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Elevated Lipid Peroxidation, Decreased Glutathione Levels and Changes in Glutathione-Related Enzymes in Rats Treated with Human PIacental Extract

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Abstract

The in vivo effects of human placental extract (1-4 ml/kg) on hepatic lipid peroxidation, blood and liver glutathione (GSH) levels and several enzymes associated with the antioxidant defence mechanism; i.e., catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase, together with some blood biochemical responses were investigated in rats. At an optimal dose level (4 ml/kg), a single acute intraperitoneal administration of the extract caused a significant enhancement (49.9%; $p < 0.001$) of lipid peroxidation with a decline in GSH level both in blood (45.1%; $p < 0.001$) and liver (61.0%; $p < 0.001$) in comparison to control animals. Activities of catalase, glutathione peroxidase and glutathione reductase were inhibited in a dose-responsive way by the treatment with the extract which also increased the activity of glutathione S-transferase in a dose-dependent manner. The extract was found to be hepatotoxic in terms of elevation of serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase, serum lactate dehydrogenase and blood methemoglobin concentration. Results of this study suggest the adverse consequences of the administration of the extract due to its substantial ability to alter normal cellular processes.

KEYWORDS: human placental extract, lipid peroxidation, antioxidant defence components, hepatotoxicity

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The *in vivo* effects of human placental extract (1-4 ml/kg) on hepatic lipid peroxidation, blood and liver glutathione (GSH) levels and several enzymes associated with the antioxidant defence mechanism; *i.e.,* catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase, together with some blood biochemical responses were investigated in rats. At an optimal dose level (4 ml/kg), a single acute intraperitoneal administration of the extract caused a significant enhancement (49.9 %; $p < 0.001$) of lipid peroxidation with a decline in GSH level both in blood (45.1 %; $p < 0.001$) and liver (61.0 %; $p < 0.001$) in comparison to control animals. Activities of catalase, glutathione peroxidase and glutathione reductase were inhibited in a dose-responsive way by the treatment with the extract which also increased the activity of glutathione S-transferase in a dose-dependent manner. The extract was found to be hepatotoxic in terms of elevation of serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase, serum lactate dehydrogenase and blood methemoglobin concentration. Results of this study suggest the adverse consequences of the adminisfration of the extract due to its substantial ability to alter normal cellular processes.

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Human placental extracts are available internationally under different trade names and implicated in diseases of diverse etiology (1-4). Previously, we demonstrated the significant suppression of carrageenin-induced inflammation by the administration of human placental extract (5). Further, a single injection of this extract has been shown to produce a significant decrease of reduced nicotinamide adenine dinucleotide phosphate (NADPH) by inhibiting the enzyme glucose-6-phosphate dehydrogenase (G-6-PDH) in different tissues in the rat (6).

A recent study suggests that at the late gestational stage, placental tissue suppresses lipid peroxide formation, lowers the concentration of lipid peroxides in the cord blood and protects the fetus from free radical-induced toxicity by stimulating the activities of some important antioxidant factors (7). These prompted us to study the precise role of human placental extract, which finds numerous clinical applications, in the light of lipid peroxidative status and antioxidant defence mechanisms *in vivo*. In view of above, we studied the effects of human placental extract on lipid peroxidation and catalase in the liver of rats, reduced glutathione (GSH) in blood and liver and different glutathione-related enzymes; *i.e.,* glutathione reductase, glutathione peroxidase and glutathione S-transferase in liver, which are known to regulate normal biochemical and physiological properties within the cell and involved in antioxidant defence mechanism *in vivo*. The levels of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum lactate dehydrogenase (LDH) and methemoglobin content in blood were also measured after the administration of the extract because these may be consid-

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ered as the markers for evaluating cellular injury in the hepatic tissue.

Materials and Methods

Preparation of human placental extract. Fresh human placentas from normal deliveries at term were collected from hospitals in a hygienic condition. Tissues were washed with cold saline to remove as much blood as possible. They were homogenised in a Teflon homogeniser containing five volumes of cold 10 mM sodium phosphate buffer (pH 7.4) followed by a series of filtration till it was clear. Benzylalcohol (1.5 % v/v) was added as preservative to this preparation and the mixture was finally filtered through membrane filter. This particular preparation, each ml of which was derived from 0.1 g of fresh human placenta, was used in our study. Some known constituents of human placental extract are human placental lactogen (HPL), corticotropin-releasing factor (CRF), fibrin stabilizing factor (FSF), lactoferrin *etc.* (8-11). Protein content of this extract was found to be in the range of 0.54-0.65 mg/ml.

