BIOCHEMICAL ANALYSIS OF GRANULATION TISSUE IN STEROID AND *CENTELLA ASIATICA* (LINN) TREATED RATS

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Summary

The present study was undertaken to investigate the effect of alcoholic extract of *Centella asiatica* Linn in normal and steroid suppressed healing in dead space wound model in rats. Albino rats of either sex were divided into 4 groups. Group 1: Control group with wound alone; Group II: Test group with wound and treated with extract; Group III: Test group with wound and treated with steroid; Group IV: Test group with wound treated with steroid and extract. Granulation tissue was created by making dead space wound and used for analyzing wound healing relevant biochemical parameters such as hydroxyproline, glycosaminoglycan content, tissue protein, lysyl oxidase and antioxidant profiles. Significant increase in hydroxyproline and glycosaminoglycans content in granulation tissue were observed. The significant elevated levels of antioxidants, protein and lysyl oxidase concentrations were observed in extract treated rats. Present results indicated that the leaf extract promotes wound healing significantly and able to overcome the wound healing suppressing action of steroid.

Keywords: *Centella asiatica***,** granulation tissue, dead space wound.

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Introduction

Centella asiatica Linn. commonly known as Indian pennywort. This plant is very useful for wound healing, ulcer protection, antimicrobial action. Both topical and oral administration of the plant extracts have been shown to accelerate wound healing[1]. Pharmacological studies with the extracts of this plant are found to have sedative, antidepressant, analgesic and anticonvulsive effects[2]. *Centella asiatica* L is reputed for its medicinal use in chronic and obstinate eczema, psoriasis, syphilis and leprosy[3]. Titrated extract of *Centella asiatica* contains three principal ingredients asiaticoside, asiatic acid and madecassic acid are known to be clinically effective on systemic scleroderma, abnormal scar formation and keloids[4] and also this extract significantly shortens the wound healing time, acting more specifically on the immediate process of healing[5]. The most beneficial effect of the ingredients appears to be the stimulation of maturation of scar by the production of type I collagen and resulting decrease in the inflammatory reaction and myofibroblast production[6]. Centella triterpenes evoke a gene expression response consistent with their prevailing medical uses in the treatment of connective tissue disorders such as wound healing and Microangiopathy[7]. Asiaticoside, isolated from *Centella asiatica* is the main active constituent, exhibits significant wound healing activity in normal as well as delayed healing models[8].

In recent years oxidative stress has been implicated in a variety of degenerative processes and diseases. These include acute and chronic inflammatory conditions such as wound healing[9]. Oxygen free radicals play a important role in the failure of ischemic wound healing and antioxidants improve the healing in ischemic skin wounds[10].

Anti-inflammatory glucocorticoids markedly affect most aspects of wound healing. When administered sufficiently early after injury, high steroid level delay the appearance of inflammatory cells, fibroblasts, the deposition of ground substance, collagen, regenerating capillaries contraction and epithelial migration[11]. Dexamethasone is a very potent anti-inflammatory glucocorticoid used in organ transplantation and skin allografts[12]. Glucocorticoids are known to suppress wound healing[13,14] . Dexamethasone treatments strongly interfere with both the synthesis and degradation of type I and type III collagen[15]. It is also a potent transcriptional inhibitor of human type VII collagen promoter activity in dermal fibroblasts, which leads to decreased anchoring of fibril formation[16]. Till today, there are not many agents which are able to successfully overcome the antihealing effects of corticosteroids. The present study was undertaken to investigate the effects of ethanol extract of *Centella asiatica* L on the different parameters of wound healing alone and in the presence of dexamethasone induced suppression of wound healing in rats.

Material and Methods

Plant material

Leaves of *Centella asiatica* were collected from the local areas of Udupi district, Karnataka, India, and were authenticated by Professor Gopalkrishna Bhat, Department of Botany, Poorna Prajna College, Udupi. A voucher specimen (No.pp525) was deposited at the Department of Pharmacognosy, College of Pharmaceutical Sciences, Manipal, India.

Preparation of ethanol extract

Leaves of *Centella asiatica* were dried in shade and powdered. The powder (75g) was extracted with 700ml of 95% ethanol in a soxhlet apparatus at 60°-75°C and concentrated. The yield was 10 –15%. The extract was stored in the refrigerator.

Animals

Healthy albino rats of either sex and of approximately the same age, weighing between 150-250g were used for the study. They were individually housed, maintained in clean polypropylene cages and fed with commercially pelleted rat chow (M/s Hindustan Lever Ltd. Mumbai) and water *ad libitum*. The experimental protocol was subjected to scrutiny of Institutional Animal Ethical Committee for experimental clearance (No.IAEC/KMC/UA/2000).

