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Gandhaka Rasayana: Possible Role as a Modulator of Fibroblast Cell Function in Wound Healing

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ABSTRACT

Background & Objective: Gandhaka Rasayana (GR), a sulfur based ayurvedic formulation is used extensively in treatment of various skin and gastro-intestinal disorders and in healing of chronic non healing wounds. The rasayana is believed to play a significant role in repairing or rejuvenating of cellular functions. In this study, we investigated the effect of GR on fibroblast function.
Materials & Methods: Conditioned media were obtained from 3T3-L1 and McCoy fibroblast cells treated with GR extract. *In-vitro* studies were carried out to study the effect of conditioned medium on wound closure and expression of metallothioneins (MTs) and tissue inhibitor of metalloproteases (TIMP 1) in A549 cells.
Results and Conclusion: GR extract was found to induce increased expression of MTs to an extent of 1.5-1.9 fold in A549 cells. Under the experimental conditions employed, the conditioned media from GR treated McCoy and 3T3 were found to upregulate expression of MTs to an extent of 1.6-2.2 and 1.5 fold respectively in A549 cells. The conditioned medium from McCoy upregulated expression of TIMP 1 in A549 cells to a limited extent of around 1.35 folds. GR appears to affect fibroblast activation and modulate the expression of proteins involved in tissue remodeling. Conditioned media from both the fibroblast cell lines facilitated gap closure in A549 cell layer.

Key words: Gandhaka rasayana, Wound healing, Metallothioneins, Conditioned medium, Fibroblast

INTRODUCTION

Wound care and management present an enormous clinical and financial burden on the healthcare system worldwide. According to a report by Velnar *et al*, [1] chronic wounds affect 120 per 10^5 people aged between 45 and 65 years and 800 per 10^5 people >75 years of age. Concomitant to the projected growth of the ageing population and predicted increase in the prevalence of chronic diseases namely peripheral vascular diseases and diabetes which are known to negatively affect wound healing processes, it is likely that wound management becomes one of the major issues of concern in the coming years. [1,2] Gupta *et al* [3] have reported the prevalences of acute and chronic wounds to an extent of 10.55 and 4.48 per 1000 of the population respectively. Prolonged period of dressing and treatment thereof, adds significant financial burden to the health-care system.

Ayurveda has documented a comprehensive wound (vrana) care management. Shodhana is an elaborate protocol including seven different therapeutic procedures to clean the wound and facilitate the formation of an healthy wound bed. Shodhana is followed by Ropana as advised by Sushruta which also

includes seven healing procedures or upakramas. These include systemic and local wound healing measures employing various rasayanas (pharmaceutical formulations) and different modes of their administration. It is interesting to note that the complex procedure of wound healing has been explained elaborately in Sushruta Samhita as three clinical stages of wounds. First stage is Shuddha varna (stage of granulation tissue formation), second stage is called Ruhyamana vrana (epithelialization) and Rudha vrana [4-6] being the stage of remodeling. Infection control in wounds is given paramount importance in modern medicine. In spite of systemic and local use of several antibiotics, issues pertaining to chronic wound healing remain a challenge. Development of antibiotic resistant strains has further compounded the problem. Although practiced widely for treatment of non healing wounds and with many untold success stories, ayurvedic approaches have not received due scientific applause. Ayurveda considers both cleansing (anti-infective measures) and healing (granulation and tissue remodelling) to be of equal importance. There is a need of systematic scientific study to validate the potential of these preparations for their acceptance on a larger scale. The approach to wound healing requires therapeutic intervention at multiple levels (cellular and molecular) to help improve wound treatment and management. Wound healing occurs after ordered sequence of events involving interactions among multiple cell populations, cellular components, cytokines, growth factors and the extracellular matrix (ECM). Many ayurvedic preparations are complex mixtures of several plant parts. Possibility of ayurvedic approaches having multipronged effect needs to be probed. Rasayanas are exclusive procedures/formulations which provide factors for initiation and formation of healthy tissues. A gandhaka based medication, Gandhaka rasayana is extensively used in treatment of various skin disorders such as psoriasis, urticaria, eczema and wound healing, gastro-intestinal disorders and sinusitis. It is used effectively in healing of abscesses and chronic non healing wounds. It is prepared in a complex mix of cow milk and various herbal decoctions which is believed to purify/ detoxify the sulfur contained in the formulation. [7-10] Antimicrobial effect of gandhaka rasayana (GR) has been reported. [11,12] However, these reports as well as the previous work done in our laboratory indicate that the antimicrobial effect if present, is exhibited at high concentrations in the range of mg/ml. Hence, it is likely that the preparation acts mainly by modulating the cellular functions. Further the spectrum of immune system disorders treated by this rasayana strengthens this hypothesis and, it appears that rasayana has significant role in repairing or rejuvenating of cellular functions. One of the first events to occur in wound is vascular damage leading to the formation of fibrin clot. Monocytes are attracted towards the damaged site and infiltrate into the damaged ECM. This is followed by differentiation of the monocyte into a macrophage (MΦ). MΦs release growth factors, cytokines, MMPs etc., promoting angiogenesis and fibroblast differentiation, migration and proliferation. Activated fibroblasts are reported to synthesize and deposit large quantities of extracellular matrix proteins (ECM), reorganizing or contracting the ECM, thus restoring the mechanical stability of injured tissue. [13-15] Metalloproteases and tissue inhibitors of the metalloproteases play pivotal role in tissue remodeling processes. A sulfur based formulation, gandhaka rasayana is likely to affect expression of proteins such as metallothioneins as well.