Treatment of animals. Male albino rats (Charles-Foster strain, CIBA, India), weighing 100-110 g, were maintained at $23 \pm 1 °C$ and allowed a standard pellet diet and water *ad libitum* till the beginning of the test. The rats were then divided in several groups of 6 each. Human placental extract in different graded doses (1-4 ml/kg) were administered intraperitoneally (i.p.) in different experimental groups. Benzylalcohol (1.5 % v/v) as vehicle was injected similarly in control animals.

Preparation of subcellular fraction. Twenty-four h after placental extract or vehicle adminstration, the animals were anesthetized with ether. After collection of blood from the heart, livers were quickly excised. All subsequent operations were carried out at 0-4 °C. The livers were minced and homogenised in ice-cold 1.15 % w/v KCl solution in a Potter-Elvehjem Teflon: glass homogeniser for 1 min to make a 10 % w/v homogenate. Then liver cytosol was prepared by differential centrifugation, first at $9,000 \times g$ and then at $100,000 \times g$ for 1 h in a Sorvall refrigerated centrifuge (4 °C). The supernatant was aspirated for the determination of its GSH, glutathione reductase, glutathione peroxidase, glutathione S-transferase and protein content.

Biochemical estimations. GSH in the liver and blood were determined by the method of Ellman (12). For this, 1 ml of cytosol or 0.02 ml of blood was mixed with 1 ml of 4 % w/v sulphosalicylic acid and the mixture was centrifuged at $1,500 \times g$ for 15 min. The supernatant was allowed to react with 0.1 mM 5,5'-dithio-bis-2-nitrobenzoic acid. The solution was then kept at room temperature for 10 min and read at 412 nm by a Shimadzu UV-3,000 spectrophotometer. Results were expressed as μ mol GSH/g wet tissue or /ml of blood.

Glutathione reductase activity was measured by a modification of the method of Carlberg and Mannervik (13). The reaction mixture (1 ml) consisted of 0.1 M potassium phosphate (pH 7.6),

0.1 mM NADPH, 0.5 mM EDTA, 1 mM oxidized glutathione (GSSG) and a suitable amount of cytosol preparation (6 mg protein/ml). The reaction mixture was incubated at 30 °C for 5 min before initiating the reaction by the addition of cytosol. The enzyme activity was determined by measuring the disappearance of NADPH at 340 nm.

The activity of glutathione peroxidase was determined by the method of Lawrence and Burk (14). The reaction mixture (1 ml) consisted of 50 mM potassium phosphate (pH 7.0), 1 mM EDTA, 1 mM NaN_3, 1 unit/ml of glutathione reductase, 1 mM GSH, 0.2 mM NADPH, 1.5 mM cumene hydroperoxide and a suitable amount of cytosol (6 mg protein/ml). The reaction mixture was incubated at 37 °C for 5 min and the reaction was then initiated by the addition of cumene hydroperoxide. The enzyme activity at 37 °C was determined by measuring the disappearance of NADPH at 340 nm.

Glutathione S-transferase activity was measured by adaptations of the method of Habig *et al.* (15). The reaction mixture (1 ml) consisted of 0.1 M potassium phosphate (pH 6.5), 1 mM GSH, 1 mM 1-chloro-2, 4-dinitrobenzene as substrate and a suitable amount of cytosol (6 mg potein/ml). The reaction mixture was incubated at 37 °C for 5 min and the reaction was initiated by the addition of the substrate. The increase in optical density at 340 nm was measured spectrophotometrically.

