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Original research article

Biochemical and functional analysis of corticotropin releasing factor purified from an aqueous extract of human placenta used as wound healer

Namrata Singh, Debasish Bhattacharyya *

Division of Structural Biology and Bioinformatics, CSIR-Indian Institute of Chemical Biology 4, Raja S.C. Mullick Road, Jadavpur, Kolkata, 700032, India

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ABSTRACT

Human placental extract constitutes of innumerable therapeutically important components mostly used in wound healing arising from the skin and burn injuries. However, there is still some bioactive present in the placental extracts yet to be characterized to better under the complex process of wound healing mediated by the placental extract. In this study, the presence of corticotropin releasing factor (CRF) in an aqueous extract of human placenta was detected and quantified by dot blot and CRF-ELISA immunoassay kit respectively. Subsequently, it was purified by immuno-affinity chromatography and quantified as 0.45 ± 0.05 μg of CRF per ml of placental extract where its molecular weight found to be 4.78 kDa by MALDI-TOF. To study functional analysis of CRF, an *in vitro* WI-38 lung fibroblast cell scratch wound model was used which indicated proliferation, motility of cells after treatment with purified CRF. Moreover, reduction in apoptosis rate of cells during closure of wound was observed from microscopy studies and FACS analysis. Also, Antalarmin, an antagonist of CRF type 1 receptor inhibited the wound closure potency of the purified component. Faster healing of wound with an elevation of IL-6 and TGF-β during early stages of repair by placental CRF was observed on excision rat model. The process of healing was accompanied by the decrease in the level of TNF-α and IFN-γ.

1. Introduction

There are numerous studies demonstrating the composition of human placental extract and their role in wound healing [1]. Though still some facts are yet to be uncovered to better understand the complexity of wound healing mediated through human placental extract. From the last few decades, characterization of the aqueous extract of human placenta which is used as a licensed drug, led to the identification of several bioactive components, for example, NADPH, a fibronectin type III-like peptide that stabilizes trypsin activity, an ubiquitin-like protein which exhibits collagenase activity and heparin-mediated inhibition of placental extract. The extract also exhibited an anti-microbial activity, *in vitro* induction of NO by mouse peritoneal macrophages together with enhancement of their adhesion property [2–9]. These findings collectively support wound healing nature of this placental extract. The aqueous extract also contains significant protease activity that may play regulatory roles in wound healing [1]. Therefore, it remains possible that biomolecules present in the extract may be proteolytically modified. Identification of each component present in the extract and elucidation of their mechanistic roles in healing and immune-stimulation are necessary for its safe and rational application.

Corticotropin-releasing factor (CRF) is a 41-amino-acid peptide synthesized in the para ventricular nucleus of the hypothalamus, abundantly expressed in placenta. It has an important role in parturition. Other members of CRF family such as urocortin have also been identified in human placenta and membranes [10,11]. Thus, based on its solubility, it is expected to be carried over in an aqueous extract of the human placenta. However, the stability and biological functionality of placental CRF in the aqueous extract of human placenta is unknown. Apart from the well-characterized

Abbreviations: CHCA, α-cyano-4-hydroxycinnamic acid; EDC, 1-ethyl-3-3-dimethylaminopropyl carbodiimide; ELISA, enzyme linked immunosorbent assay; FBS, fetal bovine serum; IFN-γ, interferon-γ; MES, 2-(*N*-morpholino)ethanesulfonic acid sodium salt 4-morpholineethanesulfonic acid; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); NBT, nitro blue tetrazolium; PBS, phosphate-buffered saline pH 7.2; pNPP, p-nitrophenyl phosphate; SGOT, also called AST or amino transferase; SGPT, also called ALT or alanine aminotransferase; TNF-α, tumor necrosis factor-α.

* Corresponding author.
E-mail addresses: namratasingh819@gmail.com (N. Singh), dbhattacharyya1957@gmail.com (D. Bhattacharyya).

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role of neuronal CRF in the homeostatic response to stress; several other actions in tissues repair and wound healing have also been attributed to CRF. It is also a potent immuno-modulator with cellular target-dependent polarity, acting to inhibit or stimulate local immune function [12,13]. Though, literature available for explaining the role of exogenous CRF from the placental extract in wound healing and cytokines involvement is scarce.

Interleukin (IL-6), transforming growth factor (TGF-β) and other pro-inflammatory cytokines are major regulators of the healing process. IL-6 is a pleiotropic cytokine involved in the growth and differentiation of various cell types including those of dermal and epidermal origin. TGF-β regulates cell proliferation, adhesion, migration, differentiation and finally induces extracellular matrix proteins [14]. Similarly, the inflammatory phase in wound healing is considered to be a preparatory process for the formation of new tissues. TNF-α and IFN-γ are effective cytokines which are mediators of the body's response to infection and key regulators of inflammatory responses [14]. Several lines of evidence indicate that IFN-γ impairs collagen accumulations and inhibits the proliferative phase of wound healing [14]. Therefore, to demonstrate the role of any bioactive molecule in wound healing and immune-stimulation, effect on these wound healing regulatory cytokines should be investigated.

The aim of the present investigation was to detect the presence of CRF in the aqueous extract of human placenta used as a wound healer. Subsequently, the role of CRF from the placental extract in wound healing was evaluated through i*n-vitro* scratch wound assay, apoptosis assay, and *in-vivo* animal model experiments. The study continued with the specific effect of placental CRF on the production of wound healing regulatory cytokines which are IL-6, TGF-β1, IFN- γ and TNF- α. Moreover, toxicity effect of placental CRF on rat model has also been reported.

2. Material and methods

2.1. Reagents

Fine chemicals were procured as follows: Annexin V-FITC Apoptosis Detection Kit, CRF, EDC, PBS, Tween 20 and Trypan blue were purchased from Sigma-Aldrich (Missouri, USA). BCIP and NBT were purchased from Promega (Madison, USA). Nitrocellulose membrane was purchased from Merck Millipore (Massachusetts, USA). CRF-EIA Kit was bought from Phoenix Pharmaceuticals (California, USA). Rabbit polyclonal IgG CRF was purchased from Santa Cruz Biotechnology (California, USA). Goat polyclonal anti-rabbit were obtained from Abcam (Cambridge, U.K). Carboxylink coupling gel was purchased from ThermoFisher Scientific (New York, USA). Dulbecco's Modified Eagle Medium (DMEM), Penicillin/streptomycin (Pen-strap), heat inactivated fetal bovine serum (FBS), MTT, trypsin, fungizone, gentamicin were purchased from Gibco-Invitrogen (Grand Island, New York, USA). Antalarmin hydrochloride was purchased from Cayman Chemical (Michigan, USA). ALP, creatinine, SGPT, SGOT and urea test kits were purchased from Span Diagnostics Ltd. (Mumbai, India). All other reagents were of analytical grade and procured locally.

