**Analysis of Low Molecular Proteins Obtained From Human Placental Extract Considered as New Strategic Biomaterial for Pulp-Dentinal Regeneration**

**Dr Ashmitha K Shetty1, Dr Swaroop Hegde2, Dr Anitha Murali3, DrAshish J Rai4, Qhuba Nasreen5**

1 Associate Professor, Ph.D Scholar, Dept of Pedodontics and Preventive Dentistry, Faculty of Dental Sciences, M .S. Ramaiah University of Applied Sciences, Bangalore-560 054,

2Professor, MDS, Dept of Conservative and Endodontics, Faculty of Dental Sciences, M .S. Ramaiah University of Applied Sciences, Bangalore-560 054,

3 Professor and Head, Ph D, Dept of Pharmacology. Faculty of Pharmacy, M .S. Ramaiah University of Applied Sciences, Bangalore-560 054,

4Assisstant Professor, Dept of Oral and Maxillofacial Surgery, AB Shetty College of Dental Sciences, NITTE University, Mangalore-575018

5Post-graduate, Dept of Pharmacology. M .S. Ramaiah University of Applied Sciences, Bangalore-560 054.

\*Contact Author E-mail: [drashmitha@rediffmail.com](mailto:drashmitha@rediffmail.com)

**Abstract:** Dental pulp infections and trauma to pulpal tissue could apparently cause inflammation and eventually cause regeneration of pulp in cellular level as process of physiological events. Pulp response to infection is similar to any other tissue response to infection in the body.

New methodologies explored in the field of regenerative dentistry involves grouping of growth factors, scaffolds and Mesenchymal Stem Cells (MSCs) .The generation of functional dentine by effective stimulation of odontoblast has not yet been achieved though there are numerous strategies have been employed in regenerative dentistry. Need for creating bioactive material that enables the pulpal tissue to heal itself instead of permanently removing the injured tissue and placing prostheses.

Human Placental Extract (HPE) was obtained by mechanical method from tissue of full term individual placenta. The pH of the solution was estimated to be favourable for osteogenesis. The protein depletion technique was employed in order to obtain low molecular weight proteins may contribute to the variation in obtaining potential proteins. HPE obtained was characterised after in-solution digestion and mass spectrometry. Sixteen proteins were identified out of which Multiple inositol polyphosphate phosphatase 1, DACT2, Proliferation marker protein Ki-67 and Zinc finger BED domain-containing protein 1 were of interest. The proteins of interest were either involved in cell proliferation directly or indirectly. Considering this information use of this extract on human dental pulp cell can be functional.

**Key words:** Human placental extract, pulp therapy, Mass Spectrometry

**Abbreviations:** MALDI TOF/TOF, Matrix Assisted Laser Desorption/Ionisation - Time of Flight Mass Spectrometry; ESI-Q-TOF-MS/MS, Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry

**Introduction:**

Endodontic material in managing the pulp involvement in case of deep dentinal caries currently involves removal of necrotic pulp tissue and replacing with inert material[1].The common disadvantage with this material will be loss of sensitivity and vitality and sometime result in secondary infection or post-operative fracture[2].

New methodologies have been explored in the field of regenerative dentistry involving grouping of growth factors, scaffolds and Mesenchymal Stem Cells (MSCs)[3].The generation of functional dentine by effective stimulation of odontoblast has not yet been achieved though there are numerous strategies have been employed in regenerative dentistry [3,4,5]. Need for creating bioactive material that enables the pulpal tissue to heal itself instead of permanently removing the injured tissue and placing prostheses.

The human placenta ensures mutual exchange of nutrients of mother and fetus, determining growth and development of the fetus thus making placenta an exceptionally a temporary organ [6] .Placental is believed to be an allogenic in nature and its geno type could be entirely foreign [7]. The human placental organ possesses an extensive collection of bioactive proteins which are necessary for growth and development of fetus. The studies in the past have suggested the placental extracts comprises of uracil, tyrosine, tryptophan, phenylalanine exhibiting strong anti-inflammatory and anti-oxidant activities [8,9,10,11].

However, at the same time, a large amount of research was little systemized and not always correlated with conventional pharmaceutical and other methods of treatment. Recent developments of cell therapy approaches along with opportunities for autobanking significantly increased the interest in the placenta as a source of biological material.

The major aim of this objective was to extract proteins effectively by chemical or mechanical method. The extract was characterized in most promising way so as to best clinical use in regeneration of dental pulp cells based on scientific literature.