The animals were divided into 4 groups of 6 animals each.

Group I: Animals served as wounded control, treated with normal saline.
Group II: Animals were daily administered the extract of *Centella asiatica* via intragastric tube at a dose of 800mg/kg body weight for 10days.
Group III: Received steroid (dexamethasone) in the dose of 0.3mg/kg,im; full dose on the day of operation and half the dose thereafter on alternate day for 10 days.
Group IV: Animals were received dexamethasone along with the extract of *Centella asiatica* for 10 days.

Experimental procedure

Dead space wound – The wounding procedures were carried out using ketamine (1ml/kg body weight) anaesthetized rats. These wounds were created by implanting two polypropylene tubes (0.5cm X 2.5cm each), one on either side in the lumbar region on the dorsal surface of each rat. On the 10th post-wounding day, the granulation tissue formed on the implanted tubes was carefully dissected out and weighed[17]. These granulation tissues were collected, dried at 60°C for 24hr and weighed and the weight was noted. The dried granulation tissue acid hydrolysate was prepared and then utilized for the estimation of hydroxyproline[18], hexosamine content[19] and hexuronic acid[20].

Granulation tissue from the other tube was collected in phosphate buffer saline for the estimation of antioxidant enzymes like Superoxide dismutase (SOD)[21], Catalase[22], reduced glutathione[23] and tissue lipid peroxidation[24]. A portion of the wet granulation tissue was used for the estimation of lysyl oxidase[25] and tissue protein[26].

Statistical Analysis

The results were analyzed using one way analysis of variance (ANOVA) with post hoc Scheffe's test. *P* values <0.05 were considered statistically significant.

Results

Hydroxyproline concentration was significantly elevated in the extract of *Centella asiatica* treated group and decreased in the case of dexamethasone treated group. The decreased hydroxyproline concentration reversed in the group IV, where the dexamethasone treated along with the extract (Table 1).

Table 1 – Effect of alcoholic extract of *Centella asiatica* in absence and presence of dexamethasone in dead space wound model. [Values are mean ± SD of 6 replications]

Treatment	Hydroxyproline (mg/g tissue	Hexosamines (mg/g tissue)	Hexuronic acid (mg/g tissue)	Tissue protein (mg/g tissue)	Lysyl oxidase (SFU)
Wounded control	14.72±4.02	10.49±2.37	12.11±3.09	41.58±3.8	1711±69
C.asiatica	46.38±6.1^{a}	28.1±6.2^{a}	29.5±5.32^{a}	67.5±6.5^{a}	3318±79^{a}
DM	10.35±3.65^{cx}	9.2±2.29^{x}	10.35±3.63^{cx}	32.16±3.59^{bx}	1556±102^{x}
DM + *C.Asiatica*	19.5±5.5^{xp}	23.6±3.4^{zp}	21.5±4.8^{yp}	51.88±2.4^{yr}	2428±76^{xr}

(SFU- Spctroflourimetric units)

P **values: ^{a}:<0.001,^{b}:<0.01,^{c}:<0.05 vs control; ^{x}:<0.001,^{y}:<0.01,^{z}:<0.05 vs *Centella asiatica;* and ^{p}:<0.001,^{r}:<0.05 vs dexamethasone**

Glycosaminoglycan contents like hexuronic acid and hexosamine concentration was significantly increased in the extract treated group and decreased in the dexamethasone treated group compared to control. There was significant increase in the glycosaminoglycan content in the extract treated along with the dexamethasone when compared to dexamethasone treated group. Tissue protein concentration was maximum and significantly increased in the case of extract treated group compared to control and decreased significantly in the case of dexamethasone treated group. This decreased protein concentration was reversed in the case of dexamethasone along with the extract treated group. Lysyl oxidase level was significantly elevated in the extract treated group compared to control group and decreased significantly in dexamethasone treated group. There was significant elevation in the enzyme level in the extract treated along with dexamethasone when compared to dexamethasone treated group (Table 1).

Antioxidant levels were significantly increased and lipid peroxidation was decreased in the case of extract treated group compared to control group. In the dexamethasone treated group there was significant decrease in the antioxidant levels and increased lipid peroxidation was observed and this effect of the steroid was significantly reversed in the presence of extract (Group IV) (Table 2).

