

POLY ANTIOXIDANT MIXTURE ACCELERATES HEALING OF EXPERIMENTAL WOUNDS IN ALBINO RATS

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ABSTRACT

With an objective to develop a wound healing moiety which can promote healing process from its every step, a preclinical study was conducted to study the effect of combination of dietary antioxidant on the pace of wound healing in rats. Four circular cutaneous punch wounds of 8mm diameter were made on the dorsal surface of the rats. Mixture of dietary antioxidants containing vitamin A,C,E and β -carotene, 50 mg each were mixed in 10 ml corn oil and administered orally at the dose 1ml/100gm body weight once daily for seven days. In other set of experiment double concentration of mixture was prepared and applied topically (20 μ l/ wound) on exposed wound twice daily for seven days. Control were received only oil in an identical manner. The oral as well as the topical application of combo antioxidant have shown significant increase in wound area contraction, collagen content and tensile strength in regenerated wound tissues as compared to their respective controls. Further these antioxidant combo treatment have shown the greater degree of epithelization, fibrogenesis and vascularization in newly generated tissues as compared to untreated animal wounds. Different doses of few antioxidant present in this mixture were tested separately to evaluate their wound healing potency, slight wound healing potential were observed in few of them however the combination is working better These finding indicates that supplementation of these dietary antioxidant combination can form an adjunct pharmacotherapy for wound repair as it is promoting wound healing by synthesizing more collagen, more contraction in wound area, better epithelization, angiogenesis as well as tissues remodeling and cross linking phase as shown by high tensile strength.

Key words: Antioxidant mixture, wound healing, .hydroxyproline, tensile strength, wound closer

INTRODUCTION

The healing of wound involves complex, continuous and integrated events to reestablish the functional and structural integrity of the affected tissues. The wound repair process is comprises of inflammation, neovascularization, granulation tissue formation and matrix remodeling phase, which occurs in an organized and regulatory manner¹. These multi phase of repair process requires concerted action of many cell types as neutrophils, macrophages² The orchestrated series of events in which these cell types interacts includes the release of cytokines, growth factors and other bioactive molecules as free radicals³. These released substance play regulatory role in local process of wound repair. Antioxidants, the first line of defense against free radical damage and are crucial for health and well being have been reported as important factor during wound repair process and for impairment of healing process in certain pathological condition^{4, 5, 6}. Further, studies have been shown for improvement of wound healing process by the different natural products enhancing the insitu level of antioxidants^{7, 8, 9}. Since several decades studies has been shown for positive effect of certain dietary antioxidant^{10, 11, 12}, none of the studies has been performed to see the combinatorial effect of these dietary antioxidant and compared with their separate wound healing effect as these antioxidant beside being antioxidant are also governs the different important phases of wound repair process, as vitamin C is collagen vitamin, vitamin A good for epithelization as well as vitamin E is goods for scar less healing, β carotene is a pro-vitamin A. In the present investigation with aim to develop a moiety which can enhance healing of wound from its every phase, efficacy of combination of dietary antioxidant for wound healing process has been performed and compared with separate effect of these antioxidant in the wound healing

MATERIAL AND METHODS

Chemicals

All the antioxidants β carotenes, Vitamin A, Vitamin C and Vitamin E were obtained from Sigma chemical company St. Louis MO, USA. Chloramin-T and Ehrlich reagent for hydroxyproline estimation were obtained from Hi-media (Bombay) India and Sigma USA respectively.

Animals

Male Sprague-Dawley rats 150-180 gm were obtained from the National Laboratory animal center of institute.

The experiments were performed following the compliance of institute animal ethics committee

Wound formation

The animals were anesthetized using ether anesthesia and hairs on the dorsal surface of the rats were shaved off and skin was cleaned with 70% ethanol. An 8mm skin biopsy punch (Acuderm, Lauderdale, FL, USA) was used to create full thickness cutaneous wound under aseptic condition by following Werner et al 1994 13 method. Four such wounds, two on either side of dorsal line in each rat, were punched and animals were allowed to recover from anesthesia and were individually caged with free access to pallet diet and water.

Treatment Protocol

The animals for antioxidant mixture treatment were divided in to two groups.

The first group received antioxidant mixture orally 1ml/100 gm body weight once daily for seven days The second group received antioxidant mixture 20 μ l /wound topically on exposed wound twice daily for seven days.

The mixture suspension was prepared in corn oil. The 10ml mixture for oral treatment contains 50 mg of each vitamin while the 10ml mixture suspension for topical treatment contains 100mg of each vitamin. Respective control group animals were received only oil in identical manner

In the third set of treatment different doses of vitamin C, E and vitamin A (50,100,300 mg/kg P.O. X seven days) were separately given to wounded rat, 10 animals in each group. Control were received only oil in identical manner.

