EFFECTS OF HUMAN PLACENTAL EXTRACT ON AGE RELATED ANTIOXIDANT ENZYME STATUS IN D-GALACTOSE TREATED MICE

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Summary

The present study was designed to evaluate the efficacy of Human Placental Extract (HPE) on D-galactose induced oxidative stress in mice. D-galactose is used for the induction of ageing in animal models. Subcutaneous injection of 5% D-galactose (0.5ml/day) produces lipid peroxidation in membranes of various tissues and decline in level of various antioxidant enzymes. The aqueous extract of human placenta (150, 300 and 450 mg/kg body weight) was given once daily, subcutaneously, to female Swiss albino mice for 15 days. Oxidative stress in brain, heart (auricle and ventricle) and liver tissues was estimated using various parameters as malondialdehyde (MDA), glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT) and protein content. HPE (450 mg/kg) administration ameliorated the decreased level of SOD and CAT in mice brain, heart (auricle and ventricle) and liver respectively. MDA level was significantly reduced in the brain, heart (auricle and ventricle) and liver, at the dose of 450 mg/kg. The increase of antioxidant defense system activity in experimental D-galactose treated animals induced by HPE might lead to decreased oxidative status provoked by D-galactose through the inhibition of the production of reactive oxygen species.

Key words: Human Placental Extract, Lipid peroxidation, Oxidative stress, Reactive oxygen species.

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Introduction

Ageing is universal but complex biological process characterized by impairment of various functions and decreased ability to respond stress. Various theories of ageing have been proposed like mitochondrial ageing theory, glycation theory of ageing, free radical induced damage, etc. D-galactose is known to accelerate the process of ageing by formation of advanced glycation end products[1]. Glycation end products increase the oxidative stress by direct radical production by chemical oxidation and degradation of glycation end products and induction of oxidative stress via- receptor binding and activation of signaling pathways[2]. D-galactose, a reducing sugar which promotes formation of free radicals hence increasing lipid peroxidation is reported in several studies for induction of ageing[3, 4].

Human placenta has been demonstrated to possess various therapeutic activities ranging from wound healing to immunomodulatory. Use of aqueous extract of human placenta in wound healing, ophthalmology, infertility, apoplexy, epilepsy etc has evolved from folk knowledge. Recent research studies reveal that human placenta is rich resource of various bio-active substances like proteins, peptides, amino acids, DNA, RNA, polydeoxyribonucleotides (PDRN), enzymes, trace elements etc[5]. In India, Human Placental Extract (HPE) is used as a wound healer in burn injuries, chronic wounds and surgical dressings[6]. Placental extract has a beneficial role as a topical agent in the management of chronic non-healing wounds[7]. Enzyme-linked immunosorbant assay (ELISA) studies revealed that cytotrophoblasts which expressed interleukin-8, a known mediator of inflammation, was suppressed by glucocorticoid present in human placenta[8]. HPE showed positive results against 5- HT induced acute inflammation, cotton pellet induced sub- acute inflammation and inhibition of platelet aggregation[9]. Aq. extract of HPE shows immuno-modulating potential in rabbit as well as in human[10]. A study showed that HPE has a peripheral analgesic property, on chemical and thermal nociception in mice, possibly mediated by an opioid mechanism[11].

In our previous study we have performed *in-vitro* antioxidant activity of HPE and have promising results. The aim of this study was to investigate the effects of aqueous extract of human placenta on age related antioxidant enzyme status in D-galactose treated mice.

Material and Methods

Chemicals: EDTA, sucrose, methionine, riboflavin, hydrogen peroxide, sodium azide, trichloroacetic acid (TCA), ascorbic acid and iron (III) chloride ($FeCl_3$) were purchase from E. Merck (I) Ltd.; Nitro blue tetrazolium (NBT) and thiobarbituric acid (TBA) from Hi- Media. All other chemicals and reagents used were of analytical grade.

Animals: Female Swiss albino mice (*Mus musculus*) of age five-month old (20-25 g) were used for the present investigation. The animals were housed in plastic cages and maintained in ambient temperature and normal light/dark cycle. They were fed standard laboratory food and water *ad libitum*. The experimental protocols were approved by the animal care and use committee of this university.