2.2. Human placental extract

An aqueous extract of human placenta was supplied by the drug house Albert David Ltd., Kolkata, India, marketed as a licensed drug under the trade name 'Placentrex'. Preparation of the extract holding the proprietary terms of the manufacturer has been previously described [1]. In short, the extract was prepared from fresh-term pooled placentae using single hot and cold water followed by sterilization under saturated steam pressure (15 psi at 120 °C) for 40 min. Under above conditions, spore-forming species such as *Clostridium tetani* were killed. This extraction was routinely tested for the presence of HIV antibody and hepatitis B surface antigen, if any, before sealing in ampoules. The final finished drug contains 1.5% benzyl alcohol (v/v) as a preservative which does not interfere with the experiments presented here. Collection, handling of the placenta and manufacturing of the drug all were carried under the guidelines of the drug controlling authority of India.

2.3. Dot blot (immuno-blot)

Placental extract (10×, 20 μl), purified placental CRF (described below, 10 μg), reference CRF and PC-12 (neuronal cell line as a known source of CRF, 10 μg both as positive controls) and BSA (10 μg as negative control) were spotted on nitrocellulose membrane strip, dried and incubated with PBS containing 0.05% Tween-20 and 5.0% skimmed milk at 4 °C for 18 h. Then the strip was washed four times with PBS containing 0.05% Tween-20 followed by incubation with CRF antibody (1:1000 dilutions with PBS) for 4 h at 25 °C. The strip was viewed for colorimetric detection using alkaline phosphatase [8,15].

2.4. Estimation of CRF using EIA kit

The protocol followed was as per manufacturer's instructions (Phoenix pharmaceuticals, India). In short, through serial dilution from a stock of 1 μg/ml of standard CRF, solutions of 0.1–100 ng/ml were prepared for the construction of a calibration curve correlating concentration of CRF and intensity of color developed at 450 nm. A linear dependency was observed (R^2 = 0.992, where R^2 = regression coefficient). Variation of reading between sets was ±5% (n = 5). Briefly, 50 μl of placental extract, standard CRF as a positive control and PBS as a negative control were added to the 96 well immune plates. Thereafter, 25 μl of rehydrated primary antibody followed by 25 μl of rehydrated biotinylated peptide to each well except the blank well were added. This immunoplate was incubated for 2 h at 25 °C with orbital shaking at 300–400 rpm. Then, wells were washed for four times with 350 μl of 1× assay buffer to remove any unbound proteins. After washing, 100 μl of SA-HRP was added to the wells and incubated for 1 h at 25 °C with orbital shaking at 300–400 rpm for binding. To avoid nonspecific binding, following incubation plate was washed again as described earlier followed by addition of 100 μl of TMB substrate solution provided in this kit into each well. The plate was incubated for 1 h with shaking. Finally, 100 μl of 2N HCl was added into each well to stop the reaction. The plate was read in an ELISA plate reader (Thermo Scientific Multiskan GO, U.S.A) at 450 nm [16].

2.5. Purification of CRF by immuno-affinity chromatography

A 2-ml column was packed with carboxy Link™ coupling gel followed by equilibration with 0.1 M MES, pH 4.7. CRF antibody (50 μg/ml, 1 ml) was applied to the gel slurry and rocked for 3 h for binding of the antibody. The antibody coupled column was equilibrated with 20 mM Na-phosphate, pH 7.5. Placental extract (20 mg/ml, 1 ml) was applied to the column, washed with buffer for complete removal of unbound fractions and CRF was eluted by 0.1 M glycine-HCl, pH 2.4 [15].

2.6. Mass analysis

Analysis was performed using a MALDI-TOF/TOF instrument (Model 4700, Applied Biosystems) operated in reflectron mode. Protein samples were dialyzed against 0.01 M Na-phosphate, pH

7.5, desalted by passing through C_4 ZipTip (Millipore) cartridge and analyzed using CHCA matrix in 50% acetonitrile and TFA [17].

2.7. Cell culture

Human lung fibroblast cells (WI-38) were grown in T-25 culture flasks in DMEM supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, fungi zone (2.5 μg/ml), gentamicin (2.5 μg/ml) and 2 mM L-glutamine at 37 °C in a 5% CO_2 in an air humidified incubator. Once grown to confluence, cells were harvested. The viability of cells was determined by trypan blue exclusion staining. Cells were diluted in DMEM medium (1:100) containing 0.4% trypan blue solution and counted in a hemocytometer to assess the number of live cells that stained blue from the total number of cells counted [17,18].

The growth of cells was measured by yellow tetrazolium MTT assay. Yellow tetrazolium MTT is reduced by metabolically active cells, in part by the action of dehydrogenases. Briefly, 1.5×10^5 cells were plated in 96 well plates and treated with purified CRF (200 ng) for 48 h. At the end of the incubation period, MTT was added at 0.5 mg/ml and incubated for 3 h. After incubation for 4 h at 37 °C, the medium was removed and 100 μl of DMSO was added to each well. Plates were agitated at 25 °C for 10 min and absorbance was recorded at 590 nm by a multi-well plate reader (Biotek-Epoch; Biotech Instruments). The average absorbance value of three replicates wells was used for each set and each experiment was repeated thrice. Here the cells without test samples but with MTT served as positive control while cells treated with hydrogen peroxide (10 μl) served as negative control. Cells without MTT served as blank [17,18].

2.8. Migration of fibroblasts

WI-38 cells were cultured in 35 mm plates until they reached confluence. The scratch *in vitro* wound was made with a 10 μl dispensing tip [19]. After creating the scratch, the media was replaced by a fresh one without serum along with purified CRF (100 ng), reference CRF (100 ng), placental extract (50 μl, 5×) and PBS (100 μl). Images were captured at stipulated time intervals and cell migration was quantified by measuring the distance between two certain points on either side of the gap using Image J software (freely downloadable at http://rsbweb.nih.gov/ij/). For statistical analysis, at least three measurements at different points were made for each image.