Wound Tissues Excision

Healed wound tissue excision was done on 7th day post wounding and complete seven days of treatment with antioxidant mixture, as well as separate antioxidant using same biopsy punch which specifically excises only the newly formed granulation tissues without any contamination with non wounded tissues, few of the sample were used directly for hydroxyproline estimation, while tissues sample for histological studies were kept in 10% buffered

formalin till further use. Skin tissue strips with centrally located regenerated tissues were excised for tensile strength measurement

Wound healing Assessment

Healing assessment was done by measuring hydroxylproline content (marker for collagen), wound contraction, tensile strength in regenerated tissues which indicates the strength in newly generated tissues, to substantiate the results histological observation were done for newly synthesized tissues.

Collagen Content

Hydroxyproline were measured as marker for collagen content by methods of Woessner (1961) (14) Briefly the excised granulation tissues were air dried in hot air oven and digested with acid to release the tissues bound hydroxyproline, released hydroxyproline were neutralized and then oxidized with chloramin-T. The oxidized hydroxyproline gives the colored complex with Eherlich reagent. The colour intensity was measured at 557 nm spectrophotometrically.

Wound Surface Area

Just before the excision of regenerated tissues, wound edges were traced on butter paper and the still open wound area was calculated

Tensile Strength Measurement

Skin strips (8mm width) with centrally located regenerated tissues (1cm skin flap at both end of central wound) were prepared. The tensile strength was measured by TKG-20 tensiometer. The total load required for breaking the regenerated tissues was expressed in Newton and tensile strength was calculated by dividing total load with cross section area of the tissues

Histological Examination

The excised wounds which were kept in buffered formalin were used for histological examination. After several steps of alcohol dehydration 4µm thick sections of paraffin embedded tissues were prepared using microtome and sections were stained with eosin and haemotoxyline, All the slides were evaluated by pathologist in double blind manner under microscope for epithelization, fibrogenesis and vascularization.

Statistical Analysis

Results are expressed as mean±S.E.M., Student's t-test .was used for statistical analysis. P< 0.05 was considered as significant.

RESULTS

Effect of Antioxidant mixture treatment on hydroxyproline content

Hydroxyproline, a marker for collagen was estimated in the wound tissues after seven days treatment with antioxidant mixture. The average value for Hydroxyproline in the excised wound tissues of orally treated animals was found to be 59.5±1.26 mg / gm of dry wound tissues. The control group of animal showed 41.0±1.7 mg hydroxyproline. Topically treated animal wound content 58.0±3.1 mg as compared to their control group 41.0±2.5 mg/ gm of dry wound Fig.1. Therefore it appears that collagen synthesis has been more pronounced following the treatment with antioxidant mixture.

Effect of antioxidant mixture treatment on wound size

Significant reduction in wound size was observed in the treated group as compared to their respective control group animals. The wound area was markedly reduced (24.0± 0.9 mm²) in the group treated with antioxidant mixture orally. The control group showed on an average the wound size of 40.0± 02.5 mm². The topical application of antioxidant mixture also resulted in significant contraction of wound measuring 24.0± 01.5mm² as compared to control 38.0±1.5mm (Fig.2).

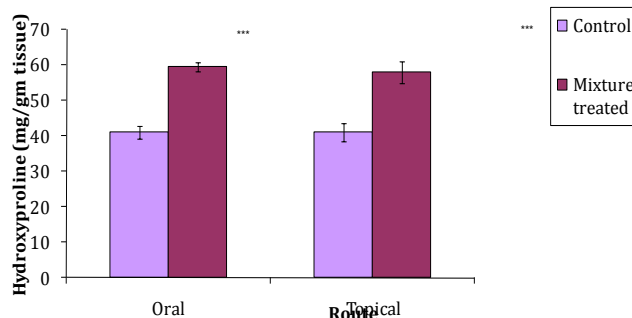


Fig. 1: Effect of antioxidant mixture treatment on hydroxyproline content

Values depicted are mean+SEM ; n=15 rats in each

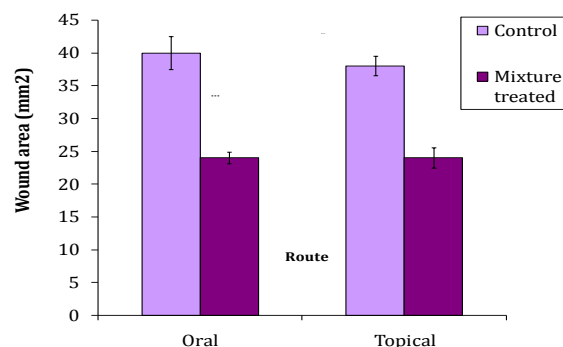


Fig. 2: Effect of antioxidant mixture treatment on wound area in rats

Effect of antioxidant mixture on tensile strength

In general the tensile strength of newly synthesized tissues were significantly enhanced following antioxidant mixture treatment by both route (Fig.3). On topical treatment as well on oral application of antioxidant mixture on an average about 30% increase in tensile strength was noticed. The relative values 11.3±0.55 and 11.6±0.23 N/cm² respectively as compared to control value 8.4±0.22 N/cm²