The present objective is critical as extraction of human placental extract has numerous proteins, lipids and glycoproteins. Thus method of extract is important because maximum protein of clinical relevance should be obtained. To confirm this mass spectrometry is done which will characterize the protein in the extract**.**

**Materials and Methods:**

Ethical committee and associated hospital of Ramaiah University of Applied Sciences, Bangalore provided approval for the study. Placenta were collected immediately following C-section delivery (n=1).Fresh full-term human placenta (40 weeks of gestation) weighing between 400-600 g within 30 minutes of delivery was obtained. Excess blood is drained and placenta will be taken for further preparation. Placenta of the patient with the history of growth restriction present, intrauterine, fetal abnormalities, hypertension, anaemia, diabetes, use of drugs or tobacco, premature labour /induced, multiple pregnancy, other medical or obstetric complications(HIV and Hepatitis) was excluded from the study. A portion of placental tissue was minced using sterile steel blade. The minced tissue was processed in food processor place in ice bath before operating at 2000 rpm for 8-10 minutes. This forms a paste-like homogenate. A broad-spectrum proteolytic enzyme inhibitor cocktail (Sigma Aldrich, catalogue number P9599) was added before processing. To the homogenate 2:1(v/v) acetonitrile is added and vortex briefly, this is allowed to stand for 30 minutes to precipitate high molecular weight proteins and highly abundant proteins. Centrifugation of this mixture at 14,000 rpm (13107xg) for 10 min at 4°C to get supernatant (~550 μL). This is separated and transferred to a new eppendorf tube and stored at -200C.

About 200µl of final extract was subjected to Mass Spectrometric analysis for characterization of proteins. UltrafleXtreme MALDI TOF/TOF (Bruker Daltonics) and Bruker Daltonics ESI Q TOF-(Maxis Impact) Mass Spectroscopy analysis was performed at Molecular Biophysics Unit, Indian Institute of Sciences ,Bangalore. In-solution digestion was achieved by addition of 1µL Trypsin and reaction is incubated overnight at 300C.

Peptide sequencing was identified MASCOT and SwissProt software. Peptide Mass Fingerprinting (PMF) searches in MASCOT software were identified at 0.2 Da peptide tolerance .Identification was based on 4 peptides matching as significant. Randomised searches after the obtained peak list is entered in the query.MS/MS analysis was for peaks obtained by ESI-Q-TOF-MS/MS (Bruker Daltonics) were listed in the query for MS/MS search using software MASCOT sequence query. Search was optimized by fixing the parameters. The data was accepted for identification of peptide at 56 or more and significant at p<0.05. SwissProt database was used for protein sequencing and protein identification. Parameter was fixed for taxa as Homo sapiens, missed cleavages was set 1; +/‒0.1 Da for peptide mass tolerance; carbamidomethyl (C) as fixed modification and oxidized methionine for variable modification.

**Results and Discussion:** Human placental extract obtained from 1ml of homogenate into approximate 30ml of aqueous extract solution. The final lyophilized 0.028 g (28mg) extract was obtained from 6ml of extract and stored at 2-30C (Fig:1).About ten proteins were characterized by MALDI TOF/TOF (Table:1)and six proteins by ESI Q TOF (Table:2)Mass Spectroscopy analysis.

**Preparation of lyophilized human placental extract:**

Fig.1: Lyophilized extract obtained from 1ml of homogenate.

**Determination of pH of the solution:** The pH of human placental extract was estimated at 7.44 as per pH digital meter. The pH of HPE was found to be alkaline and mimics the physiological pH ≈ 7.4.The pH of human placental extract has not been mentioned in any of the literature. Thus an attempt to determine the pH of the extract would useful to determine bioactivity when it is used in therapy. Pulp capping material like Biodentine shows pronounced anti-bacterial property because of its increased pH to 12.5.Disinfection of dentine and growth inhibition of bacteria was observed due to increased pH as calcium was released during the setting phase [12].The pH of the biomaterial plays a vital role as activity alkaline phosphatase is highest at a pH of 7.37.Mineralization is sensitive to pH variation. Though a higher and an alkaline pH is required for osteoinduction but the activity diminishes above 7.37 level. Moreover, at 8.0 pH mineralization diminishes in in-vitro and in-vivo. Thus pH variation is crucial at pulp and pulp-capping intersurface[13].

