Review Article

Biochemical Characterization of an Aqueous Extract of Human Placenta: A Compendium

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Abstract

Placenta supplies all the nutrients and protective agents required for development of budding fetus. Being a rich source of many biologically active components, the extract of human placenta contains amino acids, peptides, proteins, nucleotides, lipids, glycosaminoglycan, growth factors and other therapeutically active bioorganic compounds. Various extracts of placenta have been prepared, only an aqueous extract of human placenta has been shown to be of immense therapeutic value particularly in wound healing and pelvic inflammatory diseases (PID). The composition of the extract of placenta tissue depends on the method of its preparation and accordingly they show different therapeutic activities. An aqueous extract of human placenta, the trade name is 'Placentrex' is used for wound healing including post-surgical dressings and high degree of burn injuries from distant past and it contains several bioactive therapeutic molecules. The injectable preparation of the drug has potent curative effect on pelvic inflammatory diseases also. In this review the extensive biochemical characterization of this indigenous preparation of aqueous extract of human placenta (i.e. drug Placentrex) and its therapeutic efficacy has been described.

Keywords: Human placenta; Wound healing; Pelvic inflammatory diseases; Peptides; Nucleotides; Therapeutic efficacy

Introduction

Placenta, the biochemical treasure house supplies the maturing fetus with substances that the fetus itself cannot synthesize. Being the only discarded human organ, an extensive research has been done on placenta. In India, an aqueous extract of human placenta, trade name is 'Placentrex', manufactured by Albert David Ltd. is used mainly as Wound Healer (as topical preparation) and also for the treatment of Pelvic Inflammatory Diseases (PID) as injectable form. Clinical efficacy of the topical preparation of the extract is well established in various skin conditions including chronic wounds, burns, post-surgical dressings etc. [1]. Efficacy of 'Placentrex' injection in Pelvic Inflammatory Diseases (PID) is also well documented [2].

In modern pharmaceutical and biomedical research, it has become necessary to verify the pharmacological effects of the drugs derived from the natural source. This is to check whether they correspond to the ancient texts and to study the mechanism of actions and also to isolate the active principles. Research on 'Placentrex' has identified some important components like fibronectin type III [3], ubiquitin like peptide [4], corticotropin releasing factor (CRF) [5], laminin [6] and some small molecules such as nicotinamide adenine dinucleotide phosphate (NADPH) [7], polydeoxyribonucleotides (PDRNs) [8], amino acids (e.g. glutamic acid) etc. [9] that might play roles in different therapeutic activities and few more are yet to be identified. The extract has the ability for in vitro nitric oxide induction in macrophages [10]. It can stabilize serine proteases against their autodigestion by reversibly inactivating them, which enhances the efficacy of proteolytic enzymes thereby facilitates wound healing [11]. On the other hand the extract itself showed distinct proteolytic activity too. Both the properties indicate its role in modulation of enzymatic activity [12]. Moreover, recent studies revealed that the extract has an anti-biofilm property against some drug resistance bacterial strains [13].

For any drug to make its mark in the international market, certification from regulatory authority to ensure its safe use without side effects is compulsory. Exhaustive research to verify the pharmacological efficacy of the drugs derived from traditional medicine in pharmaceutical and biomedical arena is

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necessary. This would enable to confirm the traditional knowledge and to understand and verify how the specific pharmacological action of the drug is manifested. Research on the Placentrex reveals ample opportunity for discovery of potent bioactive therapeutic components whose precise mechanism of action is yet to be elucidated.

Here, the extensive research on biochemical characterization of the aqueous extract of human placenta (i.e. drug 'Placentrex') and its efficacy has been described and some of the on-going research process has been highlighted that lead to the identification of some potent components present in it that might play roles in therapeutic activity of the drug.

Placenta extract as a therapeutic agent

In 1934, Prof. V.P. Filatov, an eminent Russian ophthalmologist, proposed doctrine of 'Biogenic Stimulators'. He observed that damaged and stressed tissues develop certain compounds to survive. Filatov was convinced that any tissue of human or animal origin could be used to obtain curative effect and this tissue may not necessarily correspond histologically to the tissue affected by the pathological process. This tissue need not necessarily be grafted in the vicinity of a diseased tissue or organ. All these preliminary data enabled him to formulate an important conclusion, which became the basis of the therapeutic action of Biogenic or Biogenous Stimulators [14].