It would be interesting to explore this aspect of the rasayana and therefore, in the proposed study we aim to investigate the effect of Gandhaka rasayana on fibroblast cells and understand its possible role in wound healing processes with special emphasis to its effect on expression of metallothioneins.

MATERIALS AND METHODS

Gandhaka rasayana (GR) churna was collected from SDM Pharmacy. Metallothionein and TIMP1 kit were procured from Life Technologies, Delhi

The test cell lines A 549 lung carcinoma, 3T3-L1 and McCoy were procured from NCCS, Pune and were propagated in Ham's F12k, Dulbecco's modified Eagle's medium (DMEM) and MEM media containing 10% FBS respectively.

Energy-dispersive X-ray spectroscopy (EDS) analysis:

Gandhaka rasayana (finely ground powder) was sent to DST-PURSE laboratory, Mangalore University, Karnataka, India for EDS analysis (FESEM - Carl Zeiss SIGMA; EDS analysis-OXFORD Instruments). Spectral analysis was done using SiO2 and FeS2 as stanadards.

Solubilization of GR and cell viability studies:

Solubility of Gandhaka rasayana (GR) was tested in various solvents (5mg in 500µl) namely, ethanol, Dimethyl sulfoxide, methyl cellosolve, ethyl cellosolve, triethylene glycol, diethylene glycol, tetra hydrofuran, glycerol triacetate, ethanolamine, castor oil, coconut oil and ghee.

Effect of GR solubilized in ethanolamine on the viability of A549 cells was studied by testing the viability of cells at the concentrations of 2-120µg of GR/ml. Ethanolamine toxicity was studied at equivalent volumes (0.067-4µl/ml) present in the GR solution. The cell layer washed with PBS was subjected to Sulforhodamine B method to assess % viability. [16]

Preparation of GR extract:

To 40mg of the rasayana, (ground to fine powder form using IKA A11 basic mill), 400µl of Ham's F12k with 10% FBS was added and mixed in roto spin for 5h and kept in refrigerator overnight. The suspension was diluted ten times with the medium containing 5% FBS, mixed for 30min and filtered through 0.2µ filter. The dark brown colored filtrate was stored at -20^{0}C. Control without GR was prepared in a similar manner.

Effect on wound repair in A549 cells:

Wound of ≈2mm length was introduced into the confluent monolayer growth of test cell lines in 96 well plates with the Maxipense^{TM} 10 µl tip. The test samples (extract/conditioned medium) were added to the culture medium and incubated in CO2 incubator (Galaxy 170S, New Brunswick). Wound closure was monitored microscopically. Staining of the cells with crystal violet (0.5% in methanol filtered through Whatman No.1) was carried out 42-44 hours after introduction of the wound. The images were captured with Motic AE31 at a magnification of 10x (objective lens). Wound width was measured at the centre of the scratch. Percentage decrease in the width was calculated in comparison to control treated cell layer.

