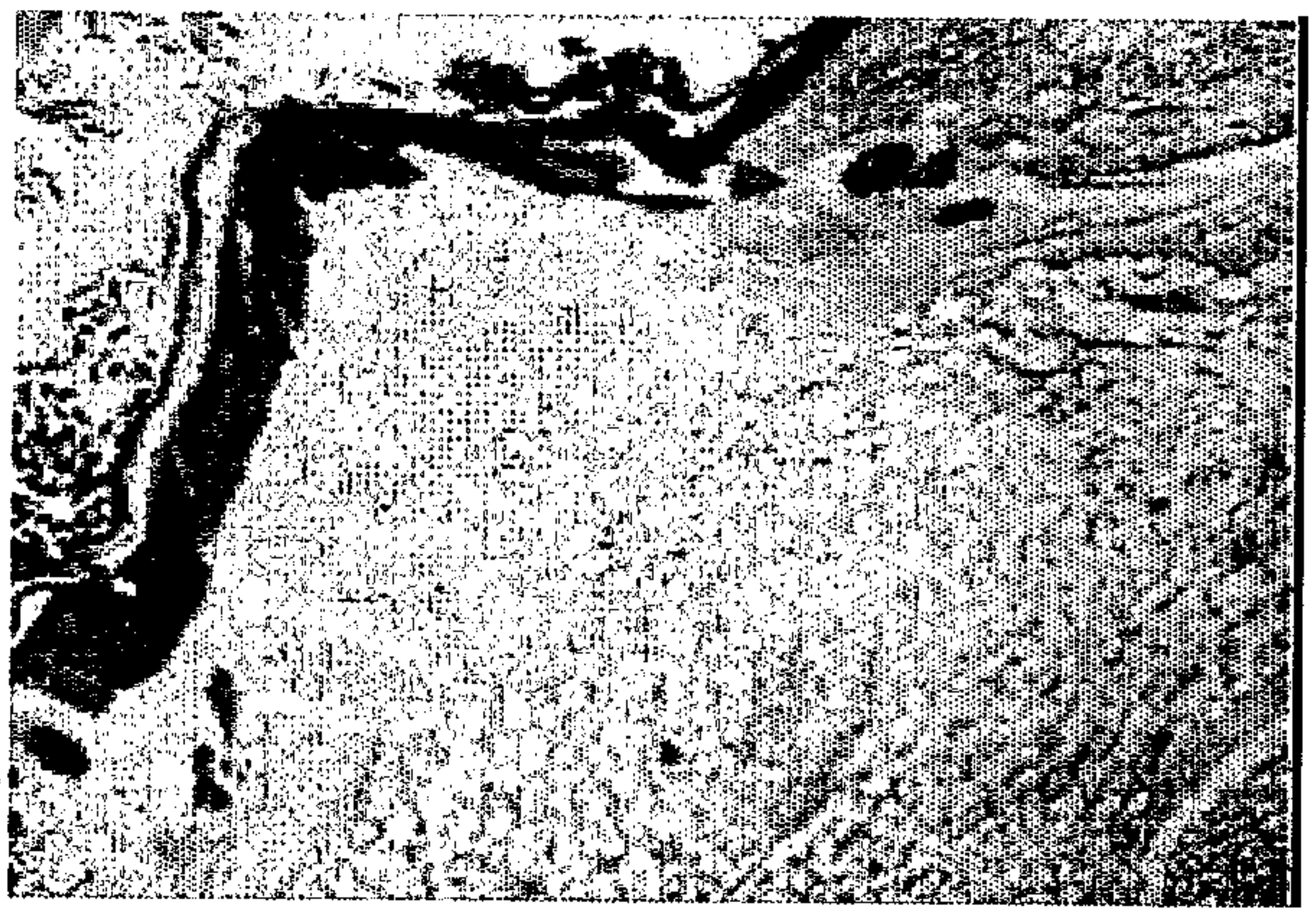

Fig. 2B

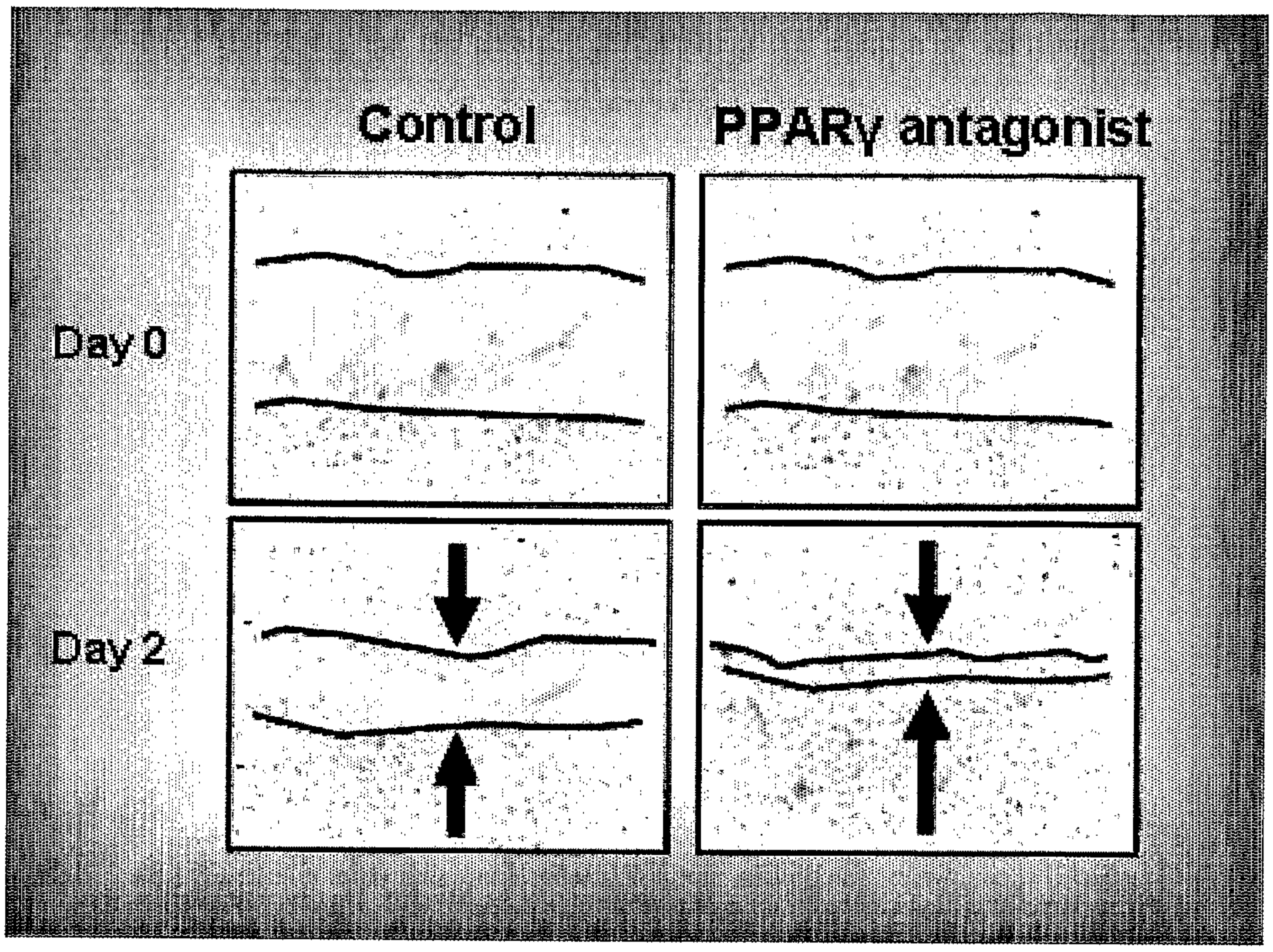


