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PENNSYLVANIA COLLEGE OF PODIATRIC MEDICINE

August 4, 1992

Mrs. Alice Overton
Managing Editor, Journal of the American
Podiatric Medical Association
9312 Old Georgetown Road
Bethesda, Maryland 20814-1621

Dear Mrs. Overton:

I am submitting for your consideration our research paper entitled "Mannose-6-Phosphate: Anti-Inflammatory and Wound Healing Activity of a Growth Substance in Aloe vera" for the 1993 William J. Stickle Awards.

Our laboratory has previously shown the importance of Aloe in wound healing and inflammation reduction. This study attempts to define the role of the two major sugar constituents in the Aloe plant, mannose and glucose. We analyzed these sugars in the phosphorylated form. The significance of this study lies in the ability of the phosphorylated sugars to fit the growth factor receptors on the surface of the fibroblast.

We continue our research with Aloe vera with the hopes of providing podiatric physicians with effective natural alternatives for wound healing and inflammation.

Should you have any questions about the work, please let me know.

Sincerely,

Robert H. Davis

Robert H. Davis, Ph.D.

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Confidential

MANNOSE-6-PHOSPHATE: ANTI-INFLAMMATORY AND WOUND HEALING ACTIVITY OF A GROWTH SUBSTANCE IN *ALOE VERA*

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ABSTRACT

Aloe vera improves wound healing and inhibits inflammation. Since mannose-6-phosphate is the major sugar in the Aloe gel, we examined the possibility of it being an active growth substance. Mice receiving 300 mg/kg of mannose-6-phosphate improved wound healing over saline controls. This dose was also shown to have anti-inflammatory activity. The function of mannose-6-phosphate in *Aloe vera* was discussed.

The ability of an organism to activate the wound healing process effectively and promptly is essential for its survival. Wound healing, a necessary safeguard against longterm infection and subsequent death, has three major phases: inflammation, proliferation, and remodeling. After injury, fibroblasts migrate toward the wound site where they proliferate and produce collagen, elastin, and proteoglycans.[1] Proteoglycans form the ground substance in which collagen and other connective tissue fibers are embedded.[2,3] These substances remodel the connective tissue (see Figure 1). The movement of individual fibroblasts within the extracellular matrix produces the forces for tissue contraction and therefore wound healing.[1] An increase in fibroblast proliferation by platelet and mononuclear phagocyte products improves wound healing.[1]

Aloe vera is a complex plant containing many biologically active substances.[7] Evidence has shown that Aloe is effective in wound healing and inflammation reduction.[4-6] This is attributed to a growth factor-like substance in Aloe which activates the wound healing and inflammation reduction process.

The objective of this experiment is to determine whether mannose-6-phosphate (M-6-P) is an active ingredient in Aloe for wound healing and anti-inflammation. It will be important to understand the role of mannose-6-phosphate (a major constituent of the Aloe leaf) and if linkage to a protein is necessary to initiate a growth response. Is wound healing a solitary event of mannose phosphate or a combined effort with other cellular substances in Aloe such as glucose phosphate? Proposing a mechanism to explain how Aloe effects wound healing and inflammation will be instrumental in determining the most efficient way to use Aloe as a growth factor-like substance.

Cells in a wound communicate with each other through substances known as growth factors.[8] Growth factors are polypeptide hormones that are stored by most cells and are secreted into local tissues. Once the growth factor has been attracted to the wound area it binds to a cell surface receptor, usually that of a fibroblast. This sequence initiates the biological response, wound healing. The recent experiments by Huang and Huang (1988) have recognized that the intracellular interaction of growth factors with their receptors may be important in generating the biological effects of these growth factors.[9] Vladsky makes the link between the IGF-II receptor and fibroblast growth factor stimulation.[9] Evidence reveals that IGF-II and mannose-6-phosphate bind to different binding sites of the same receptor on the fibroblast.[10-14] This relationship makes key the stimulation of the fibroblast surface receptor by the mannose-6-phosphate located in the mucopolysaccharide of the Aloe plant. This evidence offers a possible link between mannose-6-phosphate and growth factor stimulation of the fibroblast.