Effect of GR extract/conditioned medium (CoM):

The fibroblast cells 3T3 and McCoy were seeded at a rate of 8 lakh cells in 60mm cell culture plates in respective culture medium.After overnight incubation, GR extract 0, 3, 5 and 8µl (L0, L1. L2 and L3) were added to 2.5ml of the medium containing 4% FBS. Volume was made up with the control. The CoM was collected at 62-64h, spun at 1000rpm for 4 min at 4°C and stored at -80°C (Premium U410 freezer, New Brunswick) and used within 48h. Confluent growth of A549 with the introduced wound in 96 well plates was treated with conditioned media at a level of 10% and 4% in Ham's F12k . After 48h, the cells were washed and the cell layer was washed with PBS and treated with lysis buffer (0.25% Triton X-100, 0.1M NaCl, 0.04% 2-mercaptoethanol in 20mM Tris buffer pH 7.2) and the adherent layer was scraped. The lysates were collected, centrifuged and the supernatants were stored at -80°C. The lysates belonging to the same set were pooled and analysed for metallothionein, TIMP-1 and protein

content. Effect of GR extract on protein expression by A549 cells was also studied.

Measurement of MT and TIMP1:

Metallothionein and TIMP-1 in the A549 lysates were measured using ELISA Kits from Life Technologies, Delhi.

Estimation of protein:

The protein content of the lysate was measured by Folin-Lowry's method. [17] To 0.2ml of appropriately diluted lysate, 0.4ml of Reagent A (2.5% Na_2CO_3, 0.5% NaOH, 0.9mM K Na tartrate, 0.5mM $CuSO_4$) was added. After 10 min, 0.05 ml of 3x diluted FC reagent was added. Following incubation for 10 min, the tubes were centrifuged at 13000rpm for 5min. The supernatant was read at 750nm using plate reader. Method was calibrated by the same method using bovine serum albumin as the standard.

RESULTS

The method of preparation of GR is described in Ayurveda prakasha. Shoditha gandaka is taken, three each bhavana (triturations) are given with goksheera, caturjataka (decoction of *Cinnamomum zeylanicum, Cinnamomum tamala, Elettaria cardamomum* and *Mesua ferrea*) and gudoochi (*Tinospora cordifolia*). Similarly eight each bhavanas are given with triphala (fruit rinds of *Terminalia chebula*, *Phyllanthus emblica* and *Terminalia bellirica*), bhrungaraja (*Eclipta alba*) and aardraka (*Zingiber officinalis*) respectively. This formulation is named as gandaka rasayana and is to be taken along with sita (sugar syrup). If consumed in quantity of One karsha then it cures dhatukshaya (emaciation), 20 types of prameha (metabolic disorders especially D mellitus), agnimandya (digestive abnormality), shula (abdominal pain), koshtagata roga (abdominal disorders), kushta (skin diseases) of eighteen types, rajayaksma, pushti of bala and veerya, dehashuddi is attained if consumed after vamana (emesis) or virechana (purgation) and / other purifactory procedures. Pathya is jangalamamsa and ajamamsa. Apathya i.e. are lavana (salt), amla (sour), shaka (certain vegetables), dwidala dhanya (certain dicots) etc. are to be avoided during consumption of gandaka rasayana. It is prescribed in powder or tablet form for systemic effect (https://www.ayurtimes.com/gandhak-rasayan-benefits-uses-side-effects). The ingredients included in preparations are believed to have anti-inflammatory effects. After completion of Virechana (purgation) process and Samsarjana Krama (special dietary regimens after purgation), Jalaukavacharana (bloodletting by Leech) and Panchakarma, the patients are given Gandhaka Rasayana as palliative measure.

EDS analysis of the rasayana revealed that elemental ratio of C:O:S in GR was 1:2.66:8.76 (figure 1). Sulfur content (wt %) in GR was found to be 8.8 times more than its carbon content and 3.3 times higher than the oxygen content. The result therefore, indicated that GR as its name implies, is rich in gandhaka (Sulfur).