**Determination of protein concentration by Bradford Assay:** Protein concentration estimated was 175 μg/ml. In the present study HPE showed 1ml of the extract was estimated to have 175mg of proteins. In extract prepared by water soluble method Bradford method showed 1ml contained 50mg protein [14].Thus protein extraction method in the present study employed acetonitrile depletion which removed high molecular weight high abundant protein leaving behind low molecular weight and low abundant protein. Thus acetonitrile depletion method efficiently extracted protein form the placenta. Study on consequence of porcine placental extract on proliferation of human fibroblasts showed that protein concentration was 100-200μg/ml was adequate for proliferation of somatic cells originating from mesenchymal stem cells in vitro[15].

Table.1: Proteins identified using MALDI-TOF MS/MS

|  |  |  |  |
| --- | --- | --- | --- |
|  |  |  |  |
|  |  |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Protein | Acc. No | Mw  (kDa) | Score | Matched  peaks |
| ADP-ribosylation factor-like protein 17 | Q8IVW1 | 19.6 | 20 | 2 |
| Protein CASC10 | [Q5T4H9](https://www.uniprot.org/uniprot/Q5T4H9) | 15.1 | 25 | 3 |
| Zinc finger BED domain-containing protein 1 | [O96006](https://www.uniprot.org/uniprot/O96006) | 78.1 | 32 | 7 |
| Keratin-associated protein 15-1 | Q3L176 | 14.9 | 20 | 2 |
| Putative uncharacterized protein encoded by ZNF503-AS2 | [A6NEH8](https://www.uniprot.org/uniprot/A6NEH8) | 20.8 | 36 | 5 |
| Multiple inositol polyphosphate phosphatase 1 | Q9UNW1 | 55.6 | 31 | 8 |
| CAP-Gly domain-containing linker protein 2 | Q9UDT6 | 116.2 | 42 | 16 |
| Proliferation marker protein Ki-67 | [P46013](https://www.uniprot.org/uniprot/P46013) | 358.4 | 46 | 32 |
| 39S ribosomal protein L40, mitochondrial | [Q9NQ50](https://www.uniprot.org/uniprot/Q9NQ50) | 24.4 | 24 | 5 |
| Hemoglobin subunit beta |  | 16.1 |  |  |

Table 2: Proteins identified by ESI-Q-TOF/TOF

|  |  |  |  |
| --- | --- | --- | --- |
| Mass | Gene | Protein | Sequence |
| 16104 | **HBB\_HUMAN** | Hemoglobin subunit beta | **K.VNVDEVGGEALGR.L** |
| 71352 | **ALBU\_HUMAN** | Serum albumin | **K.LVAASQAALGL.-**  **K.DVFLGMFLYEYAR.R**  **K.DVFLGMFLYEYAR.R**  **K.EFNAETFTFHADICTLSEK.E** |
| 15306 | **HBA\_HUMAN** | Hemoglobin subunit alpha | **K.VGAHAGEYGAEALER.M** |
| 521940 | **STAR9\_HUMAN** | StAR-related lipid transfer protein 9 | **R.RQVGEAAAGR.G** |
| 83287 | **DACT2\_HUMAN** | Dapper homolog 2 | **R.AGGPLAR.G** |
| 68726 | **EWS\_HUMAN** | RNA-binding protein EWS | **R.GGPGGPGGPGGPMGRMGGR.G** |

Table.3: Biological functions of identified protein according to UniProt.

|  |  |  |  |
| --- | --- | --- | --- |
| Mass | Gene | Protein | Biological pathways  (Uniprot) |
| 19607 | ARL17\_HUMAN | ADP-ribosylation factor-like protein 17 | * They are Arf GTPase-activating proteins (GAPs) mainly involved in cell migration. Helps in protein movement. * It amends formations of vesicles contained in Golgi apparatus. |
| 78106 | ZBED1\_HUMAN | Zinc finger BED domain-containing protein 1 | * Promotes cell proliferation by binding to DNA element of the promoter region of the gene. Initiates transcription by binding to histone H1 promoter. |
| 14968 | KR151\_HUMAN | Keratin-associated protein 15-1 | * It is part of essential hair Keratin-Associated Proteins (KRTAP), by creating cross-linking bond with hair keratin residues like cysteine forming to rigid and resistant hair shaft.Keratin protein matrix with high-sulphur and high-glycine-tyrosine keratins. |
| 55654 | MINP1\_HUMAN | Multiple inositol polyphosphate phosphatase 1 | * Induces transition of chondrocytes to proliferate or causes of hypertrophy.Involves in endochondral ossification in bone development. |
| 116231 | CLIP2\_HUMAN | CAP-Gly domain-containing linker protein 2 | * Involves in control of organelle translocations specific to brain. * Helps to link dentritic lamellar body the neuronic gap junction to microtubules. |
| 358474 | KI67\_HUMAN | Proliferation marker protein Ki-67 | * It covers considerable portion of chromosome surface, thus maintaining mitotic chromosomes dispersed in the cytoplasm.Due to its high net electric charge behaves as a surfactant on the chromosome dispersing and allowing chromosomal motility independently. |
| 521940 | STAR9\_HUMAN | StAR-related lipid transfer protein 9 | * Plays role in mitosis by stabilizing the pericentriolar material. |
| 83287 | DACT2\_HUMAN | Dapper homolog 2 | * It involves in regulating signalling pathways intracellular during formation of the foetus. * It reduces TGF-beta signalling inhibits re-epithelisation of skin wounds. |
| 68726 | EWS\_HUMAN | RNA-binding protein EWS | * May cause unusual activation of target genes of fusion proteins.But generally functions as a transcriptional repressor.These proteins may be involved during the progression of tumor. |