**Fig. 3**

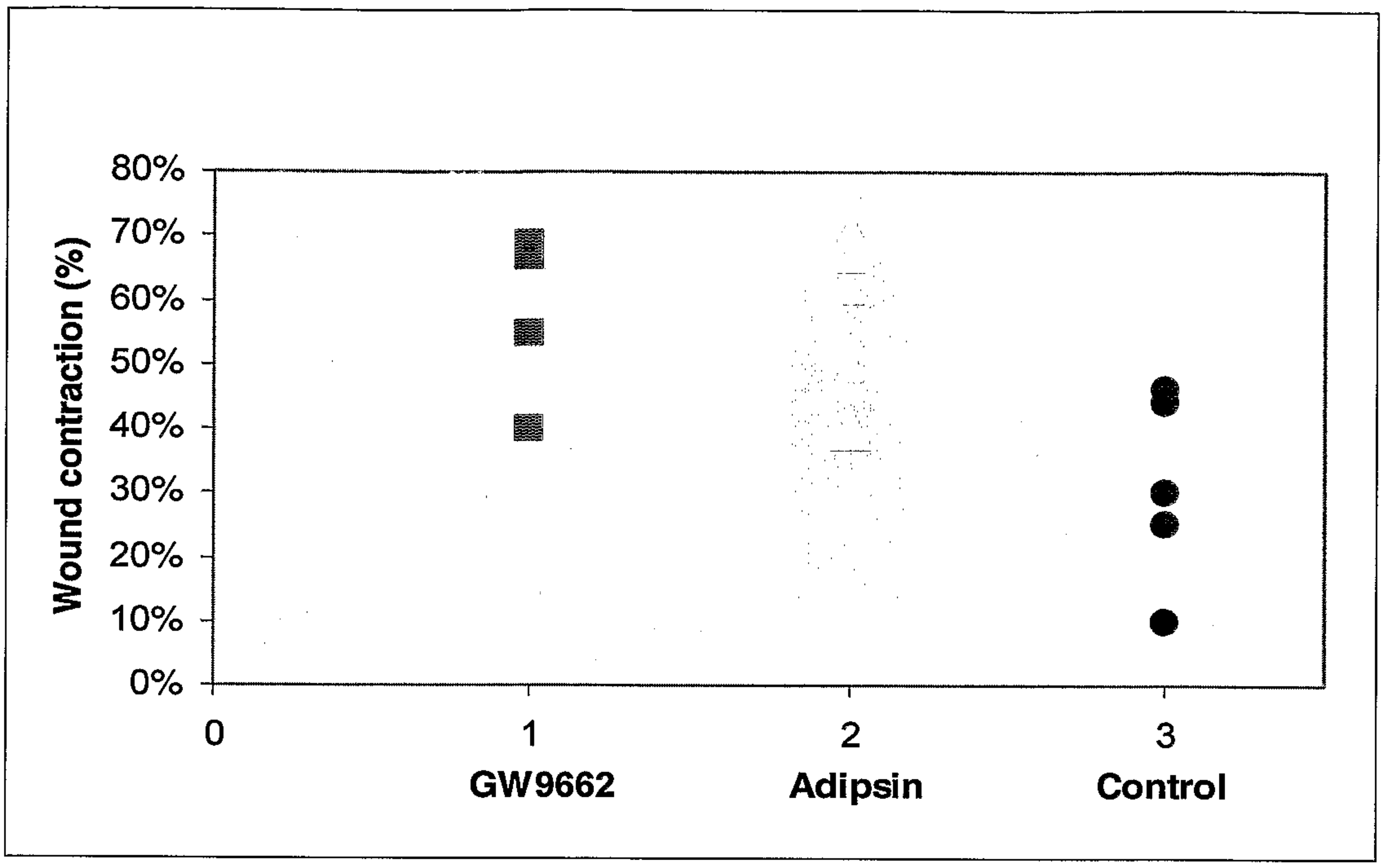
Wound contraction (%)
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
1
2
3
GW9662
Adipsin
Control