Discussion

The results of the present study clearly demonstrate that the alcoholic extract of *Centella asiatica.* possesses a definite prohealing action. An increase in hydroxyproline content of treated wounds may be due to increase in collagen concentration and stabilization of fibers[27]**.** The glycosaminoglycans are a major component of the extracellular matrix of skin, joints, eyes and many other tissues and organs. In spite of its simple structure it demonstrates remarkable viscoelastic and hygroscopic properties which are relevant for dermal tissue function. Due to an influence on signalling pathways, hyaluronic acid is involved in the wound healing process and scarless foetal healing. In clinical trials topical application of hyaluronic acid has improved the healing of wound[28]. In addition the mucopolysaccharide hyaluronic acid protects granulation tissue from oxygen free radical damage and thereby stimulates wound healing[29].

The process of maturation of collagen fibrils is catalyzed by the enzyme lysyl oxidase. Lysyl oxidase is the enzyme involved in the formation of cross-links, therefore play a very important role in the maturation process and in wound healing. The increased level of the enzyme activity may result in increased cross linking which leads to concurrent increase in the tensile strength of wounds.

The vascularity and the degree of inflammatory cells were more intense and hydroxyproline levels were significantly low in the dexamethasone treated group[30] .The daily administration of dexamethasone for a week inhibited nitric oxide synthesis and significantly increased the MDA levels in a dose dependant manner on tracheal anastomotic healing[31].

Table 2 – Effect of alcoholic extract of *Centella asiatica* for antioxidant parameters in absence and presence of dexamethasone in dead space wound model. [Values are mean ± SD of 6 replications]

Treatment	SOD U/mg protein	Catalase k/sec/mg protein	Reduced glutathione ugm/mgprotein	Lipid peroxidation-MDA in nm/mgprotein
Wounded control	1.60±0.61	0.008±0.0065	0.014±0.0047	0.0661±0.02
Centella asiatica	4.91 ± 1.1^{a}	0.412 ± 0.18^{a}	0.409 ± 0.052^{a}	0.016 ± 0.009^{a}
Dexamethasone	0.91 ± 0.23^{ax}	0.00361 ± 0.00031^{ax}	0.006 ± 0.0015^{ax}	0.0871 ± 0.04^{x}
Dexamethasone + *Centella asiatica*	2.01 ± 0.71^{xp}	0.148 ± 0.09^{xp}	0.048 ± 0.012^{xp}	0.058 ± 0.016^{x}

***P* values: ^{a}:<0.001,^{b}:<0.01,^{c}:<0.05 vs control; ^{x}:<0.001,^{y}:<0.01,^{z}:<0.05 vs *Centella asiatica;* and ^{p}:<0.001,^{r}:<0.05 vs dexamethasone**

An increase in the levels of antioxidant enzymes was observed in granulation tissue. These enzymes are known to quench the superoxide radical and thus prevent the damage of cells caused by free radicals[32]. Antioxidant status in the dexamethasone treated group was very poor and there was increased MDA level in dead space wound granulation tissue. It suggests that bad wound healing status in dexamethasone treated animals may be due to imbalance in the oxidant and antioxidant systems. Dexamethasone has down regulated the expression of intracellular adhesion molecule (ICAM-1), which is important in the migration of leucocytes from the circulation to the wound site, and significantly impairs the healing of intestinal anastomosis in rats[33]. Steroids inhibit the fixation of sulfate in glycosaminoglycans and thus prevent the synthesis of chondroitin sulfates, which are rapidly synthesized in the healing wound[34]. Glucocorticoids are also known to suppress the fibroblast proliferation. In our study, we also found that dexamethasone enhance the catabolism of proteins, as evidenced by decrease in protein content which has resulted in the reduction in mass of granulation tissue. The effect of decreasing the concentration of protein in the granulation tissue was reversed by the addition of *Centella asiatica* extract. The plant extract antagonized the action of dexamethasone, mainly in collagen synthesis, maturation. Thus it has the potential for antagonizing the antihealing effect of steroids in patients receiving steroid therapy.

Scientific studies proved that, triterpenes from *Centella asiatica* stimulate extracellular matrix accumulation and also able to stimulate glycosaminoglycans synthesis in rat experimental wounds[35]. Asiatic acid was the only component responsible for the collagen synthesis stimulation[36]. These active constituents of the plant also helped in the reversal of steroid suppressed wound healing in our study. Natural antioxidants from plants strengthen the endogenous antioxidant defenses from reactive oxygen species ravage and restore the optimal balance by neutralizing the reactive species. They are gaining immense importance by virtue of their critical role in disease prevention. *Centella asiatica* has impressive antioxidant property and stimulatory effect on the cellular antioxidants and immune system which can be exploited for prophylactic use against a number of human ailments such as cardiovascular diseases and stress related disorders.

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