**Identification of Potential Proteins of Interest**:

**MINP1\_HUMAN Multiple inositol polyphosphate phosphatase 1:** Multiple Inositol Polyphosphate Phosphatase (MIPP-1) acts as an enzyme involved in [inositol phosphate metabolism](https://en.wikipedia.org/wiki/Inositol_phosphate_metabolism). It catalyses higher inositol polyphosphates to Inositol (1,4,5) Trisphosphate-3 (Ins(1,4,5)P3) which in turn is a second messenger which consecutively regulates calcium internal storage in the cell. (Ins(1,,4,5)P3)plays a crucial role cellular processes like metabolism, contraction ,proliferation and neural activity[16].For calcium mobilization from Ins (1, 4, 5) P3 sensitive calcium pools which are linked to calcium channels, Ins (1, 4, 5) P3 should bind to receptors associated with it. Calcium release mediated by Ins (1, 4, 5) P3 can be regulated by various drugs and physiological processes [17]. It is observed that MINPP1 invivo participated in homeostatic regulation of and inositol pentakisphosphate InsP5 and inositol hexakisphosphate InsP6 which are concerned cell growth and differentiation of chondrocytes[18].MIPP1 is highly expressed in liver, placenta and kidney. It is observed that human MIPP1 is clinical significant as an increased transformation of inositol phosphate is important in ossification of bone in normal as well as pathological situation such as osteoarthritis and healing of fracture[19].

**DACT2\_HUMAN:** Dapper homolog (DACT) 2 belongs to DACT gene family. DACT gene involve in Wnt Signaling pathway [20].The two signalling system in animals are Wnt signalling and Transforming Growth Factor beta (TGF-β) signalling. They control various processes required for embryonic development, cell differentiation, proliferation and migration. Its role is also significant in cancer, tissue regeneration and stem cell behaviour. DACT 2 is specific in modulating TGF β-dependent wound healing [21].

**Proliferation marker protein Ki-67:** Ki-67 involves in wound healing in early stages of cell proliferation by association with nuclear antigen synthesis in cell cycle phases. Considered as marker for keratinocyte and remodeling of epithelium [22].During mitosis is seen that the proteins are relocated to the surface, but Ki-67 protein is present during all the phase of cell division that is G1, S, G2 and mitosis it is not present at the resting phase G0.Thus Ki-67 makes an excellent cell proliferative maker [23].

**Zinc finger BED domain-containing protein 1(ZBED 1):** ZBED genes controls range of functions in vertebrate tissues .Specifically ZBED1 is associated with cell proliferation. It controls Ribosomal protein gene essential for cell proliferation. ZBED1 is also identified as human-Ac, hDREF and TRAMP [24].hDREF behaves as a transcriptional factor in the process of cell proliferation. It is linked with up regulation of RP genes. Consequently up-regulated RP genes modulate the transition of G1-S phase of cell proliferation. The expression of hDREF reaches peak during S phase in normal human fibroblast. Thus hDERF plays a significant task in cell cycle progression [25].

**Conclusion:** Characterization of low molecular weight proteins in HPE provided insight into the therapeutic proteins. The pH of the solution was also favourable for osteogenesis. The proteins of interest were either involved in cell proliferation directly or indirectly at molecular level. Therefore, owing to these properties of HPE, regeneration of pulp tissue in damaged teeth may possibly preserve the pulp and prevent more radical treatment. This study provides substantial potential of human placental extract in vital pulp therapy.

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