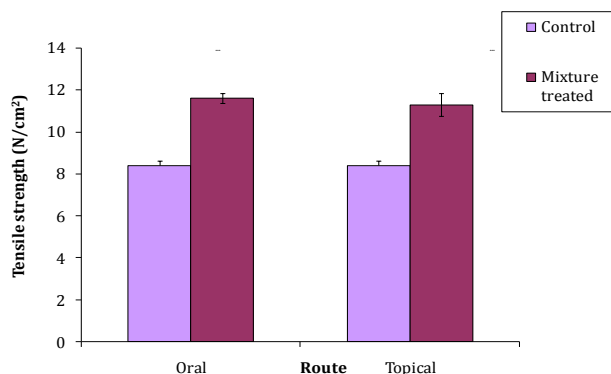


Fig. 3: Effect of antioxidant mixture treatment on tensile strength in rats

Values depicted are mean±SEM; n=15 rats in each group;

Effect of antioxidant mixture on histological pattern of wound tissues

Histological wound tissue observation in haemotoxyline and eosin stained 6µm thin sections were analyzed in both the treated and control groups. Wounds harvested from animal treated either topically or orally with antioxidant mixture demonstrated greater degree of epithelization, collagen deposition and enhanced neovascularization compared to respective controls Fig. A,B,C,D).