**Fig. 4**

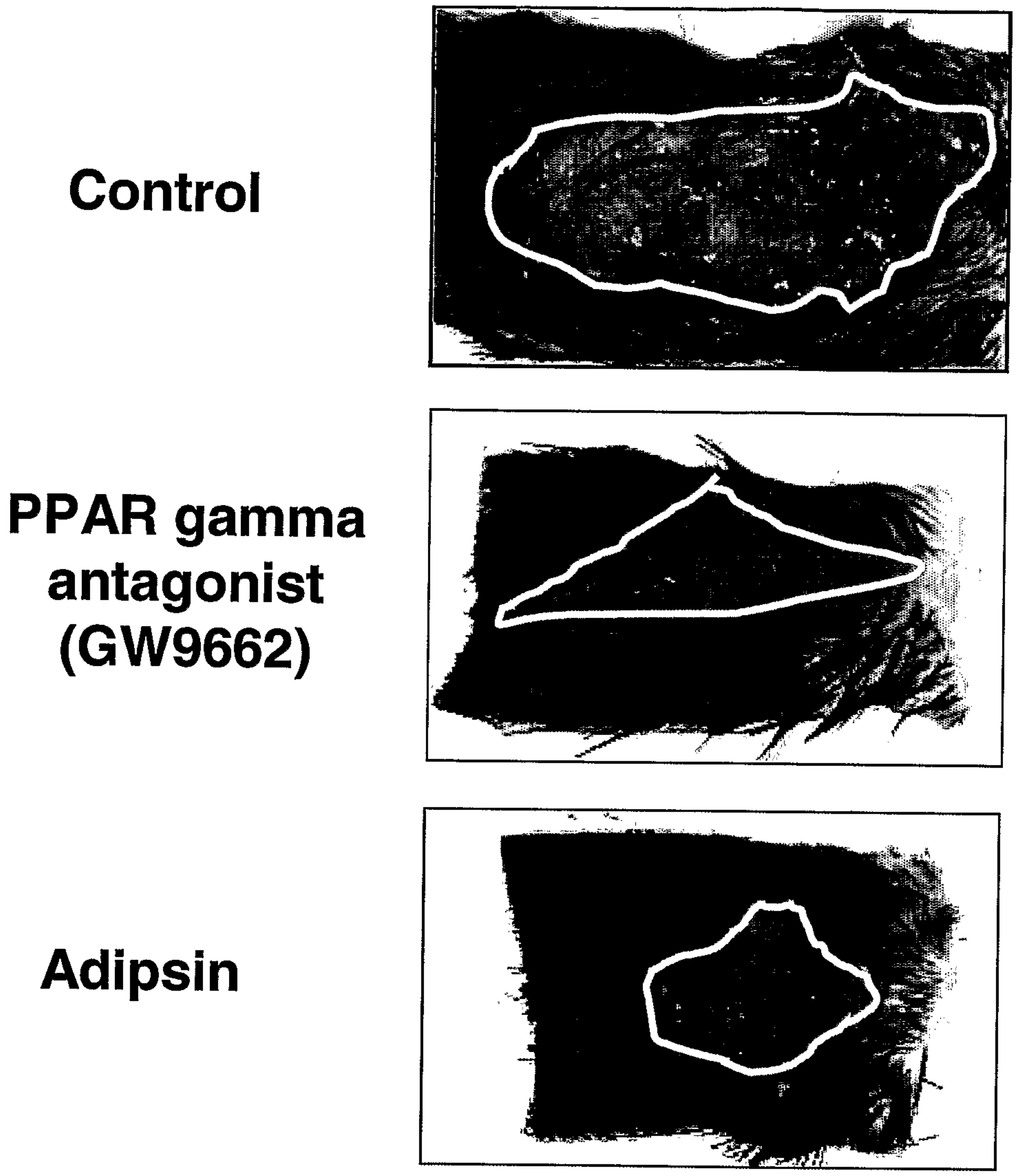


**Fig. 5**

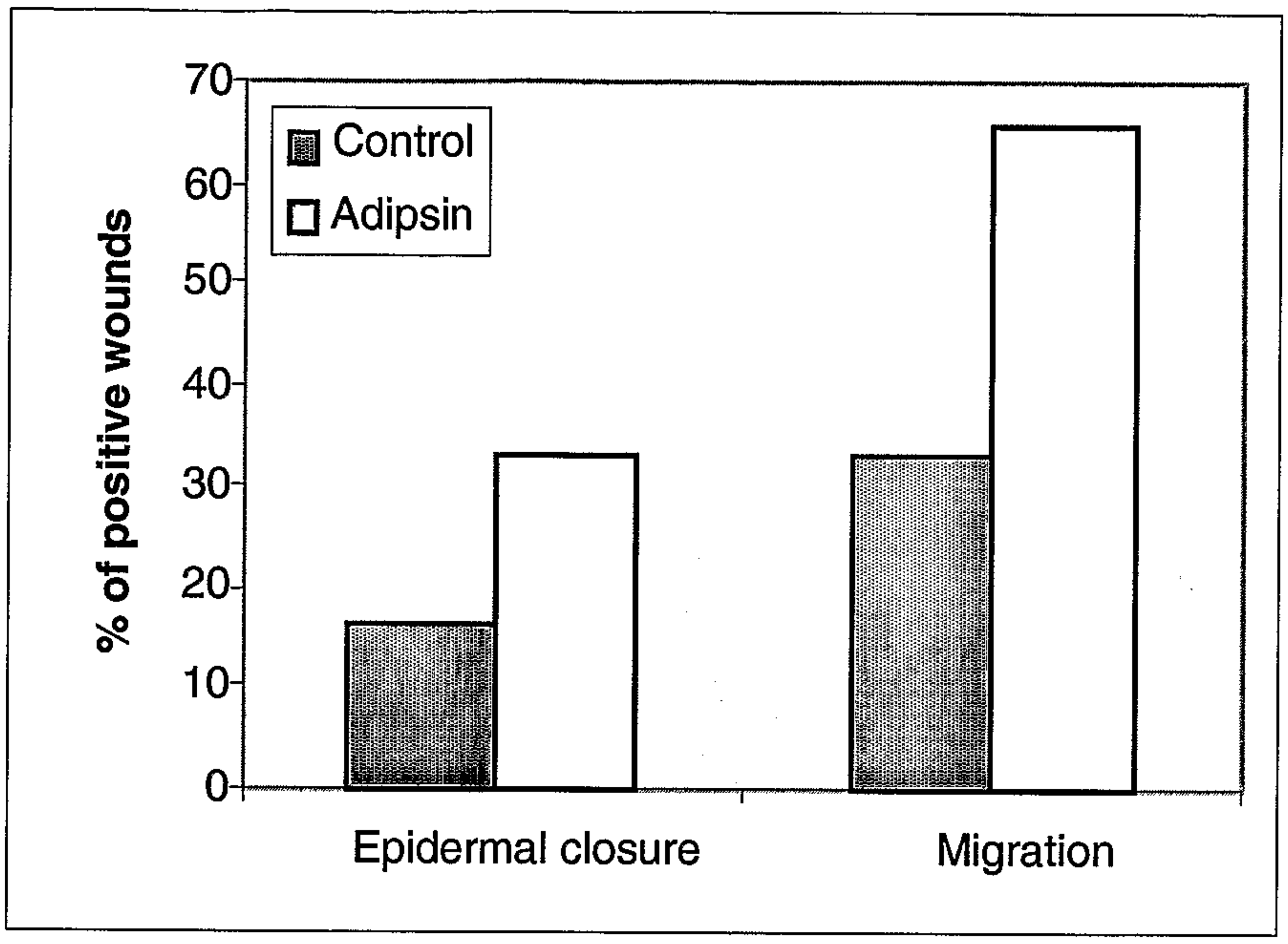
70
60
50
40
30
20
10
0
% of positive wounds
Control
Adipsin
Epidermal closure
Migration