Filatov had outlined general protocols for preparation of placental extract and initiated modern placenta therapy [15]. The various preparations of placenta as described by Filatov include aqueous extract, lipid extract, immunoglobulins and tissue coagulants. Of these only the aqueous extract of placenta acts as potent biogenous stimulator. This aqueous extract has the following actions in the body: accelerate cellular metabolism, aids in absorption of exudates, stimulates regeneration process and increase physiological function of organ/s. A variety of biologically active substances such as hormones, proteins, glycosaminoglycans, nucleic acids and polydeoxyribonucleotides (PDRNs) have been identified in the extract. It is also a rich source of many bioactive peptides, vitamins, amines, micronutrients, lipids and enzymes. Such extracts are used as a licensed drug for wound healing in India and abroad and also as biological dressing in post surgical operation and in burn injury [1, 2].

Clinical evaluation of the aqueous extract of human placenta (i.e. Placentrex) has shown that the drug acts as a potent wound healer with anti-inflammatory and immunotropic effect [1]. The injectable form of placental extract was found to be very effective, inexpensive and excellent stimulant of granulation tissue, moreover superior to the dressing of povidone iodine [16]. Aqueous placental extract has long been used as a cosmetic supplement for skin care and skin pigmentation. It has been reported that menopausal symptoms and fatigue in middle-aged women improved after the treatment with the aqueous placental extract [17]. An aqueous extract of human placenta has anti-inflammatory and anti-platelet aggregation activity [18]. Human placental dressing was found to be effective in clinical wound healing for chronic varicose ulcers. Later, this finding was supported by Subramanian et al [19]. Thus, the potency of placental extract with therapeutic properties has earned global recognition.

Preparation of the indigenous aqueous extract of human placenta

In India, several studies have been made on aqueous extract of human placenta (i.e. 'Placentrex') as wound healer. The manufacturing procedure of the indigenous drug holding confidentiality of the proprietary terms is as follows: fresh placentae were stored in ice and portions were tested for HIV antibody and Hepatitis B surface antigen. Single hot and cold aqueous extractions were done after incubating dissected and minced placenta at 900C and 60C respectively. This was followed by sterilization of the extract under saturated steam pressure (15-lbs/sq inch at 120OC for 40 min). After filtration and addition of 1.5% (v/v) benzyl alcohol as preservative, ampoules were filled then were sterilized once again under the said condition for 20 min. In the first sterilization, the extended duration of heat treatment essentially completed precipitation of a number of macromolecules like proteins. This is apart from adding safety margins to the temperature, time or both to destroy most resistant spore-producing species like Clostridium tetani. The terminal sterilization step was to maintain sterility of the products after they were filled and sealed in ampoules. Each milliliter of the drug was derived from 0.1 g of fresh placenta. A single batch was prepared from the pool of several placentae. Carried over bioactive components in the extract depends on the method of its preparation [7].

Bio-chemical characterization of the drug 'Placentrex' (an aqueous extract of human placenta)

Major findings with the aqueous extract of human placenta (i.e. drug 'Placentrex') include minimal batch variation of extract using conventional independent spectroscopic and chromatographic techniques e.g. UV-absorption spectra, FT-IR, TLC, HPTLC and HPLC etc. as well as by newly developed method for fingerprinting of multi-component drugs using fluorescence Excitation-Emission-Matrix (EEM) plots [20]. This consistency reflects standardization of the manufacturing process of the drug.

Evidence for existence of free NADPH (cofactor for enzymes) in 'Placentrex' has also been shown using thin layer chromatography and reversed-phase HPLC. Biological functionality of the fluorophore in the drug has been confirmed by enzymatic assay [7, 21]. It has been reported that reduced form of NADP (NADPH) in aging human skin cells in vitro increases synthesis of collagen involving filaggrin and keratin [22].

Anti-microbial property against a large number of pathological micro-organisms (to prevent secondary infections to wounds) has also been shown. The extract has an effective inhibitory role on the growth of different microbes particularly growth of clinically isolated bacteria, e.g. E. coli from urine and blood culture and S. aureus from pus. The extract has both bacteriostatic and fungistatic activities. Dose-dependent response of the extract was observed. A mixture of polydeoxyribonucleotides appears to be the causative agent. Partial protection of the wound from secondary microbial

infection is thus indicated. Though the mechanism of such growth-inhibitory activities has not been studied, it is predicted that the PDRNs present in the extract enter the microbes and interfere with their replication machinery [23].