Liver catalase activity of the supernatant obtained by centrifugation of the crude homogenate (10 % w/v) at $700 \times g$ for 10 min at 4 °C was determined by the method of Cohen *et al.* (16). The supernatant was mixed with 1/100 volume of ethanol and incubated for 30 min in an ice-water bath and then 1/10 volume of 10 % w/v Triton X-100 was added. The mixture was again diluted 100-fold to obtain an enzyme sample (2.4 mg protein/ml). The sample (0.1 ml) was mixed with 1 ml of 6 mM H_2O_2 in an ice-bath and after 3 min, 0.2 ml of 6 N H_2SO_4 was added. Then the mixture was kept at room temperature for 5 min and 1.4 ml of 0.01 N $KMnO_4$ was added and mixed well. The absorbance of the mixture was determined at 480 nm within 1 min.

Lipid peroxidation was determined according to the method of Ohkawa *et al.* (17). To 0.2 ml of 10 % w/v homogenate 0.2 ml of 8.1 % w/v sodium dodecylsulphate, 1.5 ml of 20 % acetic acid solution adjusted to pH 3.5 with 20 % w/v sodium acetate and 1.5 ml of 0.8 % w/v thiobarbituric acid solution were added. The mixture was diluted to 4 ml with distilled water and then heated for 60 min in a boiling water bath. Then it was cooled at room temperature and 1 ml of distilled water followed by 5 ml of a mixture of n-butanol and pyridine (15:1 v/v) were added. The mixture was shaken vigorously and centrifuged at $1,500 \times g$ for 15 min. The absorbance of the organic layer was measured at 532 nm. Results were expressed as nmol of malondialdehyde (MDA) formed/g wet weight.

SGOT and SGPT were determined by the methods of Reitman and Frankel (18) whereas LDH was estimated by the method of Wroblewski and La Due as described by Varley *et al.* (19). Methemoglobin content in blood was measured according to Evelyn and Malloy (20). Protein was estimated by the method as

indicated by Lowry *et al.* (21).

Results were analysed statistically using the Student's *t*-test.

Results

There were no significant variations in different parameters due to the administration of different doses of the vehicle in control animals. Therefore, data from the control subjects were combined and average values were mentioned in the Tables as control figures.

Administration of human placental extract to rats after 24 h of treatment showed marked alteration in hepatic lipid peroxidation and the enzymatic profiles of antioxidant defence system (Tables 1 and 2). Both blood and liver GSH level were suppressed by the extract in a dose-dependent manner. A significantly low level was attained with the highest dose of the extract (Table 1). The extract

Table 1 Effects of human placental extract on glutathione (GSH) levels and lipid peroxidation in rats

| Treatment (i.p) | GSH | | Thiobarbituric acid reactive substance (TBARS) in liver (n mol MDA/g) |
	Blood (μ mol/ml)	Liver (μ mol/g)	
Control (vehicle)	0.93 ± 0.61	3.21 ± 0.42	340.42 ± 6.37
Placental extract 1 ml/kg	0.90 ± 0.22 (3.2)	3.10 ± 0.37 (3.4)	347.31 ± 7.12 (2.0)
Placental extract 2 ml/kg	0.85 ± 0.31 (8.6)	2.56 ± 0.26 (20.2)	362.15 ± 5.34 (6.3)
Placental extract 3 ml/kg	0.64 ± 0.19* (31.1)	1.87 ± 0.42**(41.7)	475.26 ± 6.17* (39.6)
Placental extract 4 ml/kg	0.51 ± 0.23**(45.1)	1.25 ± 0.18**(61.0)	510.39 ± 4.12** (49.9)

Values indicate means ± SEM (n = 6)
*$p < 0.01$ and **$p < 0.001$ when compared with the control group.
Parentheses indicate % change *versus* control.

Table 2 Effects of human placental extract on catalase, glutathion peroxidase, glutathione reductase and glutathione S-transferase activity in the liver in rats

Treatment (i.p.)	Catalase (μ mol H_2O_2 decomposed/ min/mg protein)	Glutathione peroxidase (μ mol NADPH oxidized/min mg/protein)	Glutathione reductase (n mol NADPH oxidized/min mg/protein)	Glutathione S-transferase (n mol glutathione conjugate formed/ min/mg protein)
Control (vehicle)	7.85 ± 2.3	260.4 ± 6.3	41.3 ± 4.8	970.3 ± 82.5
Placental extract 1 mg/kg	7.80 ± 1.1 (0.6)	255.2 ± 7.5 (1.9)	39.6 ± 3.1 (4.1)	999.7 ± 65.7 (3.0)
Placental extract 2 mg/kg	6.57 ± 2.4 (16.3)	219.7 ± 5.0 (15.6)	36.7 ± 2.9 (11.1)	1329.5 ± 50.1* (37.0)
Placental extract 3 mg/kg	5.18 ± 3.2*(34.0)	161.2 ± 9.1*(38.0)	31.5 ± 4.2 (23.7)	1587.6 ± 36.5** (63.6)
Placental extract 4 mg/kg	4.79 ± 4.7*(38.9)	156.0 ± 8.3*(40.0)	29.8 ± 1.9*(27.8)	1649.8 ± 42.6** (70.0)