**Fig. 6**

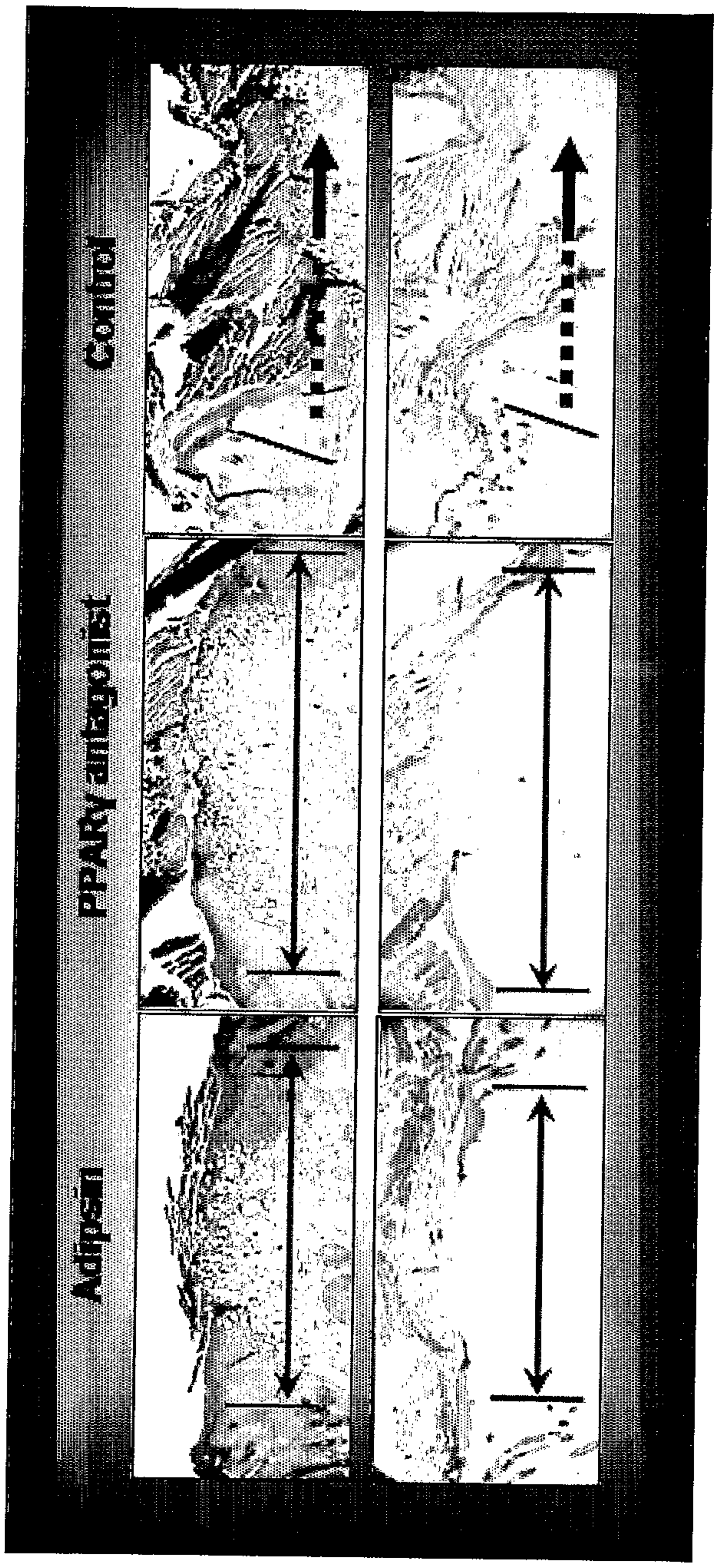


Fig. 7

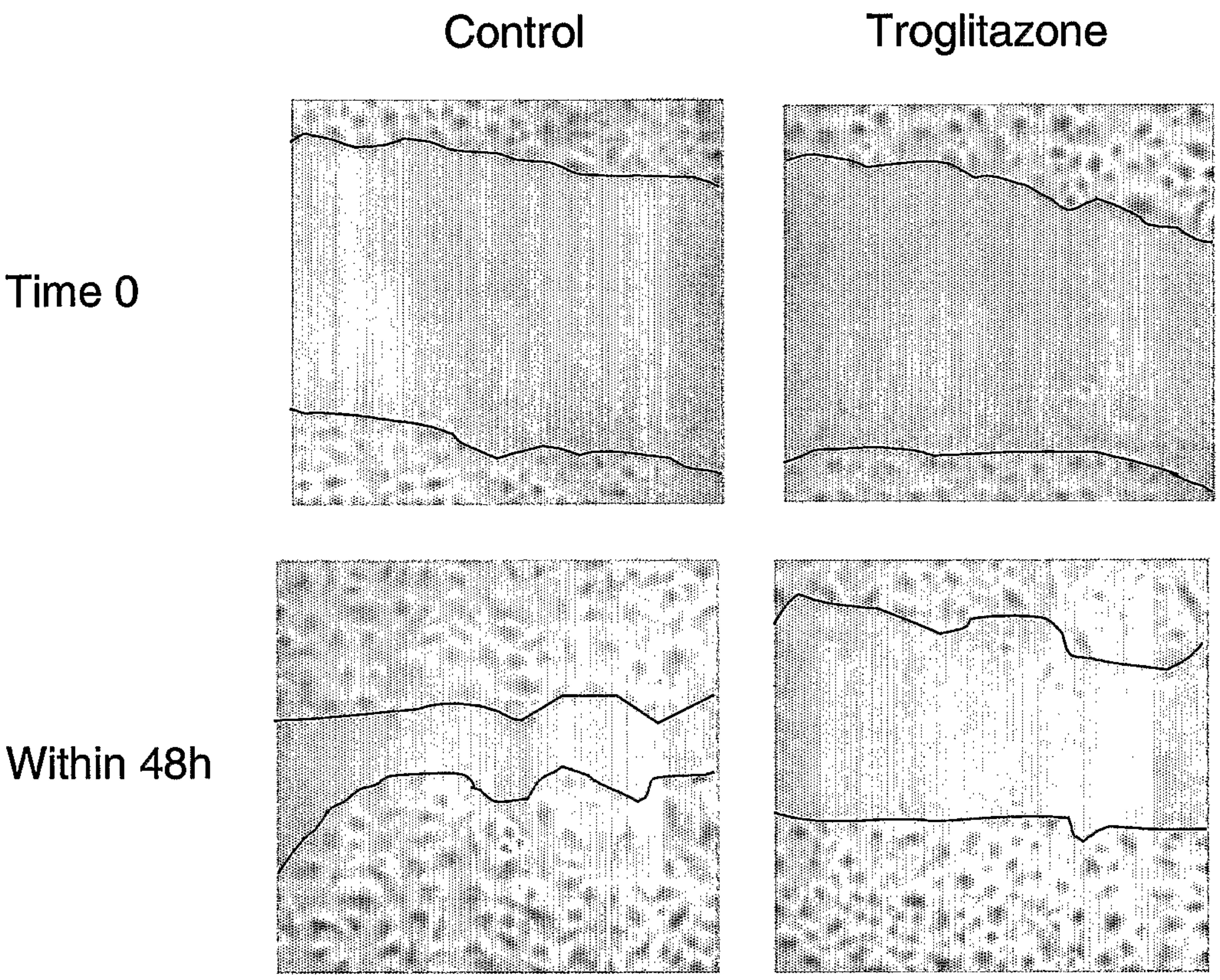