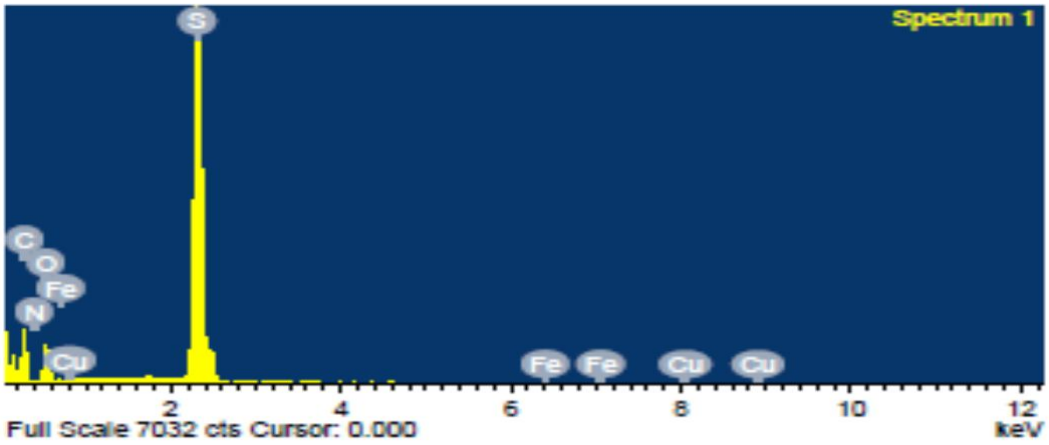

Figure 1: EDS spectrum of Gandhaka Rasayana

GR extraction was initially carried out in alcohol and chloroform (100mg in 1ml for 5h). The dried extracts could not be completely solubilized in DMSO, leaving behind yellow colored residue. Partly solubilized alcohol extract exhibited cytotoxic effect on A549 cells. The extract obtained using chloroform as solvent, did not exert significant effect on the viability of A549 cells (results not shown). Sulfur is known to be insoluble/sparingly soluble in most of the organic solvents. Sulfur being the major active component of the formulation, various solvents including Dimethyl sulfoxide, methyl cellosolve, ethyl cellosolve, triethylene glycol, diethylene glycol, tetra hydrofuran, glycerol triacetate, ethanolamine, castor oil, coconut oil and ghee were used for solubilization of GR. GR was found to be sparingly soluble in these solvents with the exception of ethanolamine, which could solubilise GR to an extent of 30mg/ml (it was found that sulfur could also be solubilized in ethanolamine to an extent of 10mg/ml) . Ethanolamine although is a component of many biomolecules including phospholipids, it was found to be toxic in its free state. When exposed to concentrations of 1.2 µl /ml and 2 µl /ml, cells appeared shrunk, and lost 49% and 81% of viability respectively. Wound closure studies at 10-20 µg GR/ml (ethanolamine) appeared to exhibit positive effect on wound closure in comparison to the cells treated with its respective ethanolamine control (0.33-0.7 µl/ml). However, in comparison to untreated A549 cells, the gap closure was not significantly different. As toxicity of ethanolamine could be hampering or interfering in wound closure, it was decided to employ fetal bovine serum as the extractant. Finely ground GR powder was extracted in medium containing FBS. The filtrate (GR extract) obtained from was dark brown in color in comparison to the control filtrate which remained pink. Conditioned media harvested from McCoy and 3T3 fibroblast cells treated with GR extract were studied for their effect on wound closure in A549 cells. The wounds created in each well were observed after 48h. CoM (L3), obtained from the fibroblast cells treated with GR extract applied to A549 cells at a dosage of 10%, was found to facilitate wound closure as shown in figures 2 and 3.