Figure - 4

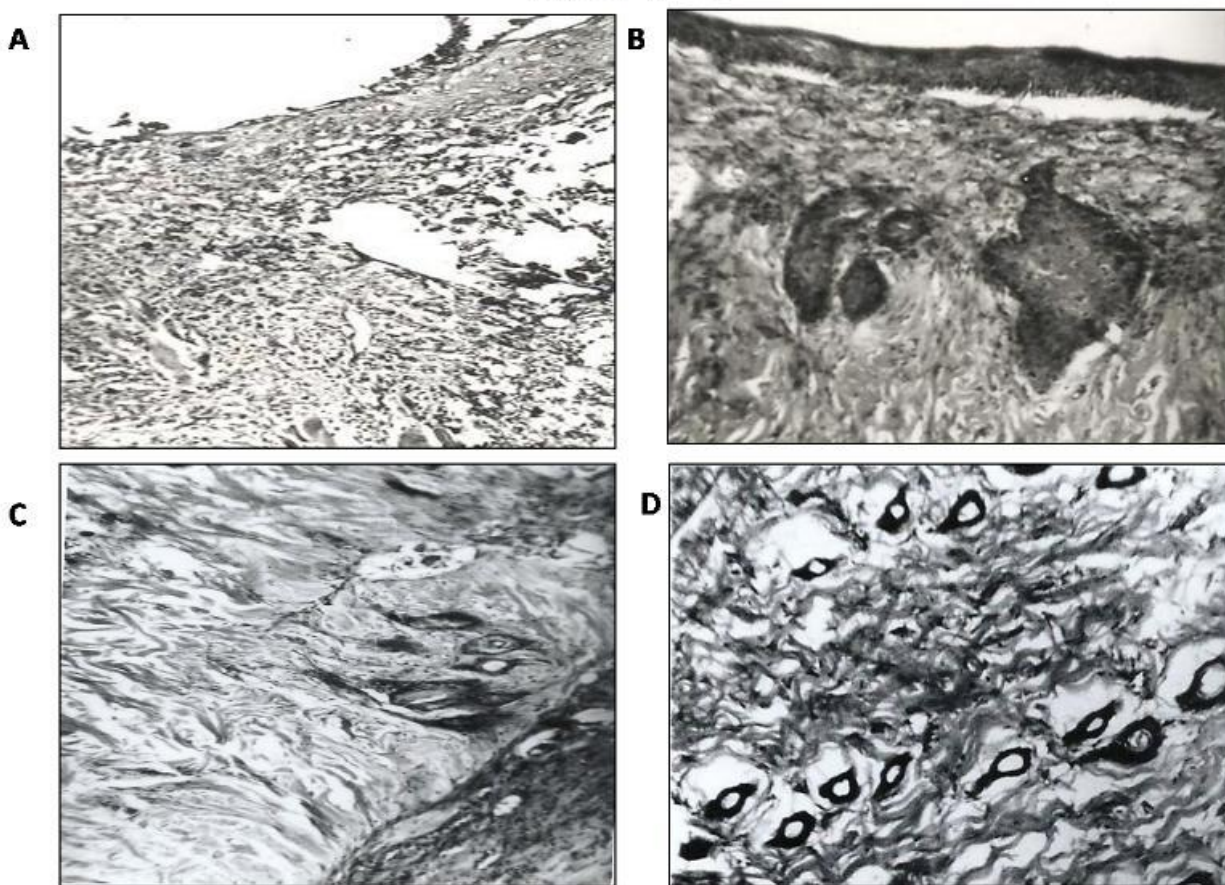


Fig 4: Histological patterns of haematoxyline and eosin stained regenerated wounds tissues sections (6µm thick) (A) Control rat wound showing non epithelized wound surface. (B) Antioxidant mixture treated wound showing wound surface with well organized thick epithelium.(C) Antioxidant mixture treated rat wound showing fibroblast ingrowth, collagen alignment (D) Antioxidant mature treated rat wound showing angiogenesis (new blood vessel formation).

Effect of Different dose of Vitamin C, E and A on wound healing parameters

Wound healing parameter were assessed in rats treated separately with different doses of vitamin C, E and A. Animal treated with 100 and 300 mg/kg dose of vitamin C were showing significant increase in collagen content and slight increase in tensile strength.

However none of the vitamin E treated animal are showing increase in collagen content though the 100, 300 mg/kg P.O. dose are showing better wound closer. The Vitamin A treated animal were showing slight increase in collagen content as well as better wound closer as compared rats.(Table-1)

DISCUSSION

Wound healing is a complex biological process which involves cascade of cellular events as inflammation, granulation tissue formation, angiogenesis, matrix remodeling and epithelization, which are governed by concerted action of many cell type and can be modulated or affected by certain bioactive molecules as free radicals, growth factors and cytokines, there are many work which have show to promote healing of wound by enhancing any of these factor in the healing process and hence the total outcome as the healed wound. In the present investigation we have tried an unique combination of dietary supplement which enhances the healing process from its every phase. Oral as well as topical application of antioxidant mixture significantly enhanced the collagen formation as it is evident from significant increase in hydroxyproline level as

compared to control wounds. This might be mainly due to the presence of vitamin C as it has been reported to be required for collagen biosynthesis (17, 18). Addition of ascorbic acid have also been shown to increases the collagen production in human skin fibroblasts (19).The increased content of wound collagen correlates with increasing tensile strength (10) Better epithelization seen after antioxidant mixture treatment may be attributed to presence of vitamin-A as it is established epithelial growth promoting vitamin(21, 22). Better healing in treated group is may be due to presence of vitamin E which is reported to be beneficial for UV damaged skin (23, 24, 25) and have shown to accelerate dermal as well bone healing by enhancing the angiogenesis and reducing the scar formation (26).The β carotene part of antioxidant mixture is being helpful due to its efficient antioxidant and anti-inflammatory effect (27) as free radicals are known to impairs the healing of wounds (5) Similar to our finding where combination of antioxidant are better for wound healing as compared to single use of antioxidant, studies have been reported for the combined application of various antioxidant for more potent photo protective as compared with their alone use(28). Further our finding lend strong support for the study that depletion of antioxidant delays healing process, it seems this combination which have supplement to help every steps of healing working well synergistically to enhance the healing outcomes, the obvious reason being that mixture containing combinations of substance which promotes healing process at various phases. Further in view of beneficial effect of this dietary combination of antioxidant on cutaneous wound repair it may be clinically exploit as adjunct pharmacotherapy.

Table 1: Showing wound healing potential of different oral doses of Vitamin C, E and vitamin A.

TREATMENT DETAIL	HYDROXYPROLINE (mg/kg dry wound)	WOUND AREA (mm ²)	TENSILE STRENGTH (N/cm ²)
Control	41.0±2.5	28±2.3	8.4±0.19
Vitamin C (P.O. X 7 day)			
50 mg/kg	44.16±1.95	25.0± 3.5	8.9 ±0.32
100mg/kg	52.6± 2.2*	23.95± 2.9	9.0± 0.29
300mg/kg	51.95± 1.95	20.0± 1.5*	9.3 ±0.31#
Vitamin E (P.O. X 7 day)			
50 mg/kg	39.5± 3.2	24.5± 3.1	8.4 ±0.23
100 mg/kg	40.33 ±1.5	22.9± 2.2#	8.5± 0.29
300 mg/kg	46.13± 2.2	20.0 ±2.2**	8.9± 0.25
Vitamin A (P.O.X 7 day)			
50 mg/kg	42.0 ±0.53	19.0 ±2.5**	8.3 ±1.5
100mg/kg	49.25± 0.12	19.5 ±0.24**	8.95±± 0.23
300 mg/kg	50.19± 1.98*	18.3± 0.12*	8.99 ±1.5

Values are mean ± SEM, n=10 animals; * p< 0.05, # p< 0.02, ** p < 0.01

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