Test drug: Marketed formulation of Human placenta (Placentrex)

Experimental design: Animals were divided in to following groups (n = 8 per group).

I. Control group: received subcutaneous injections of 0.5 ml sterile water per day for 15 days.
II. D-galactose treated group: Ageing accelerated group received subcutaneous injections of 5% D-galactose 0.5 ml per day for 15 days.
III. D-galactose + HPE co-treated group: received subcutaneous injections of 5% D-galactose and HPE 150 mg/kg body weight, 0.5 ml per day for 15 days.
IV. D-galactose + HPE co-treated group: received subcutaneous injections of 5% D-galactose and HPE 300 mg/kg body weight, 0.5 ml per day for 15 days.
V. D-galactose + HPE co-treated group received: subcutaneous injections of 5% D-galactose and HPE 450 mg/kg body weight, 0.5 ml per day for 15 days.

All animals had free access to food and drinking water during the study. Animals were sacrificed after 24 hours of completion of the treatment by cervical dislocation. Different region *viz*; brain, heart (auricle and ventricle) and liver were dissected out with chilled scissor for biochemical estimations.

Preparation of homogenates: Different tissues were perfused *in situ* immediately with cold 0.9% NaCl and thereafter removed, and was rinsed in chilled 0.15 M Tris–KCl (pH 7.4). They were then blotted dry, weighed and homogenized in ice cold 0.15 M Tris–KCl buffer (pH 7.4) to yield a 10% w/v homogenate. Aliquot of this homogenate was used for assaying lipid peroxidation, while the homogenate for analyzing the specific activities of SOD, CAT and GPX was prepared accordingly.

Assessment of oxidative stress in tissues

Estimation of lipid peroxidation: The extent of lipid peroxidation in tissues was assessed by measuring the level of malondialdehyde (MDA) according to the method of Ohkawa et al.[12]. Briefly, the reaction mixture containing 1 ml of trichloroacetic acid (15%) and 2 ml of thiobarbituric acid (0.38%) were added to 1ml (10%) tissue homogenate. The reaction mixtures were heated for 60 min at 90^{o}C, cooled and centrifuged at 6900 rpm for 15 min. The absorbance of supernatant was measured at 532 nm against blank, which contained all reagents except homogenate. MDA was quantified and expressed as µmol of MDA per mg of wet tissue.

Estimation of superoxide dismutase: The SOD activity was measured by the method of Beauchamp and Fridovich[13]. The homogenate [10 mg/ml] was prepared in 0.25 M sucrose containing 1mM EDTA. The reaction system contained phosphate buffer (pH 7.8), 0.3 ml EDTA (10 mM), 1.2 ml methionine (130 mM), 0.6 ml NBT (750 µM) and tissue homogenate. Reaction was initiated by adding 0.4 ml riboflavin (60 µM) and placing all the tubes in front of fluorescent tube (18 W) of 30 cm length and 2.5 cm thickness and kept in a light proof chamber to avoid interference of external light. The blue colour was measured spectrophotometrically at 560nm.

The amount of SOD source required for half inhibition was considered to contain one unit of SOD.

Estimation of catalase: Catalase (CAT) activity was determined in tissues according to the method by Sinha[14]. In this method dichromate in acetic acid is reduced to chromic acetate when heated in the presence of hydrogen peroxide (H_2O_2), perchromic acid is formed which is an H_2O_2 unstable intermediate. The chromic acid produced is measured calorimetrically at 570 nm. Briefly, 50 µl of supernatant was added to 750 µl of phosphate buffer (0.1 M, pH 7.5). Then, 200 µl of H_2O_2 substrate (50 mM) was added. Exactly 1 min later, the reaction was stopped by adding 2 ml of dichromate (5%) in acetic acid (300 ml). After that, the tubes were kept at 100°C for 10 min. After cooling with tap water, changes in absorbance were recorded at 570 nm. H_2O_2 was quantified using a calibration curve and the CAT activity was expressed as µmol of H_2O_2 consumed per mg of protein.

Glutathione peroxidase (GPX) activity: Hydrogen peroxide was used as the substrate. Sodium azide (1 mM) was added to the reaction mixture in order to inhibit remnant CAT activity. One unit of glutathione peroxidase (GPX) was defined as the amount of enzyme decomposing 1 µmol H_2O_2 per min, at 25°C and pH 7.0. Results are expressed as unit of GPX activity per mg protein[15].