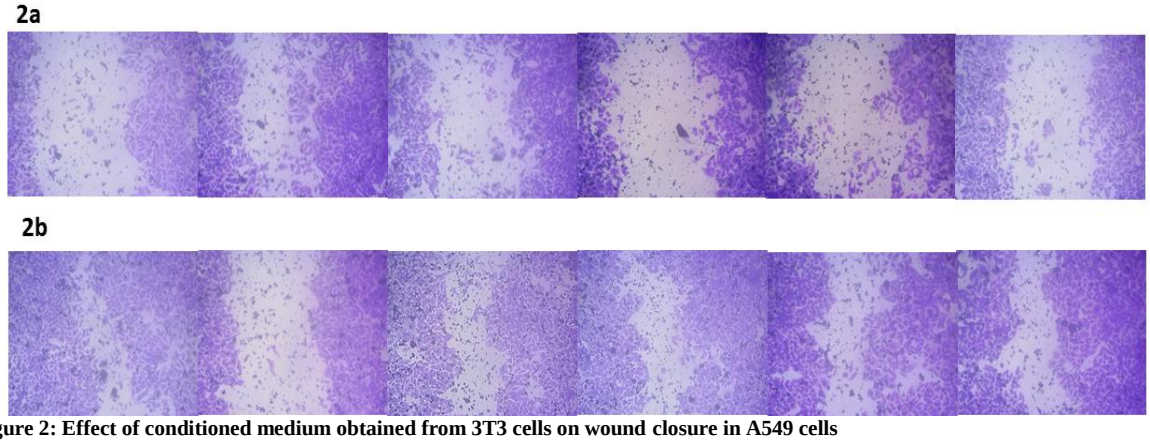

Figure 2: Effect of conditioned medium obtained from 3T3 cells on wound closure in A549 cells
1a: Conditioned medium obtained from untreated cells; 1b: Conditioned medium obtained from cells treated with GR extract (L3).

Conditioned media obtained from 3T3 and McCoy fibroblast cell lines were found to be effective in wound closure in A549 cell layer, resulting in an overall decrease in the wound gap to an average extent of 28% and 37% respectively in comparison to the A549 cells treated with CoM obtained from respective controls.