MATERIALS AND METHODS

Wound Healing Assay

Adult male ICR mice (30 g, 15 animals/group) were anesthetized with ether and shaven on both sides of the back. A 6 mm wound was made on each side of the vertebral column. Anterior to posterior wound diameter measurements were made using a caliper on days 0,4, and 7. Mice received daily subcutaneous injections of a mannose-6-phosphate solution at dosages of 30, 150, and 300 mg/kg respectively. Control mice received daily injections of saline on a 10 mg/kg basis. Another group of animals was given a 150 mg/kg dose of glucose-6-phosphate to evaluate its effects on wound healing and inflammation. Glucose-6-phosphate served as a control for mannose-6-phosphate.

Ear Swelling Croton Oil Assay

Each mouse was given a 0.01 ml dose of croton oil (25 ug/ul) on day seven. The dose was applied topically to the right ear to induce inflammation. The left ear of each mouse was used as an internal control. After 6 hours, a sterile 6 mm biopsy punch was used to remove a specimen from each ear of every mouse. These specimens were then weighed using a Mettler balance. Standard errors for each mean value were calculated. The Student's "t" Test was used to determine significant differences between treatment and control groups. An analysis of variance was also calculated to determine the statistical significance of the study as a whole.

RESULTS AND DISCUSSION

Wound Healing

On day seven, control animals receiving saline demonstrated an average decrease in wound diameter of 2.8 mm (42.1%). The group receiving 30 mg/kg of mannose-6-phosphate had a average wound decrease of 3.1 mm (43.8%), while animals injected with 150 mg/kg had a wound diameter decrease of 3.7 mm (47.3%). Neither response is considered significantly different from the control group ($p>0.10$). Mice receiving a dose of 300 mg/kg of the mannose-6-phosphate had an average decrease of 4.3 mm (50.7%). This is considered significant ($p<0.01$). Animals that received the 150 mg/kg of glucose-6-phosphate had an average decrease of 3.1 mm (40.3%), which is not significant ($p>0.10$). These values are summarized in Table 1.

Inflammation

The average ear weight difference between the treated right ear and the control left ear of the saline control group was observed to be 7.3 mg. The glucose-6-phosphate group had a ear weight difference of 7.0 mg. The groups that were administered mannose-6-phosphate at doses of 30, 150, and 300 mg/kg were observed to have differences of 6.7, 5.6, and 5.5 mg respectively. The group that received the dose of 300 mg/kg is the only group considered to be significantly different from the control group ($p=0.05$).

Mannose-6-phosphate and glucose-6-phosphate are the main constituents of the polysaccharide chain in Aloe. Our experiments have shown that mannose-6-phosphate demonstrates wound healing and anti-inflammatory activity in a dose response fashion (Figures 2&3). Furthermore, we concluded that glucose-6-phosphate does not improve wound healing or reduce inflammation. Therefore, our evidence suggests that mannose-6-phosphate is a major structural constituent that stimulates wound healing and anti-inflammation. Our data may implicate a structural blueprint of the mucopolysaccharide in Aloe vera (Figure 4). This figure is a possible representation of a lock and key mechanism between the IGF-II/M-6-P receptor on the fibroblast and the active polysaccharide chain in Aloe.

Gowda has reported that there is approximately a six to one ratio of mannose to glucose in the Aloe polysaccharide chain.[15] We believe that the protein is non-covalently attached to the polysaccharide chain via a glucose binding site. The polysaccharide can dissociate from the protein in the same manner as it dissociates from the IGF-II/M-6-P receptor.

Current theory suggests that mannose-6-phosphate needs to be protein linked in order to yield a wound healing or an anti-inflammatory response.[16] Our data demonstrates that free mannose-6-phosphate effectively reduces inflammation and heals wounds. Our laboratory has previously shown Aloe vera extract improves wound healing and reduces inflammation.[4-6] A comparison of data would suggest that mannose-6-phosphate linked to a protein (thus forming a mucopolysaccharide) may yield even greater wound healing and inflammation reduction.