2.9. Apoptosis of fibroblasts

WI-38 cells were cultured in 60 mm plates until the surface was completely covered. Thereafter a small area was disrupted and a group of cells was destroyed or displaced by scratching a line through the layer with a tip. The culture medium was replaced with serum free medium and cells were stimulated with purified CRF (100 ng) and standard CRF (100 ng positive control) and PBS (negative control) for 24 and 48 h. Apoptosis of cells was measured by Annexin V-FITC apoptosis detection kit. The kit detected apoptotic cells by flow cytometry utilizing Annexin V-FITC as a fluorescent probe which measured early apoptotic phosphatidylserine binding. Early stages of apoptosis involve cellular changes that include loss of phospholipids asymmetry. At the onset of apoptosis, phosphatidylserine translocates from the internal to the external side of the plasma membrane. Consequently, phosphatidylserine becomes available to bind with Annexin V in presence of Ca^{+2}. While propidium iodide binds to DNA was also monitored to indicate damage of the cell membrane and progression to apoptosis. After 24 and 48 h of incubation, cells were trypsinized with 0.25% typsinwashed twice with PBS and collected by centrifugation (1000 rpm, 5 min). Collected cells were suspended in 400 μl of binding buffer at the density of 1×10^6 cells, and stained with Annexin V-FITC (4 μl) and PI (3 μl) in the dark for 15 min at 25 °C. Then the cells were analyzed using Beckton-Dickinson FACS Array apparatus (BD Pharmigen) using CELLQuest software at 550 nm (reference filter 620 nm). The level of induction of apoptosis was assessed by depicting cells stained by PI (y-axis) *versus* Annexin V (x-axis) [17].

2.10. Animals

Female Sprague Dawley rats (8–12 wks, 120–140 g) were housed at 22 ± 3 °C under 12 h light/dark cycles. Food and water were supplied *ad libitum* during the experimental period. The purpose and protocol of the experiment were approved by the institutional animal ethics committee. The experimental protocol ensured minimum suffering of the animals. At the end, animals were euthanized by ketamine overdose (200 mg/kg). Experiments were carried out following the Organisation for Economic Cooperation and Development guidelines [20].

2.11. In vivo wound model

To evaluate the potential role of placental CRF in wound healing, skin wound was created on the inner thigh surface of rats after being anesthetized by diethyl ether, shaving and cleaning the exposed region with 70% ethanol using a sterile biopsy punch of 5 mm diameter. Purified CRF (200 μg/ml that was equivalent to 500 μg/kg body weight in 500 μl), standard CRF (200 μg/ml, 500 μl) and placental extract (5×, 500 μl, positive control) and PBS (500 μl, negative control) were administered *via* intra-peritoneal route to the animals on every alternate day till 10th day. Each wound site was digitally photographed with a Sony cyber shot W830 20.1MP Digital Camera (Tokyo, Japan) at the indicated time intervals. Wound area was measured by tracing the wound on an mm^2 scale graph paper on 0, 2, 4, 6, 8 and 10th days post wounding. Wound areas were expressed as percentage of the initial wound areas. The area of the wound created on the first day was treated as 100%. Relative wound size was determined by plotting the wound area *versus* days. Formation of scab on the wounded area leaving no raw wound behind served as end point of complete epithelization.

2.12. Quantification of cytokines by ELISA

Purified CRF from the placental extract in 500 μl of PBS was administered subcutaneously at 500 μg/kg body weight per rat. A control group received the same volume of PBS. Blood was collected from the animals by retro-orbital puncture every alternate day for 8 days. Serum was obtained after centrifugation of blood at 2000*g* for 20 mins at 4 °C and was used for cytokine analysis. The release of cytokines (IL-6, TGF- β, IFN-γ and TNF-α) in serum were determined by capture enzyme-linked immunosorbent assays (ELISA) using micro-titer plates. Plates were coated with respective rabbit IgG antibody (1:1000 dilutions) in carbonate-bicarbonate buffer, pH 9.6 for 24 h at 4 °C. After being coated, plates were washed and blocked with 1% BSA in PBS for 1 h at 25 on plates. Samples wereC. In brief, 100 μl of serum from treated and control rats were loaded on plates. Samples were applied in duplicates and plates were incubated for 4 h at 25 °C with mild shaking. Then the plates were washed with PBS containing 0.005% Tween 20 to remove uncaptured samples. Thereafter, secondary antibody conjugated with alkaline phosphatase was added (1:5000 dilution). After incubation for 1 h, antigen-antibody binding was revealed by incubation with the substrate *p*-NPP. The plates were read in an ELISA plate reader (Thermo Scientific Multiskan GO, U.S.A) at 405 nm. Fresh culture medium as a blank well, the absence of any test sample and primary antibody in wells constituted three negative controls for

each set. For determination of the concentration of IL-6, TGF- β, IFN-γ and TNF-α in the cell, calibration curves were constructed with 10 – 100 ng/ml of the standard compounds after dilution in PBS containing 0.005% Tween 20. Linear dependency between concentration and absorbance was observed for IL-6, TGF- β, IFN-γ and TNF-α (R^2 = 0.941; 0.939, 0.953 and 0.961 respectively). Variation of absorbance for each point was ± 5% (n = 5) [15].

2.13. Toxicity studies

2.13.1. Acute toxicity

Rats were grouped into six. Purified pool of CRF in 500 μl of PBS was administered subcutaneously at 0, 100, 250, 500 and 1000 μg/kg body weights per rat. In this study, in vivo wound healing was optimized with a dose of 500 μg of CRF/kg body weight of rat. For this reason, a range of 0–1000 centered on 500 μg/kg was applied to rats for toxicity study. The control group received the same volume of PBS. Symptoms of morbidity and mortality were followed. Animals were weighed on 0, 7th and 14th day. At the end of the experiment, blood was drawn by retro-orbital puncture. Biochemical analysis was performed Standard diagnostic kits (Span diagnostics Ltd., India) were used for estimation of SGPT, SGOT, ALP, urea and creatinine spectrophotometically as per manufacturer's protocol [15].

2.13.2. Sub-chronic toxicity

Ten rats were grouped into two. Purified CRF at doses of 100, 500, 1000 μg/kg body weight of rat in 500 μl of PBS were administered for the measurement of sub-chronic toxicity. The control group of rats received an equal volume of PBS. Samples were administered daily subcutaneously to each rat for 30 consecutive days at the same hour and the symptoms for morbidity and mortality were followed. Body weights of the animals were recorded weekly. On the 31st day, blood was collected followed by isolation of serum for analysis [15].