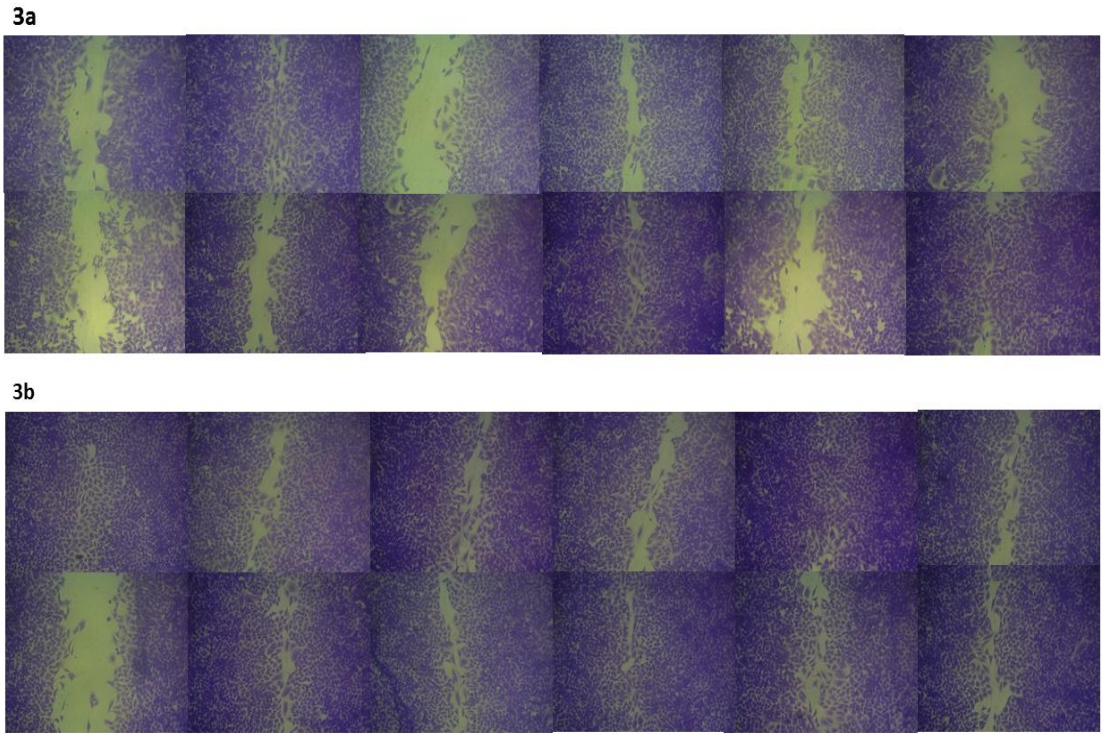

Figure 3: Effect of conditioned medium obtained from McCoy cells on wound closure in A549 cells
1a: Conditioned medium obtained from untreated cells; 1b: Conditioned medium obtained from cells treated with GR extract (L3)

The fibroblast cell lines treated with GR extract and A549 treated with the conditioned media were subjected to lysis. This lysate is a complex mixture comprised of not only cellular components, but also includes extracellular matrix components. As shown in Figures 4 and 5, increased expression of MTs to an extent of around 1.5 fold was observed under the influence of CoM harvested from 3T3 cells treated with high concentration (L3) of GR extract in comparison to respective controls (L0). However, expression of TIMP 1 was found to be down regulated.

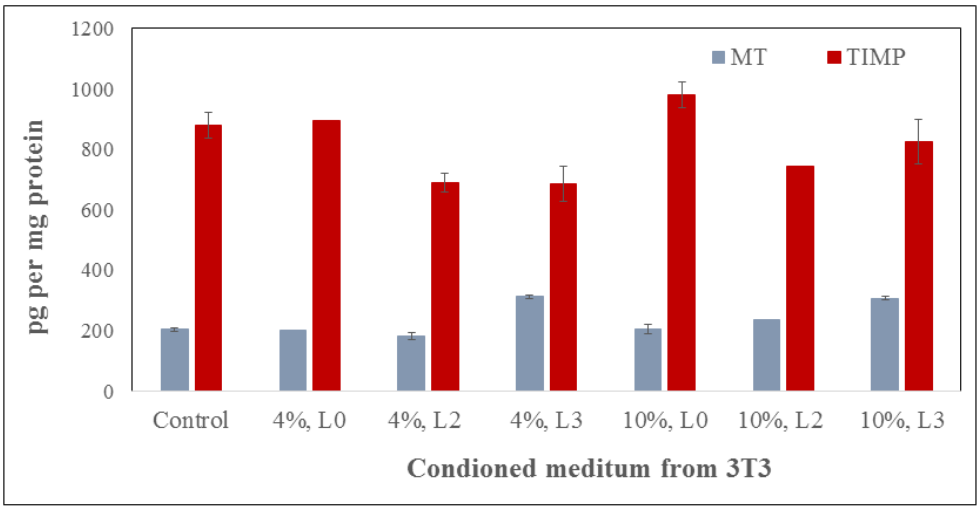

Figure 4: Influence of 3T3 CoM on the Expression of MT and TIMP-1 in A549 cells.

CoM from McCoy (L3 treated) applied at a level of 4% was found to increase the MT level by 1.6 fold, while at a dosage of 10%, 2.2 fold increase was observed. Increase to an extent of 1.2-1.35 fold was affected in the expression of TIMP-1 under the influence of CoM applied at a level of 10%.