Estimation of protein: Total tissue protein was estimated using method by Lowry, et al. for calculation of specific enzyme activity i.e. enzyme activity/ mg protein[16].

Statistical analysis: All experimental data were expressed as mean ± SD and statistically assessed by one-way analysis of variance (ANOVA). The differences between test animals and controls were analyzed using Scheffe test. $P < 0.01$ was considered to be significant.

Results

The reactive species mediating oxidative stress in organism are thought to be free radicals. Lipid peroxidation reactions are chain reaction driven by oxygen free radicals in which one radical can induce the oxidation of a comparatively large number of substrate molecules. The malondialdehyde level is one of the indices of lipid peroxidation. The MDA levels in the brain, heart and liver were significantly higher in the D-galactose treated group as compared to the normal control mice. Administration of human placental extract (150, 300, 450 mg/kg) once daily for 15 days, caused a significant reduction in the levels of MDA in the brain, heart (auricle and ventricle) and liver as compared to ageing group. These values were even lower than the control group (Table. 1).

Table 1: Effect of HPE on D-galactose induced lipid peroxidation in mice. Values are expressed as malondialdehyde (nM of MDA/mg tissue).

Organ	Control	D-galactose	HPE treated (mg/ kg body weight)		
			150	300	450
Liver	1.53 ± 0.9	2.16 ± 1.4$^{\#}$	2.01 ± 1.4*	1.96 ± 0.9*	1.49 ± 1.01*
Brain	1.24 ± 1.2	1.82 ± 1.2$^{\#}$	1.78 ± 1.34*	1.59 ± 1.32*	1.24 ± 0.9*
Heart (Auricle)	0.95 ± 1.5	1.68 ± 0.8	1.42 ± 1.22*	1.40 ± 1.2*	1.02 ± 1.5*
Heart (Ventricle)	0.97 ± 0.7	1.75 ± 1.1$^{\#}$	1.65 ± 1.0*	1.30 ± 1.4*	1.00 ± 0.9*

Values are Mean ± SD, n=8, $^{\#}p < 0.001$ highly significant compared to control, *$p < 0.005$ significant compared to D-galactose treated mice.

D-galactose challenge animals caused a significant depletion in SOD, CAT and glutathione peroxidase levels, in mice liver, brain and heart (auricle and ventricle) when compared to control group. In the estimation of SOD the tendency of the blue formazan to form a fine precipitate rather than a uniform blue solution makes precise colorimetric readings difficult. Addition of a small amount of detergent minimizes the precipitation of the blue formazan. The SOD activity was measured by the method described by Beauchamp and Fridovich[13]. All the doses of HPE reduced the depletion of SOD and CAT levels as compared to D-galactose treated mice. At the higher dose of 450 mg/kg, the depletion of SOD and CAT levels were significant compared to accelerated ageing group in brain, heart (auricle and ventricle) and liver (Table. 2 and 3).

Table 2: Effect of HPE on D-galactose induced depletion in superoxide dismutase (SOD) in mice. Values are expressed as Units of SOD/ mg protein.

Organ	Control	D-galactose	HPE treated (mg/ kg body weight)		
			150	300	450
Liver	37.45 ± 1.0	3.69 ± 0.6$^{\#}$	9.00 ± 1.4*	15.98 ± 1.5*	21.56 ± 1.2*
Brain	22.54 ± 1.1	6.39 ± 0.4$^{\#}$	8.56 ± 1.2*	12.67 ± 1.3*	14.98 ± 0.9*
Heart (Auricle)	15.47 ± 1.2	4.75 ± 0.7$^{\#}$	5.89 ± 1.0*	7.00 ± 1.1*	7.90 ± 1.2*
Heart (Ventricle)	25.25 ± 1.1	5.55 ± 0.9$^{\#}$	10.11 ± 0.9*	12.34 ± 1.2*	15.56 ± 1.2*

Values are Mean ± SD, n=8, $^{\#}p < 0.001$ highly significant compared to control, *$p < 0.005$ significant compared to D-galactose treated mice.

Table 3: Effect of HPE on D-galactose induced depletion in catalase levels in mice. Values are expressed as mU/mg protein.