A Possible Mechanism of Action of M-6-P in Aloe

It is well established that IGF-II and M-6-P bind to the same receptor on the fibroblast.[10-14] These two ligands bind at separate binding sites within the IGF-II/M-6-P receptor.[9] However, the exact effect of these ligands binding to their individual binding sites is still unclear. There are several possible mechanisms of action of these ligands. One possible theory is that the binding of either ligand is capable of activating fibroblast proliferation. This would indicate that free M-6-P is a growth substance capable of yielding the same response as IGF-II. In Aloe, M-6-P is part of a polysaccharide chain which is attached to a protein. This may be important in understanding how Aloe produces its wound healing and anti-inflammatory activity.

Another possible theory of fibroblast activation by the binding of the ligands is that they work through cooperativity. Nolan reports that the binding of a ligand at one binding site is capable of influencing ligand binding at the other binding site of the same receptor.[9] It is therefore possible that the binding of M-6-P to its binding site preferentially increases the affinity of IGF-II to its binding site.[17,18] This would increase the rate of endocytosis of IGF-II. In this manner, IGF-II is delivered at a higher rate to the cells and thereby increases fibroblast activity and the wound healing response.

Further experiments are required to clarify the exact mechanism of the IGF-II/M-6-P receptor in yielding fibroblast activation. One possible experiment would utilize an antibody against the IGF-II binding site. In the past, research using a receptor antibody has been unable to identify the exact mechanism of ligand binding.

Nissley's group used an antibody that blocked the entire IGF-II/M-6-P receptor.[19] This not only blocked the binding of IGF-II to the receptor, but it also blocked M-6-P binding as well. They reported no decrease in autocrine growth. Nissley then concluded that the IGF-II/M-6-P receptor is not involved in autocrine growth. This experiment failed to clarify the IGF-II/M-6-P receptor mechanism for two reasons. First, the entire receptor was blocked and not the individual IGF-II binding site. Therefore, M-6-P was also blocked from binding to the receptor. Second, IGF-II is apparently capable of binding to cell surface binding proteins other than its receptor. Nolan reports this phenomenon.[9] Nolan and his associates found that cells lacking the IGF-II/M-6-P receptor still bound sufficient levels of IGF-II to yield a growth response. This may be the reason that Nissley found no reduction in autocrine growth.

An important experiment may be to produce an antibody specific for the IGF-II binding site within the IGF-II/M-6-P receptor. This antibody should also be specific for the cell surface binding proteins. If this antibody is successful in blocking all IGF-II binding sites, the exact role of M-6-P will become clear. If a growth response still occurs with M-6-P treatment, then M-6-P alone is capable of stimulating the fibroblasts and is a growth substance. If no growth response is observed, then M-6-P functions only to increase IGF-II binding and it does not directly stimulate fibroblast activation.

In summation, we are convinced that M-6-P in Aloe directly or indirectly stimulates fibroblast activation. Therefore, it is clear that M-6-P is an important factor in the wound healing process and plays a significant role in the biological activity of Aloe vera.

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TABLE 1. EFFECT OF MANNOSE-6-PHOSPHATE ON WOUND HEALING AND TOPICAL CROTON OIL INDUCED INFLAMMATION OVER 7 DAYS

EFFECT OF MANNOSE-6-PHOSPHATE ON WOUND HEALING AND TOPICAL CROTON OIL INDUCED INFLAMMATION OVER 7 DAYS

	DOSES (MG/KG x 7)	WOUND HEALING MM	WOUND HEALING % Dec.	EDEMA MG	EDEMA % Dec.
Saline	10	2.8 ± 0.2	42.1 ± 2.0	7.3 ± 0.8	———
Glucose-6-PO4	150	3.1 ± 0.2	40.3 ± 1.7	7.0 ± 0.8	4.1 ± 0.5
Mannose-6-PO4	30	3.1 ± 0.2	43.8 ± 1.9	6.7 ± 1.0	8.2 ± 1.2
	150	3.7 ± 0.3	47.3 ± 2.4	5.6 ± 0.7	23.3 ± 2.9
	300	4.3 ± 0.2	50.7 ± 1.6*	5.5 ± 0.7	24.7 ± 3.1**