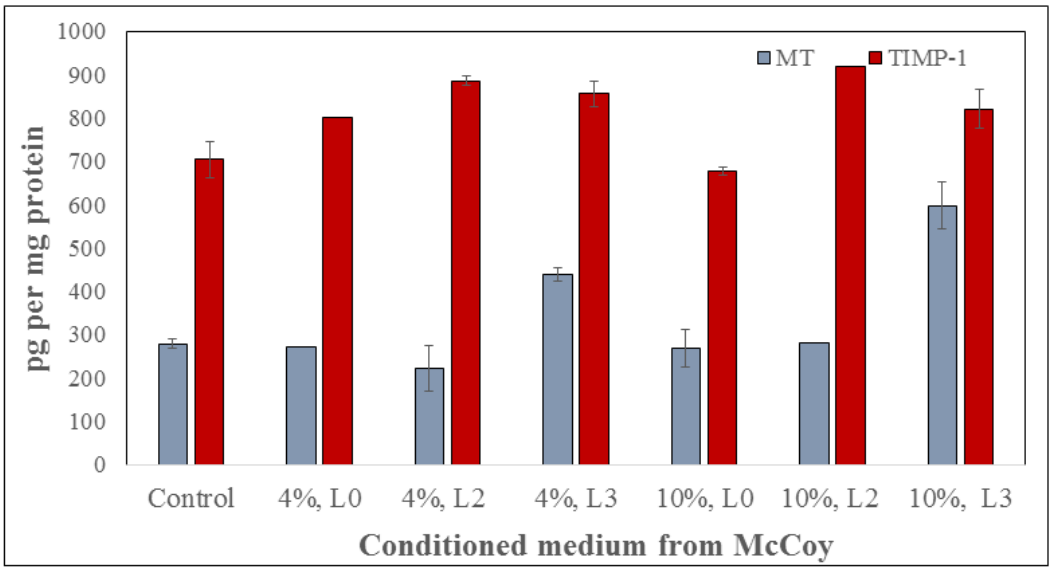

Figure 5: Influence of McCoy conditioned medium on the Expression of MT and TIMP-1 in A549 cells

Study was also carried out to find the direct effect of GR extract on expression of MTs in A549 cells. A549 cells upregulated expression of MTs by approximately 1.5 to 1.9 fold as shown in figure 6.

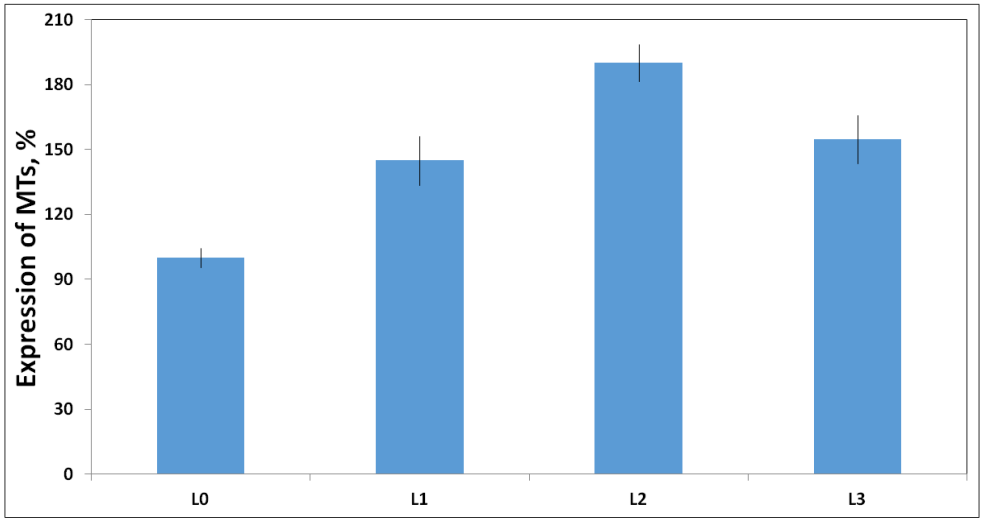

Figure 6: Expression of Metallothioneins by A549 cells

Thus, GR extract and conditioned medium obtained from GR extract treated fibroblasts were found to induce increased expression of these cysteine rich low molecular weight metal binding proteins.

DISCUSSION

The use of herbal medicine in wound care has been very encouraging and several researchers around the globe have started to publish their results. [18-19] Gandhaka rasayana is one such preparation which finds use in wound repair.

Sulfur is the major component of GR and is known to be insoluble in the inert organic solvents which are employed as extractants in laboratories. Serum albumin is known to act as a carrier of many insoluble non-polar components. Complexation of albumin with sulfur has been reported by Jarabak and Westley. [20] Pulverised GR was therefore subjected to extraction in medium containing bovine serum albumin. GR

extract was added to the fibroblast cells and the conditioned media obtained from these McCoy and 3T3 cells were studied for their effect on wound closure in A549 cells. As shown in figures 2 and 3, conditioned media exerted positive effect on wound closure.