2.14. Statistical analysis

All values were expressed as error bars (mean ± standard deviation) obtained from independent experiments represented by 'n' while each experiment was done in triplicate and the mean value was accepted. Statistical significance was determined using student – *t*-test and $p < 0.05$ was the significance level.

3. Results and discussion

3.1. Identification, quantification and purification of CRF from the human placental extract

CRF is a component of hypothalamic-pituitary-adrenal axis and is also present in human placenta, where its secretion after synthesis is raised more than 20 fold in 5 weeks preceding parturition [21,22]. The presence of CRF- like activity in placental extract is reported [23]. But presence or abundance and functionality of CRF in the extract may vary depending on the procedure followed [24]. To detect the presence of CRF in this placental extract, a dot blot assay using CRF-antibody was performed. This antibody is capable of detecting full length as well as proteolytically fragmented peptides of CRF containing epitopes. A dot blot strip exhibited distinct color spot for the placental extract, PC-12 cell extract and standard CRF (both as a positive control) but no spot for BSA (negative control) when developed against CRF antibody (Fig. 1A, a-d). Once the presence of CRF in the extract was detected, it was quantified by CRF EIA kit using manufacturer's protocol. The concentration of CRF in the extract was calculated with reference to a calibration curve of using standard CRF. The placental extract also demonstrated linear dependency of absorbance against concentration (R^2 = 0.961). The concentration of CRF in the placental extract was estimated to be 0.45 ± 0.05 μg/ml (n = 4 where n represents number of independent experiments).

The placental extract being very dilute, it was applied directly to a CRF antibody coupled to an immunoaffinity matrix for single step purification with good recovery yield. Developed profile was similar to a typical immunoaffinity chromatogram (Fig. 1B). Elution of CRF was confirmed by dot-blot experiments (Fig. 1B, Inset). Pooled fractions were quantified to contain 38 μg of CRF that was recovered from 100 ml of the extract. This yield suggested that the methodology was reasonably efficient. The purity of CRF was verified from the mass spectrometric analysis that showed the presence of a single component of 4785 Da (Fig. 1C). The preparation may contain other components that failed to ionize under the detection conditions or impurities of low abundance or fragmented products of CRF. However, the abundance of such components was beyond detection limit in the MS analysis. Whereas, the molecular weight of the standard CRF that appeared as a single peak of 4755 Da (Fig. 1C, inset). Data revealed the difference of 30 Da between molecular weight of standard CRF and purified CRF from placental extract which might be due to the remaining salt content in purified fractions or alteration in one amino acid residue (Fig. 1C). Also, Shibasaki et al., 1982 reported that the molecular size of the placental CRF-like substance is slightly larger than that of adrenocorticotropin [23].

3.2. In vitro scratch assay

To demonstrate the effects of placental CRF on the migratory capacity of cells involved in wound healing, if any, *in vitro* scratch wounding assay was performed [19]. In this model, the gap at the initial stages was mainly covered by cells that moved to close it rather than by cells only to proliferate. Cells were treated with placental extract, placental CRF, standard CRF or PBS and viewed for 0–72 h. The results were a combination of proliferation and motility expressed as% of the distance remained open at the particular time point (Fig. 2). At 0 h, relative wound size was represented as 100%. At 24 h, 77.08 ± 3.56% of the initial gap was still open in control, while 36.93 ± 1.17, 41.09 ± 3.56 and 25.12 ± 1.17% of the gaps were open in the sets treated with placental CRF, standard CRF and placental extract respectively (n = 3). Corresponding values after 48 h were 12.75 ± 0.35, 13.78 ± 0.25 and 9.82 ± 0.35% respectively as compared to 55.42 ± 0.65% for the control (n = 3). This data suggested that placental CRF in μM range promoted cell motility and exerted an inductive effect on migration of cells during wound healing. This, in turn, indicated placental CRF accelerates wound healing. Data obtained favors the earlier report that indicated local injection of CRF dramatically reduces acute inflammation and enhances wound healing [25]. In the context of wound healing, one report suggested that dermal fibroblast cells cultured in a medium that was deficient in growth factor showed stimulation of cell proliferation with neuronal CRF [26].

To check whether the enhancement of migration of fibroblast cells of the purified fraction was due to placental CRF, antalarmin (100 nM), an antagonist of CRF_1 (type 1) was pre-incubated with cells at 37 °C for 1 h [27]. Subsequently, the cells were treated with placental component as mentioned above. The rate of migration of fibroblasts cell line was followed till 48 h in the presence of placental CRF. It was comparable to the control sets where PBS was applied (Fig. 3). This suggested that the functional placental component was indeed CRF and it had a positive role in wound closure. This study also shows that placental CRF belongs to CRF subclass 1.