**Fig. 8**

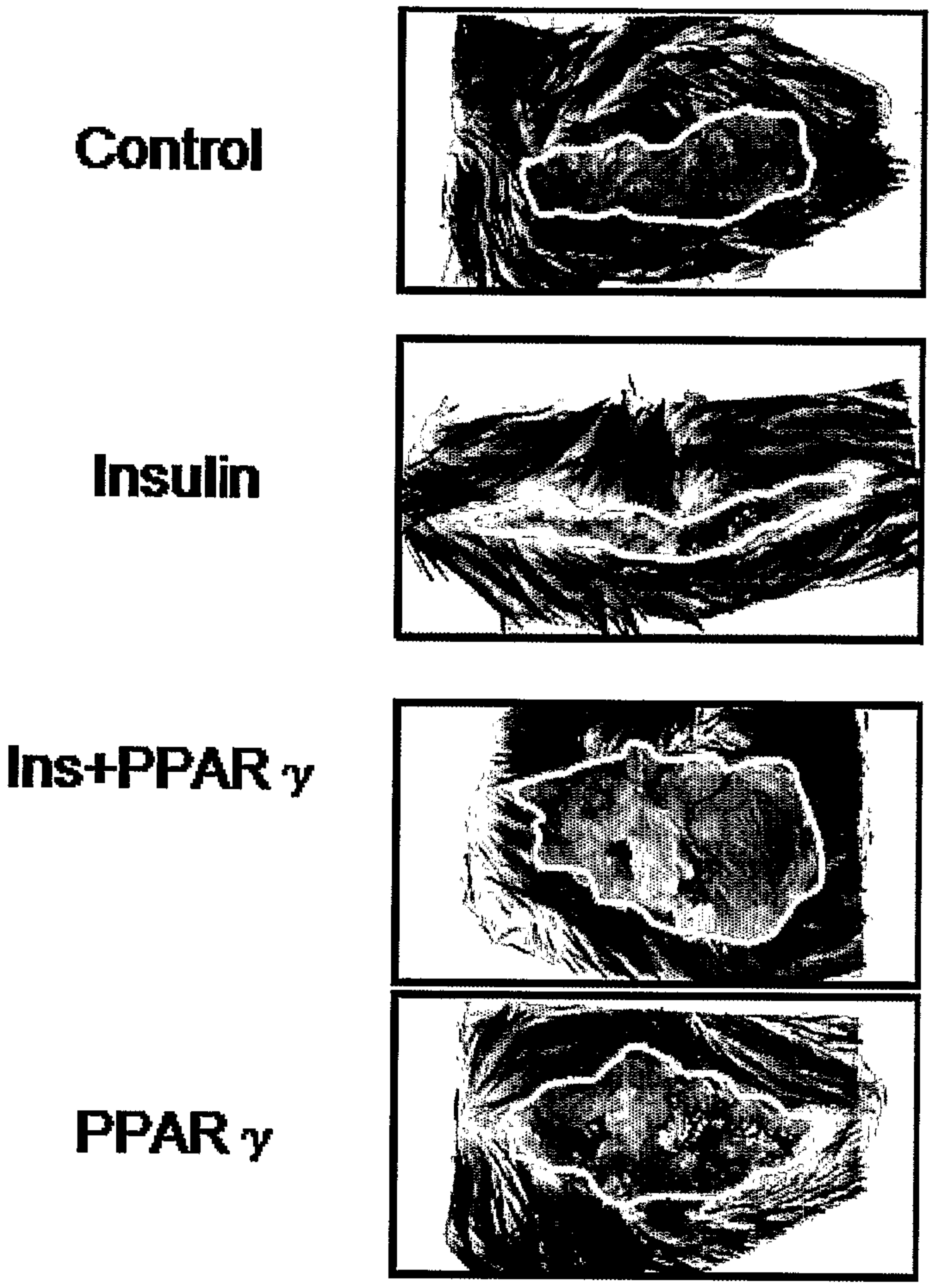


Fig. 9

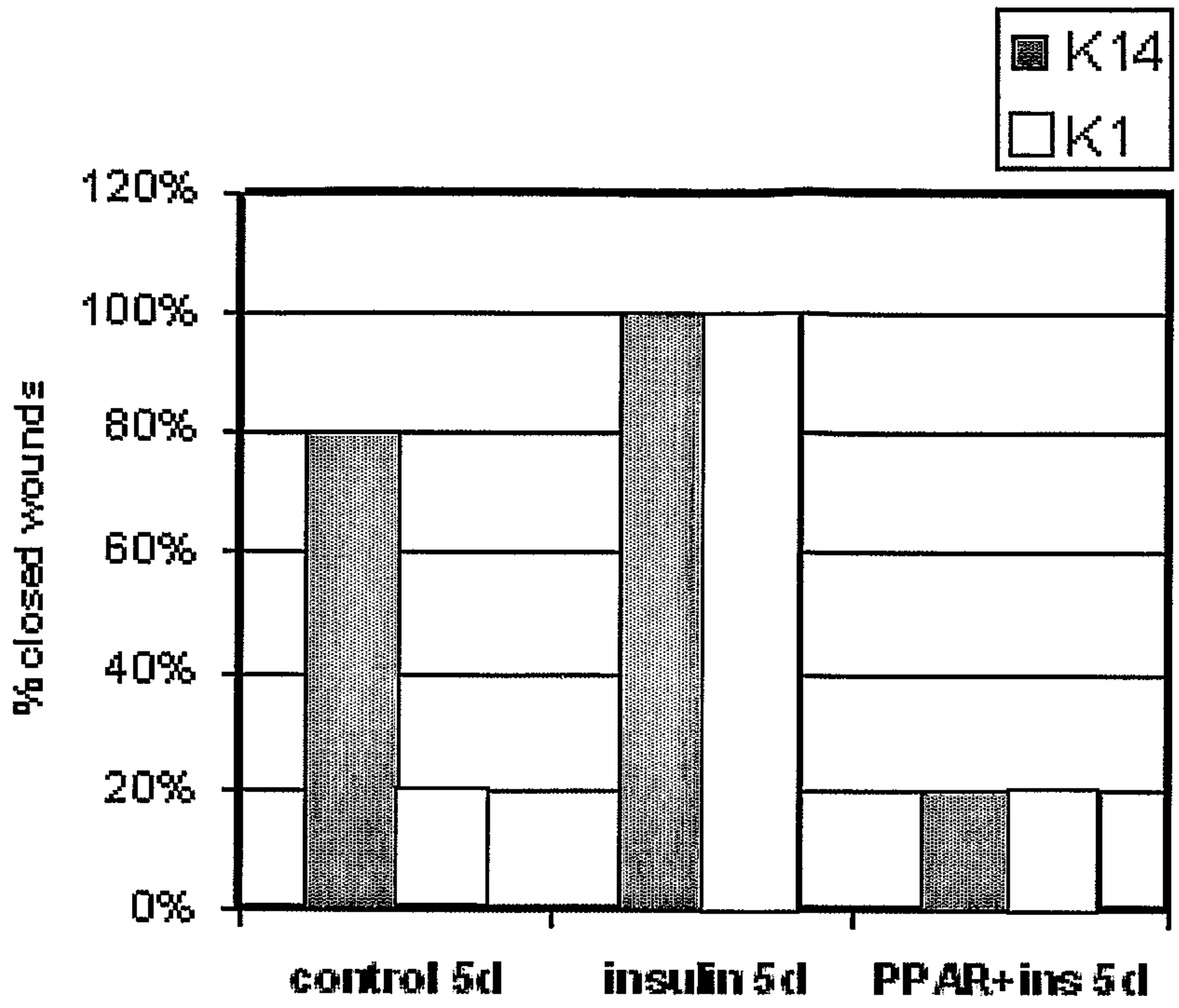
K14
K1
120%
100%
80%
60%
40%
20%
0%
% closed wounds
control 5d
insulin 5d
PPAR+ins 5d

**Fig. 10**