Crosstalk between Membrane metalloproteases (MMPs), tissue inhibitors of metalloproteases and metallothioneins (MTs) are known to play pivotal role in repair and regenerative processes. MTs, the cysteine rich low molecular weight peptides influence tissue remodeling to a significant extent while MMPs which comprise of around 23 proteases in mammals are mainly involved in the homeostasis of ECM. [21-22] Accumulation of MTs in tissues comprising of proliferating cells and in healing tissues implies possible role of MTs in injured tissues undergoing repair. Fibroblast based therapies are being considered for effective treatment of wounds. [23-24]

Conditioned media from both 3T3 and McCoy were found to induce increased expression of MTs (figures 4 & 5). Both the fibroblast cell lines treated with GR extract were also found to express higher amount of MTs. MTs are low molecular weight proteins which are rich in sulfur containing aminoacid cysteine. Being a sulfur based formulation, the possibility of GR as an inducer of metallothioneins expression was considered in the study. Induction of metallothioneins is reported to accelerate wound healing by promoting cell proliferation and, re-epithelialation by matrix remodeling. Metallothioneins are being considered for use as potential vulnerary aids. However, excessive response is reported to promote keloidogenesis in healing wounds. [25] Concommitant to their growth enhancing effects, potential role of MTs in carcinogenesis has been reported by Cherian *et al.* [26] In the present work, at the higher concentrations of the modulating agents, MTs were upregulated and the increase was limited to a factor of around 1.5 to 2.2 folds in both cases (direct effect of GR extract on fibroblasts and influence of CoM on A549 cells).

TIMPs are endogenous inhibitors of metalloproteases and hence, they play a pivotal role in remodeling of tissues / reepithelialization of the wound by regulating degradation and deposition of extracellular matrix proteins. TIMP-1 is reportedly upregulated in proliferating cells in normally healing wounds. [27-29] However, according to the report of Salonurmi *et al* [30] over expression of TIMP-1 lead to negative effect on wound healing in animal models. Thus a balance in the expression of these proteins is critical in wound healing. The CoM from GR treated McCoy increased the expression of TIMP-1 by A549 cells to a relatively lesser extent (1.2-1.35 times). TIMP-1 is known to inhibit a wide array of proteases and is reportedly expressed in epithelial cells of healing excisional / burn wounds, and also in wound fibroblasts. A fine balance between TIMPs and respective metalloproteases is critical, as excess protease activity can lead to a chronic non-healing wound. [31] A549 cells responded differentially to the conditioned media of 3T3 and McCoy with regards to TIMP-1 expression. 3T3 is derived from mouse, while A549 is of human origin. McCoy cell line is believed to have been derived from human synovial fluid in 1955. However, later studies claim that the cells are of mouse origin. [32] In the present study, the CoM from McCoy modulated A549 cells to a higher extent in comparison to CoM from 3T3 cells. The CoM obtained from GR extract treated McCoy cells was found to induce A549 cells to upregulate production of MTs to a significant extent. Gandhaka rasayana was also found to enhance expression of metallothioneins by the fibroblast cells.

CONCLUSION

Increased expression of metallothioneins was observed in fibroblasts treated with GR and also in A549 cells cultured in presence of CoM obtained from GR extract treated fibroblast cells. The

results indicate the possible mechanism of Gandhaka rasayana by fibroblast activation and by modulation of the proteins involved in tissue remodeling. GR is a complex mixture of herbo-minerals prepared by subjecting gandhaka suspended in cow's milk to heat treatment, followed by 64 bhavanas (triturative procedures). In addition to its basic principles of treatment, Ayurveda emphasizes on not introducing adverse effects. Unique combination of thermal processing and sequential herbal treatments are believed to transform the inorganic constituents present in ayurvedic preparations to complex ionic or colloidal forms which probably neutralizes the toxicity of the minerals and/ acclimatizes the toxic principles into therapeutic components (http://shodhganga.inflibnet.ac.in/bitstream/10603/38973/7/07_chapter%203.pdf).

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