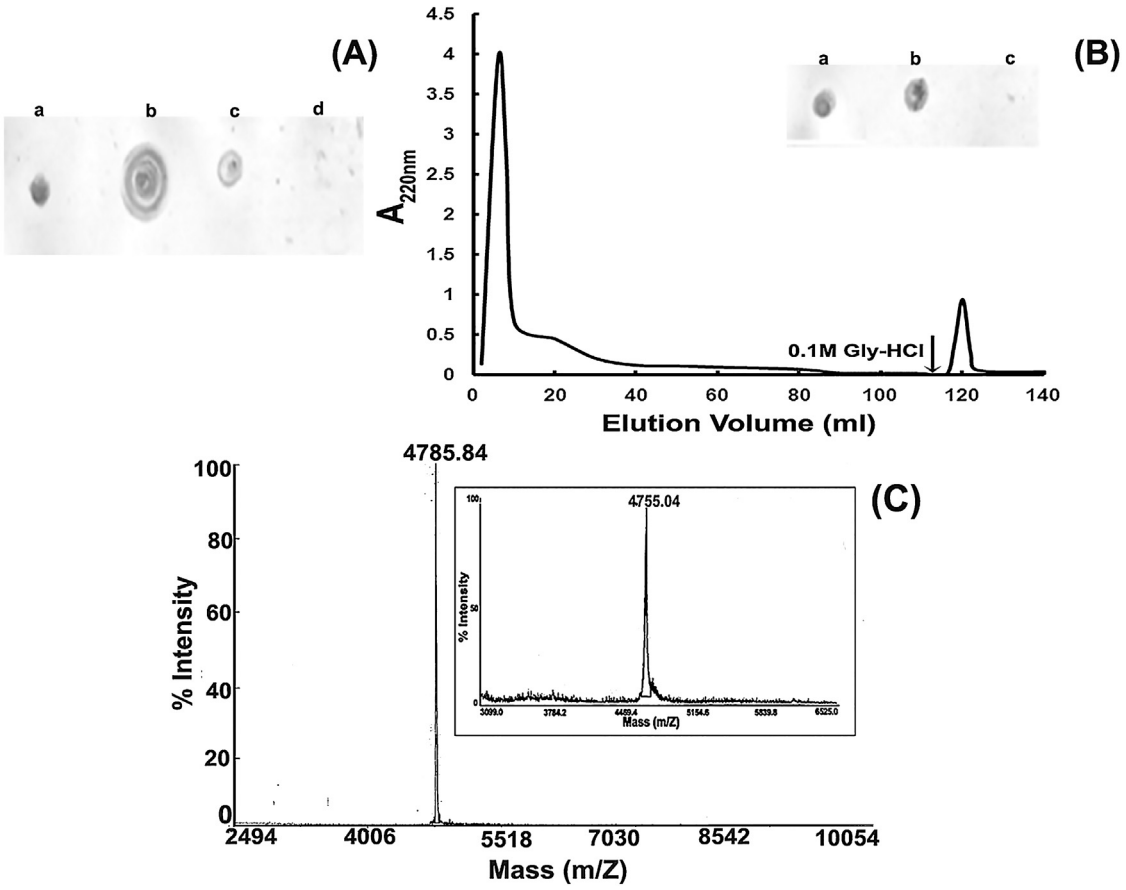

Fig. 1. Identification, purification and quantification of CRF in placental extract. (A) Immunoblot. (a) Placental extract, (b) PC-12 cell lysate, (c) standard CRF, (d) BSA. (B) Immuno-affinity chromatogram indicated elution of a single bound fraction. The arrow indicates application of elution buffer 0.1 M gly-HCl, pH 2.4. Insets: Immunological cross reactivity between anti-rabbit IgG (CRF) and (a) Placental CRF (10 μg), (b) Placental extract (10 μg), (c) CRF standard (10 μg) and (d) BSA (50 μg). (C) Mass spectra of purified CRF from placental extract. Inset: Mass spectra of standard CRF.

3.3. Measurement of apoptosis

Apoptosis is crucial to normal wound healing particularly in the removal of inflammatory cells and in evolution or generation of granulation tissue to scar. When cells proliferate rapidly during tissue restoration, cell growth is balanced by apoptosis. There are evidences supporting the fact that the cascades of cellular events occur during the healing of the cutaneous wound and other tissue repair processes are tightly regulated and controlled by a distinct pattern of cellular apoptosis. In an early progression of normal wound repair, cellular infiltration and proliferation must be sufficient and explicit. Rapid increase in cell proliferation is allowed by an initial decrease of apoptosis. The fibroblasts migrate, proliferate and synthesize components of extracellular matrix participating in the development of granulation tissue. Measurement of apoptosis was followed by *in-vitro* scratch treatment with placental CRF (10 μg/ml) and PBS as a negative control. Apoptosis and necrosis percentage in fibroblast cells were assessed during healing with time after treatment with placental CRF. This estimation is directly related to tissue repair and wound healing. Results showed that the *in-vitro* scratched WI-38 plates followed by treatment with placental CRF indicated a decrease of apoptosis from 72 ± 5 to 43 ± 7% after 24 h and 56 ± 8 to 14 ± 2% after 48 h when compared with the controls (Fig. 4). Evidence also indicated some necrosis in 48 h indicating that CRF decreased apoptosis and initiated tissue repair. This data confirmed that placental CRF helped in proliferation and healing.

3.4. Excision wound model

While monitoring the time course, it was observed that the wound closure in untreated mice was significantly delayed as compared to ones where placental CRF, standard CRF and placental extract were applied. Relative wound size in control rats were 65.34 ± 2.8% on the 2nd day of injury which healed to 48.26 ± 3.2% on the 8th day. Complete epithelization and healing were observed on the 15th day (data not shown). Relative wound sizes in rats treated with placental CRF and standard CRF reduced from 68.09 ± 6.3 to 14.12 ± 5.0% and 64.07 ± 7.5 to 10.14 ± 4.0% respectively from 2nd day to 8th day of wound formation. Similar closures of wounds were 55.09 ± 4.6 to 12.43 ± 5.9% for the placental extract. These values were comparable. Contraction of wound size in rats treated with placental extract was even faster as compared to CRF treated rats probably due to the presence of several potent bioactive components involved in healing in it. This experiment strongly supported the initiation of wound healing by placental CRF (Fig. 5 and inset).

3.5. Effect of purified CRF on cytokines

Of the innumerable cytokines, IL- 6 and TGF- β are intimately associated with wound healing. IL-6 plays crucial roles in inflammation, mostly at the early phase of creation of wound. IL-6 along with other pro-inflammatory cytokines and growth factors are produced locally in both skin cells and resident immune cells. It is a pleiotropic cytokine which has a significant role in the growth and