PDRNs are mixture of DNA fragments of different molecular weight. A spectrofluorimetric method of quantitation of PDRNs in 'Placentrex' by using ethidium bromide (EtBr) had been described. It had been demonstrated by thin layer chromatography (TLC) followed by reversed phase HPLC that EtBr binds specifically with the PDRN fraction of the multi-component aqueous extract of human placenta (i.e. Placentrex). The binding specificity of EtBr has been verified by the analysis of emission spectra of the extract. PDRN content of the extract has been estimated based on the resultant fluorescence emission (after background correction) with respect to the standard calibration curve of calf thymus DNA (CT-DNA). Estimation of PDRN in a large number of batches of placenta extract (n = 100) has been done. The statistical analysis of the estimation was found to be significant and the lower and upper levels of PDRN were 158.30 and 239.03 µg/ml of the extract respectively [24].

'Placentrex' also has the ability for in vitro Nitric Oxide (NO) induction in mouse peritoneal macrophages [10]. NO acts as a biological signaling and effector molecule capable of diffusing across membranes and reacting with a variety of targets. Once induced, production of NO within the tissue can induce an environment that is toxic to invading microorganism. It promotes inflammatory mediation of repair mechanism and wound matrix development followed by remodeling. NO mediated cellular signaling possibly provides enhancement of wound repair by increasing tissue oxygen availability through angiogenesis [25].

Rapid migration of neutrophils to the wound site is a prerequisite to the wound healing process. Gel filtration analysis of placental extract gave the initial cue about the presence of migration-promoting factor of the extract. HPLC analysis of the extract revealed glutamate to be the predominant free amino acid. Recent studies show that glutamate induced phenotypic neutrophil chemotaxis, as seen in the time lapse- and transwell assays. Glutamate was also found to induce chemokinesis of the neutrophil, though the stimulation of chemotaxis was more pronounced [9].

Glutamate identified in Placentrex at an optimum concentration induced phenotypic neutrophil chemotaxis, as seen in the time lapse and transwell assays. The glutamate induced chemotaxis was accompanied by polarization of the actin cytoskeleton, and by polymerization of F-actin. These data indicate that glutamate has a strong chemotactic functionality in the neutrophil, which could be of interest both therapeutically and in further investigation of the molecular basis of chemotaxis [26].

Another important finding was isolation and purification of a peptide of around 7.4 kDa from the extract. Derived partial amino acid sequence from mass spectrometric analysis showed its homology with human fibronectin type III. Under nondenaturing condition, it formed aggregate, the elution pattern of which was identical to that of fibronectin type III as confirmed by reverse-phase HPLC. Immuno-blot of the peptide showed strong cross reactivity with reference human fibronectin type III-C [3]. It draws special attention because its partial amino acid sequence showed homology with 10th type-III fibronectin peptide that also contains the 'RGD' signature sequence endowed with cell adhesion properties [3]. The importance of fibronectin in cutaneous wound healing is well documented as a general cell adhesion molecule by promoting the spread of platelets at the site of injury. It also helps in the adhesion and migration of neutrophils, monocytes, fibroblasts, and endothelial cells into the wound region, and the migration of epidermal cells through the granulation tissue [27].

Recently, the presence of corticotropin releasing factor (CRF) in 'Placentrex' 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±0.05µg of CRF per ml of placental extract where its molecular weight found to be 4.78kDa 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. 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 [5].

Immuno-blots revealed presence of laminin in 'Placentrex' (70 ± 0.257 lg/ml; n=3). It was purified using immuno-affinity chromatography. SDS-PAGE and SE-HPLC indicated a protein with some small peptides. Since placental laminin existed in its truncated form, its roles in cellular migration, differentiation and wound healing were verified. Induction of cellular migration and motility in rat fibroblasts were enhanced by placental laminin as observed from scratch wound assay. Promotion of neuronal differentiation of PC12 cells by placental laminin was observed by phase contrast microscopy. Confocal images showed presence of laminin on the cell surface and along the axonal processes. Significant interaction between integrin receptors and laminin responsible for cellular differentiation was demonstrated from co-localization experiments. Union between integrin receptor and its synthetic antagonist revealed retarded pattern of neurite outgrowth in laminin treated cells. Animal model studies revealed faster wound healing in the presence of placental laminin. Induction of re-epithelialization and angiogenesis in wound area by cellular proliferation and adhesion were observed. The cytokine levels showed an initial rise and gradual fall over the duration of wound healing on application of the fragmented laminin. Thus, roles of placental laminin in neuronal differentiation and wound healing were indicated [6].