Organ	Control	D-galactose	HPE treated (mg/ kg body weight)		
			150	300	450
Liver	98.9 ±1.7	21.0 ± 2.1#	35.56 ± 0.8*	38.51 ± 1.4*	40.45± 1.2*
Brain	95.2 ± 1.2	34.0 ± 1.8#	60.78 ± 1.4*	73.44 ± 1.0*	79.56 ± 1.6*
Heart (Auricle)	198.0 ± 1.9	73.8 ± 2.3#	90.34 ± 1.5*	99.55 ± 0.9*	120.21 ± 0.8*
Heart (Ventricle)	207.0 ± 0.8	110 ± 1.6#	132.41 ± 1.3*	146.00 ± 1.2*	148.99 ± 1.7*

Values are Mean ± SD, n=8, #p < 0.001 highly significant compared to controls, *p < 0.005 significant compared to D-galactose treated mice.

Glutathione peroxidase activity decreased in all the test tissues in accelerated ageing group. In HPE treated group, there was significantly higher activity of glutathione peroxidase as compared to accelerated ageing group as well as control group. All changes were highly significant (Table. 4).

Table 4: Effect of HPE on D-galactose induced depletion in glutathione peroxidase levels in mice. Values are expressed as mU/mg protein.

Organ	Control	D-galactose	HPE treated (mg/ kg body weight)		
			150	300	450
Liver	39.5 ± 1.0	15.0 ± 0.8#	25.0 ± 1.2*	35.51 ± 1.4*	35.98 ± 1.5*
Brain	32.8 ± 1.3	19.6 ± 1.1#	21.8 ± 0.9*	24.67 ± 1.2*	25.61 ± 1.4*
Heart (Auricle)	26.0 ± 1.1	12.7 ± 0.9#	15.9 ± 1.3*	18.67 ± 1.0*	26.45 ± 1.2*
Heart (Ventricle)	34.4 ± 0.9	23.9 ± 1.0#	24.7 ± 1.9*	28.43 ± 1.7*	33.98 ± 0.8*

Values are Mean ± SD, n=8, #p < 0.001 highly significant compared to controls, *p < 0.005 significant compared to D-galactose treated mice.

Discussion and Conclusion

Although, HPE is used in various cosmetic preparations but there is no clear report in literature that it can be used for age related problems. Human placenta has been demonstrated to possess various therapeutic activities like wound healing, immunomodulatory, anti-inflammatory, anti-microbial, peripheral analgesic property etc. In this regard, we previously found and reported antioxidant potential of HPE. The study was performed on inhibition of oxygen derived free radicals (ODFR) *viz*., hydroxyl radical, reducing power, superoxide anion scavenging assay, nitric oxide scavenging assay and anti-lipid peroxidation by HPE. The results obtained suggest that HPE is a potential source of natural antioxidants[17]. L-Trp is one of the main bioactive peptide present in human placenta. L-Trp effect is based on the suppression of lipid peroxidation in the oxidative stress status. Study suggests that L-Trp isolated from human placental extract showed higher inhibitory activity than mannitol and DMSO on the Fenton reaction-induced degradation of 2-deoxy-D-ribose and on cytochrome P-450-dependent lipid peroxidation than uracil. On the other hand, the inhibitory effect of L-Trp on the Fenton reaction-induced protein oxidation was smaller than that of uracil[18]. It is for this reason that we decided to explore the effect of HPE on age related antioxidant enzyme status in D-galactose treated mice.