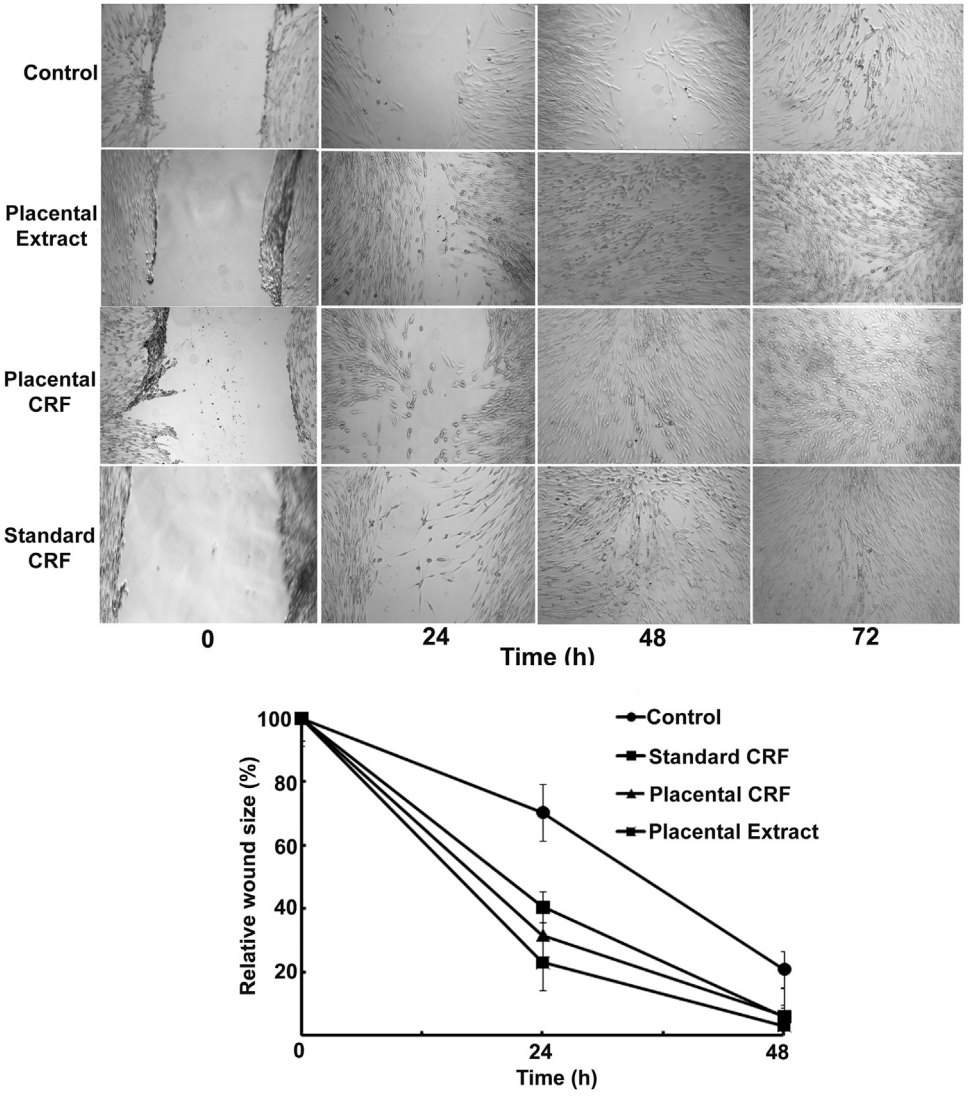

Fig. 2. Placental CRF induces the motility of WI-38 cells in an *In-vitro* scratch assay. WI 38 cells were stimulated with placental CRF, placental extract, standard CRF and PBS. Images were captured at 0, 24, 48 and 72 h after disruption of a small area of the cell layer. The remigration of the cell was estimated by measuring the distance on each image with the program Image J, and expressed the distance as% of the average. Results represent the average of three independent experiments.

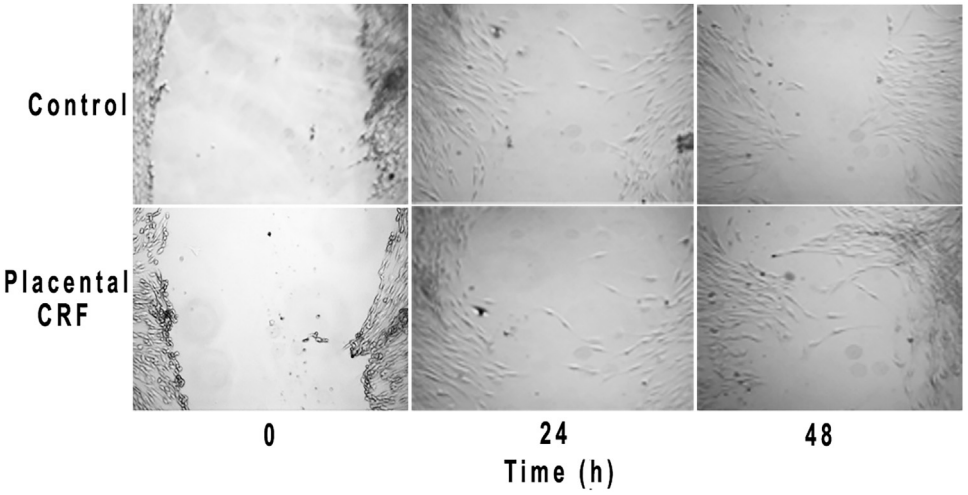

Fig. 3. In the scratch wound healing experiment, WI 38 cells were pretreated with antalarmin, an antagonist of CRF type 1 receptor. Images were taken for 0, 24 and 48 h after incubation with PBS as control or placental CRF.

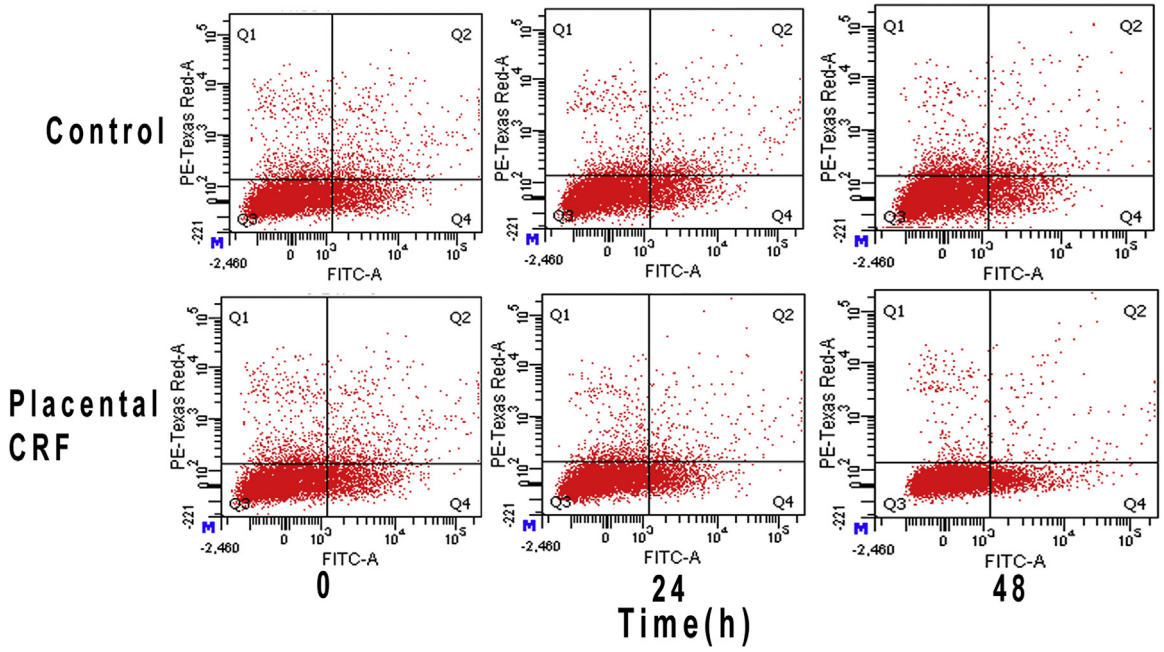

Fig. 4. FACS analysis for apoptosis measurement of *in vitro* scratched WI-38 cells. Upper panel: Control (0, 24 and 48 h) and lower panel: Placental CRF (0, 24 and 48 h).