Proteases play important roles in wound healing by regulating the balance between tissue degradation and regeneration [28]. Slow removal of the necrotic tissue due to insufficient protease activity delays the onset of healing. Human placenta extract shows stabilization of trypsin against autodigestion as one of the peptides of the extract binds very strongly with the protease. Inhibition of esterolytic activity by trypsin in presence of peptide fraction of the extract indicated blocking of the catalytic site of the enzyme. Rayleigh scattering, size-

exclusion HPLC, fluorescence resonance energy transfer, and surface plasmon resonance show that fibronectin type III-like peptide present in the extract (i.e. drug Placentrex) interacts with trypsin. The peptide–trypsin complex is dissociated in presence of high concentration of substrates. Thus, regulation of trypsin activity by the placental extract is evident [11].

On the other hand the 'Placentrex' exhibits strong gelatinase/collagenase activity in zymography. 2-D gel electrophoresis of the extract with gelatin zymography in the second dimension displayed a single spot, identified as ubiquitin-like component upon MALDI/TOF MS/MS analysis. Immunoblot indicated presence of ubiquitin and absence of collagenase in the extract. Collagenase activity of the ubiquitin-like component was confirmed from the change in solubility of collagen in aqueous buffer, degradation of collagen by size-exclusion HPLC and atomic force microscopy. Quantification with DQ-gelatin showed that the extract contains 0.04 U/ml of collagenase activity that was inhibited up to 95% by ubiquitin antibody. Bioinformatics studies suggest that placental ubiquitin and collagenase follow structurally divergent evolution [4]. This thermostable intrinsic collagenase activity of 'Placentrex' might have wide physiological relevance in degrading and remodeling collagen as it is used as a drug for wound healing and pelvic inflammatory diseases.

Wound healing can be broadly categorized into three overlapping phases both in terms of time and space cleansing or debridement, following which proliferation occurs to provide a platform for tissue regeneration and finally differentiation occurs. During debridement, extensive 'hydrolytic activity' ensures proper cleaning of the wounded tissue. The last two stages of healing require extensive 'synthetic activity' and minimal hydrolytic activity. Trypsin and similar proteolytic enzymes help in debridement and prevent keloid formation during wound healing and therefore regulation of its activity is an important criterion. Trypsin, collagenase etc. were reported to be effective in wound healing as a debriding agent. These agents remove foreign bodies and necrotic tissues and reveals healthy, bleeding tissues so that the wound can heal.

Proportionate mixing of 'Placentrex' with some proteolytic enzymes and subsequent evaluation of its efficacy in wound healing, may be another promising avenue of the future study leading to the development of an effective wound healer with debridement potential. This multifaceted character of this aqueous extract of Human Placenta encourages further investigation following which the biochemical basis of its mode of action is likely to be highlighted.

Conclusion:

Placenta considered as a 'treasure house' for biologically active compounds. Components of the extract warrant individually examined to provide a broader perspective of a part of its multi facet character. Initial studies on such extract revealed presence of a functional peptide in relatively high abundance. Fibronectin type III-like peptide [3], Laminin [6] identified in the extract finds special importance in wound healing as these are vital components of tissue matrix. Identification of Ubiquitin like peptide [4], CRF peptide [5], NADPH [7] and Polydeoxyribonucleotide fragments (PDRNs) [24] further strengthen the claims of Placentrex as a wound healer and an anti-inflammatory agent.

Interaction of these peptides with other bio-molecules may be followed so that the knowledge could be applied for designing and developing newer formulations by supplementing these purified components. Important therapeutic properties like immunomodulation and DNA damage repairing ability of the drug 'Placentrex' needs to be investigated. With successful continuation, synergistic effect of different components could be followed resulting in an efficient formulation for wound healing as well as for PID.

While development of synthetic drugs has reached a near-saturating level, it is now globally accepted that newer drugs are to be derived from natural sources. Being the only discarded human organ, use of human placenta is being encouraged for alleviating human diseases using traditional knowledge. Once all the active components are identified and a proper biochemical basis of their actions is defined, these components may be used even for newer purposes. This review provides rational and scientific foundation for such applications.

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