D-galactose is a reducing sugar, which reacts with free amino groups of proteins, to form in soluble aggregates called as advanced glycation end products[19]. The later produced fifty fold more free radicals than non glycated proteins[20]. These free radicals are superoxide radical generated by protein bound Amadori products in the presence of transition metal ions such as iron. The superoxide radicals are dismutated to hydrogen peroxide and results in generation of lethal hydroxyl radicals[21]. One of the mean through which advanced glycation end products modulates cellular function is through binding to specific cell surface receptor molecules and activate intracellular signal transduction mechanism which evoke formation of free radicals[22]. D-galactose intoxication elicited a significant decline in the antioxidant enzymes (SOD and CAT). In states of oxidative stress, glutathione (GSH) is converted to GSSG and depleted leading to lipid peroxidation. Therefore, the role of GSH as a reasonable maker for the evaluation of oxidative stress is important. To prevent lipid peroxidation, it is very important to maintain the level of GSH. GSSG is reduced to GSH by glutathione reductase, which is NADPH-dependent. It plays a role in maintaining adequate amounts of GSH[23]. Reduced glutathione results in significant elevation in MDA. Increased malondialdehyde is an indication of increased lipid peroxidation in D-galactose treated mice that results due to increased oxidative stress. Increased level of lipid peroxidation in treated mice indicates ageing associated changes[24]. Since during ageing there is increased production of ROS, hence increased lipid peroxidation[25]. In a recent study Deshmukh, et al., also demonstrated increase of lipofuscin (a pigment, characteristic feature of the ageing tissues) in different brain areas of mice treated with 5% D-galactose for fifteen days. Our results showed that in HPE treated group, there was significantly low level of lipid peroxidation indicating that HPE is protecting the membrane lipids from free radical attack. There was significantly low level of lipid peroxidation indicating that HPE is protecting the membrane lipids from free radical attack.

Although increased lipid peroxidation may also be due to formation of advanced glycation products by D-galactose. The decrease of superoxide dismutase, catalase and glutathione

peroxidase activities in D-galactose treated group must be due to non enzymatic glycation of these catalytic proteins. Sakurai, et al., demonstrated decrease of superoxide dismutase activity in erythrocyte during glycation[26]. Decrease in antioxidant enzymes has also been claimed due to decreased rate of transcription caused by oxidative damage to DNA, RNA polymerase and transcription factors[27]. Thus the decrease in antioxidant enzymes in D-galactose treated groups might be due to oxidative modification or post translational modifications of the proteins caused by increased free radicals[28]. It is demonstrated that in ageing tissues, there is an accumulation of enzyme molecules that are devoid of catalytic activity but still retain antigenic determinants[29].

The superoxide anion, hydrogen peroxide and the hydroxyl radical are the major reactive oxygen species (ROS) which function in concert to induce LPO of cell membrane lipids. The natural cellular antioxidant enzymes include SOD, which scavenges the superoxide ion by speeding up its dismutation, CAT, a heme enzyme which removes hydrogen peroxide and GPX[30], a selenium-containing enzyme, which scavenges hydrogen peroxide and other peroxides. The ROS scavenging activity of SOD is effective only when it is followed by the actions of CAT and GPX, because the dismutase activity of SOD generates hydrogen peroxide from the superoxide ion, which is more toxic than oxygen-derived free radicals and requires to be scavenged further by CAT and GPX[31]. Apart from its own toxicity, hydrogen peroxide, in the presence of iron, leads to the generation of toxic hydroxyl ions[32]. Excess of SOD in relation to the activities of hydrogen peroxide removing enzymes like CAT and GPX, are known to induce deleterious tissue effects[33]. The enzyme SOD has a number of functional groups (NH2 and epsilon amino groups of lysine and SH group of cysteine[34]. These groups are highly prone to oxidative damage. Conversion of SH groups into disulphides and other oxidized species (e.g. oxyacids) is one of the earliest observable events during the radical-mediated oxidation of proteins[35]. There was highly significant increase in GPX activity in HPE treated group as compared to the control in almost all regions examined. GPX is preferentially involved in lipid peroxide metabolism. Hence, the results suggest that HPE also stimulates the metabolism of the lipid peroxides. HPE appears to mitigate this stress-induced perturbation by tending to normalize SOD activity and reverse the inhibitory effects of stress on CAT and GPX. These effects would, therefore, lead to decreased LPO, which would then result in attenuation of the adverse effects of oxidative stress. In our study we observed the increased level of superoxide dismutase, catalase and glutathione peroxidase in treated mice as compared to D-galactose treated mice group. The increase in the antioxidant enzyme level was due to scavenging the endogenous metabolic peroxides generated in the various tissues.

In conclusion, our study demonstrated that D-galactose induces marked oxidative stress in mice brain, heart (auricle and ventricle) and liver. All these results suggest that human placenta is highly protective against oxidative damage and ageing induced by D-galactose. It is having multidimensional role. It itself scavenges free radicals, it balances the antioxidant enzyme system and it stimulates metabolism of oxidative wastes like lipid peroxides.

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