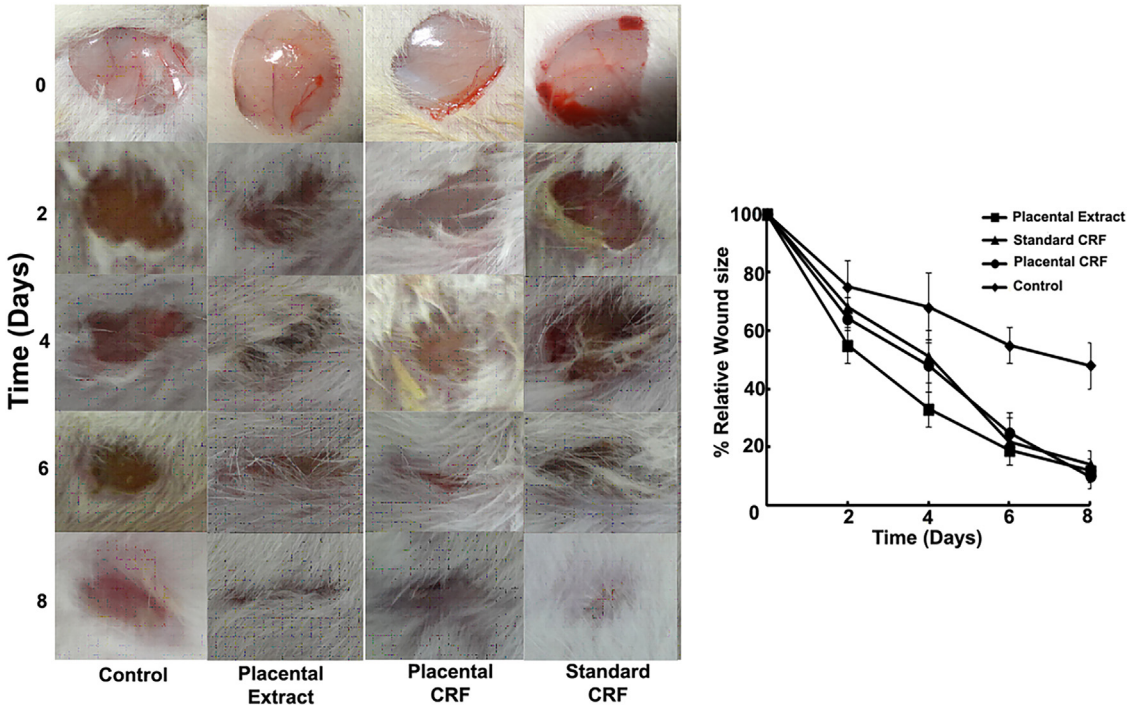

Fig. 5. Photographic representation of rate of contraction measured in percent of wound area on different post-excision days of control, placental extract (5×, 500 μl), and placental CRF and standard CRF (200 μg/ml) treated rats.

differentiation of several cell types mostly of dermal and epidermal origin and acts as a major regulator of the healing process [18]. When cells are subjected to an *in vitro* wounding, IL-6 is readily identified in cutaneous wounds and in the supernatant of keratinocyte cultures [28].

TGF-β is another major regulator of wound healing. It is a growth factor involved in a number of processes such as inflammation, fibroblast proliferation, and collagen synthesis and remodeling of the newly formed extracellular matrix [14]. The previous report indicated that the treatment with exogenous TGF-β1 has improved the process of healing [29]. The result indicated that the subcutaneous injection of placental CRF, standard CRF (serving as a positive control) in wounded rats increased the levels of IL6 to a comparable extent. As shown in Fig. 6A, when placental CRF (200 ng/ml) was injected to wounded rats on every alternate day induced the production of IL6 from 20 ± 2.1 to 36 ± 3.2 ng/ml on the 2nd day of wound formation. Results observed were comparable with the positive control data [standard CRF (200 ng/ml)] which was 19 ± 2.8 to 32 ± 3.1 ng/ml on the 2nd day. Later this elevation of IL-6 decreased gradually to the basal level in the CRF treated rats with healing. A

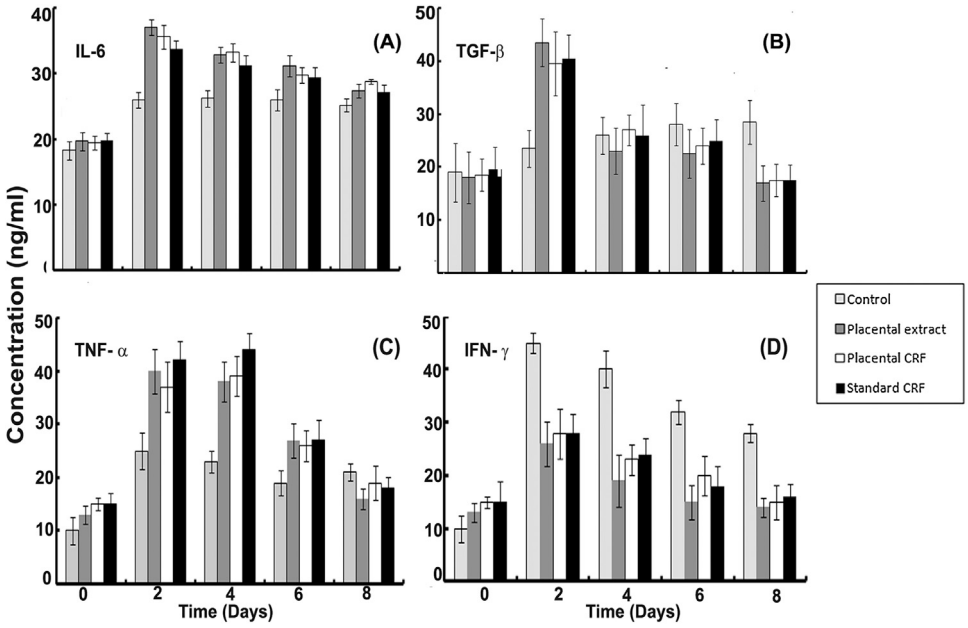

Fig. 6. Induction of cytokines level during wound healing mechanism. (A) IL 6 (B) TGF- β (C) TNF-α (D) IFN-γ with dose of placental extract (5×, 100 μl), and placental CRF and standard CRF (200 μg/ml).