* $p < 0.01$ ** $p = 0.05$

FIGURE 1. WOUND HEALING PROCESS

WOUND HEALING PROCESS

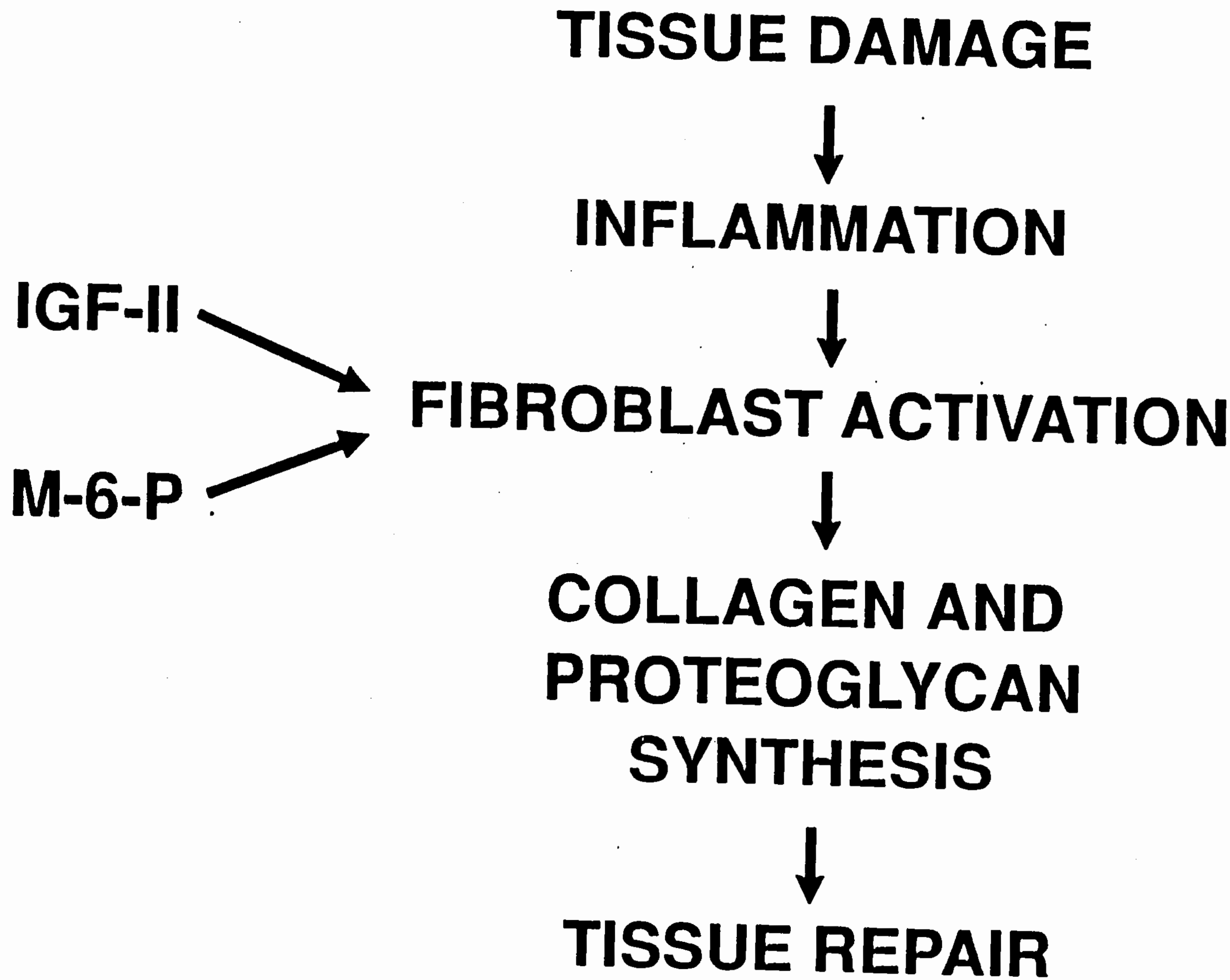

FIGURE 2. EFFECT OF MANNOSE-6-PHOSPHATE ON WOUND HEALING

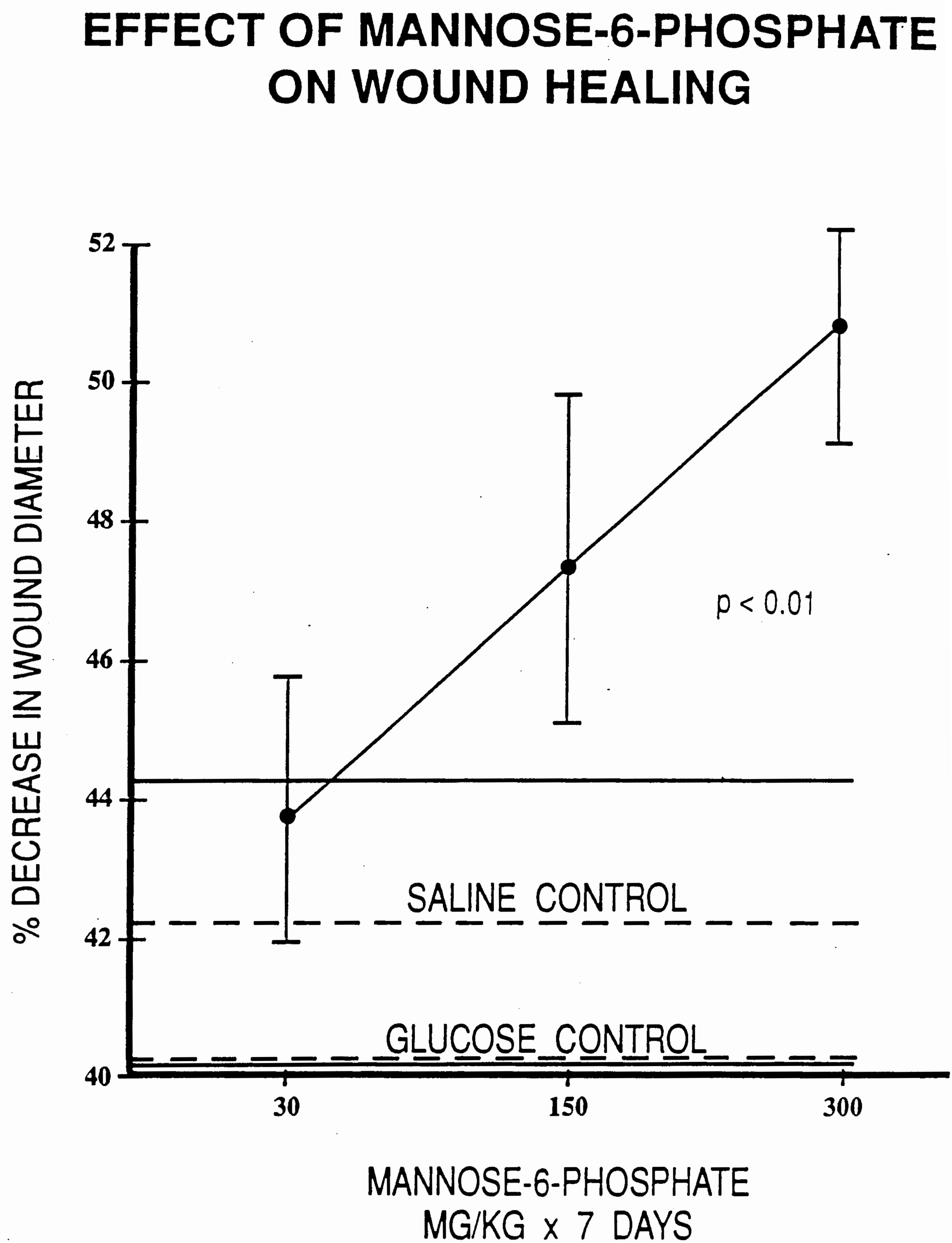
EFFECT OF MANNOSE-6-PHOSPHATE
ON WOUND HEALING
% DECREASE IN WOUND DIAMETER
52
50
48
46
44
42
40
p < 0.01
SALINE CONTROL
GLUCOSE CONTROL
30
150
300
MANNOSE-6-PHOSPHATE
MG/KG x 7 DAYS

FIGURE 3. EFFECT OF MANNOSE-6-PHOSPHATE ON TOPICAL CROTON OIL INDUCED INFLAMMATION

EFFECT OF MANNOSE-6-PHOSPHATE ON TOPICAL CROTON OIL INDUCED INFLAMMATION