Similar effect was observed with placental extract which induces IL6 production from 21 ± 3.1 to 38 ± 4.7 ng/ml on the 2nd day of creation of wound (Fig. 6A).

Similar to IL6, levels of TGF- β in control rats was slightly increased from 22 ± 2.7 to 24 ± 5.1 ng/ml on the 2nd day of injury which later remained constant due to delayed in healing. While, placental CRF induced TGF- β from 18 ± 2.7 ng/ml to 25.5 ± 2.7 ng/ml. Rats treated with placental extract showed maximum elevation due to multiple healing components present in it [1–9]. Alike IL-6, it was observed that after the sixth day of wound formation, rats treated with placental CRF, standard CRF and placental extract, release of TGF-β was gradually decreased indicated that healing reduced their induction (Fig. 6B).

Another major process involved in the early stages of wound healing is inflammation [30]. During the initial stage of wound repair, the inflammatory response occurs where recruitment of cells is done to act against potential bacterial contamination of the wound and stimulates cytokine secretion to activate the processes of dermal and epidermal repairing. Hence, to estimate the effect of placental CRF on inflammation during healing mechanism, TNF- α and IFN-γ were analyzed in the serum of excision rats. It was observed that levels of TNF-α significantly increased after 2nd of injury in placental CRF, placental extract, and standard CRF treated rats compared to control sets (Fig. 6C). The level of TNF- α was 42 ± 3.6, 37 ± 4.7, 40 ± 3.4 ng/ml in placental CRF, standard CRF and the placental extract treated rats respectively, which was significantly higher than the control (25 ± 3.2 ng/ml). A similar effect was observed for IFN-γ, where its elevation in serum increased on the 2nd day of injury after treatment with placental CRF, placental extract, standard CRF treated samples but maximum level of expression was observed in controls. Later, IFN-γ levels decrease in treated rats whereas inflammation was still observed in controls (Fig. 6D). These data indicated that wound healing led to the reduction in inflammation and suggested that CRF has anti-inflammatory activity. Previous literature also indicated that during the early phase of wound repair, pro-inflammatory cytokines are predominantly expressed in polymorphonuclear leukocytes, and are reduced during progression of wound healing with gradual disappearance of the inflammatory phase [30]. Highest levels of all these cytokines were seen at early 24 h after wounding (Fig. 6). After completion of the proliferative phase of healing, levels of these cytokines returned to the basal level as compared to the controls. Results showed that the level of both the cytokines in serum of rats treated with placental CRF and standard CRF were significantly increased during the 2nd day of injury as compared to the rats treated with PBS. The rise of cytokine levels was later reduced to the optimum level on healing. In control set of animals, cytokine level increased slowly and remained approximately constant till 10th day of injury due to delayed in healing. This data showed the placental CRF induced IL 6 and TGF-β during healing. In the context of cytokines biology in healing process, pro-inflammatory cytokines also play a vital role in healing. Therefore, impact of placental CRF on TNF-α and IFN-γ was also evaluated. Results indicated decrease in level of both the cytokines from the day of injury as compared to the control.

3.6. Toxicity studies

3.6.1. Acute toxicity

Acute toxicity measurements revealed that neither any sign of motility and morbidity appeared in any animal after administration of CRF. There was no alteration in the clinical observations for body weight, behavioral patterns or food and water consumption among treated and control animals. Under the conditions of acute toxicity testing, LD_{50} value for purified CRF was estimated to be greater than 1000 μg/kg body weight (Table 1). Moreover, measurement of toxicity indicated safety efficacy of administration of placental CRF on rats during healing showed LD_{50} value to be greater than 1000 μg/kg of body weight (Table 1).

3.6.2. Sub chronic toxicity

During analysis of sub chronic toxicity, no significant changes in biochemical parameters were observed in the serum of treated rats. The body weight of treated and untreated sets of rats increased at comparable rates. The level of serum enzymes viz. SGPT, SGOT and ALP in Placental CRF treated rats did not show detectable variations as compared to the control groups (PBS treated rats). The mark-

Table 1
Biochemical parameters evaluating toxicity, if any, in rats treated with purified CRF in an excision in vivo wound model.

Biochemical markers	Acute toxicity			Sub – chronic toxicity		
	Control (n = 6)	Treated (n = 6)	Control (n = 5)	Treated (n = 5)		
		1000 μg/kg		100 μg/kg	500 μg/kg	1000 μg/kg
SGPT (1U/L)	27.80 ± 3.10	29.65 ± 2.45	26.56 ± 5.70	25.54 ± 5.75	27.76 ± 4.56	26.54 ± 6.30
SGOT (1U/L)	28.79 ± 1.15	25.73 ± 4.28	31.85 ± 4.92	29.87 ± 4.68	32.34 ± 2.80	28.93 ± 3.10
ALP (1U/L)	222.56 ± 1.20	245.00 ± 6.09	253.65 ± 7.56	268.13 ± 3.65	252.00 ± 5.80	298.07 ± 6.65
Urea (mg/100 ml)	53.89 ± 1.44	62.67 ± 4.70	61.53 ± 7.52	52.56 ± 2.36	61.79 ± 6.50	365.92 ± 2.70
Creatinine (mg/ml)	0.96 ± 0.08	1.11 ± 0.06	1.17 ± 0.05	1.13 ± 0.02	1.19 ± 0.07	1.15 ± 0.02

n = number of animals tested.

ers of renal functions viz. urea and creatinine levels also remained statistically unaltered (Table 1).

4. Conclusion

This study investigates the stability and biological functionality of placental CRF in the aqueous extract of human placental. Presence of intact CRF in terms of the amino acid chain has been confirmed by mass spectrometric analysis of the purified peptide. Subsequently, the role of purified CRF from placental extract in cell proliferation, apoptosis, wound healing was elucidated along with cytokine release and inflammation in vitro and in vivo. In addition, this study indicated that administration of CRF induced insignificant toxicity to excision wounded rat during healing.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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