Values indicate means ± SEM (n = 6)
*$p < 0.01$ and **$p < 0.001$ when compared with the control group.
Parentheses indicate % change *versus* control.

Table 3 Effects of human placental extract on some blood biochemical indices in rats

Treatment (i.p.)	SGOT (I.U./L)	SGPT (I.U./L)	LDH (I.U./L)	Methemoglobin (g/100 ml of blood)
Control (vehicle)	60.7 ± 4.8	27.4 ± 2.5	720.4 ± 69.3	1.21 ± 0.46
Placental extract 1 ml/kg	67.5 ± 5.7 (11.2)	29.3 ± 3.1 (6.9)	792.1 ± 75.1 (9.9)	1.30 ± 0.21 (7.4)
Placental extract 2 ml/kg	72.3 ± 5.2 (19.1)	32.4 ± 3.6 (18.2)	880.5 ± 90.3 (22.2)	1.27 ± 0.26 (4.9)
Placental extract 3 ml/kg	76.8 ± 6.3 (26.5)	36.1 ± 4.7* (31.7)	1010.3 ± 132.7* (40.2)	1.58 ± 0.51* (30.5)
Placental extract 4 ml/kg	80.3 ± 7.1*(32.2)	38.9 ± 4.1**(41.9)	1087.8 ± 175.1** (50.9)	1.65 ± 0.59* (36.3)

Values indicate means ± SEM (n = 6)
*$p < 0.01$ and **$p < 0.001$ when compared with the control group.
Parentheses indicate % increase *versus* control.

also enhanced lipid peroxidation in a dose dependent manner in the liver based upon the direct measurement of thiobarbituric acid reactive substance (TBARS); MDA in fresh tissue homogenate (Table 1). Catalase, glutathione peroxidase and glutathione reductase were inhibited significantly by the application of the extract and in each case the inhibition was observed in a dose-dependent fashion (Table 2). There was a substantial stimulation of glutathione S-transferase activity in response to the placental extract. The highest enzyme activity was achieved with the highest dose of the extract (Table 2). Activities of different serum enzymes; SGOT, SGPT and LDH, were found to be elevated slightly in a dose-dependent manner with a concurrent increase in blood methemoglobin level following placental extract administration (Table 3).

Discussion

In our present investigation, we observed that human placental extract induces a significant elevation of lipid peroxidation. It is well known that active oxygen species interacts with unsaturated fatty acids present in phospholipid to initiate lipid peroxidation which is the major factor influencing the breakdown and turnover of biomembranes (22). The most important sources of O_2^- *in vivo* in most aerobic cells are electron transport chains of mitochondria and some components of the chain do leak a few electrons onto oxygen while passing the great bulk of them onto the next component in the chain and this leakage produces a univalent reduction to give O_2^- (23). Thus, with a greater stimulation of mitochondrial respiration by placental extract, there is a possibility of an increased leakage of free electrons leading to an increased rate of production of O_2^- and hence H_2O_2 by mitochondria. This could be attributed to the enhanced lipid peroxidation that reconcile well with the known effect of placental extract on the acceleration of mitochondrial respiration.

It is established that enhanced lipid peroxidation is followed by increased GSH oxidation to form oxidized glutathione (GSSG) (24). In general, intracellular reduction of GSSG to GSH is mediated by glutathione reductase. The activity of glutathione reductase that has been found to be inhibited is consistent in keeping with the lower level of GSH that is observed in our study which is again supported by our previous result of inhibition in

the activity of G-6-PDH limiting the supply of NADPH for maintaining the GSH in a reduced state (6).

The decreased concentration of lipid hydroperoxide in the placenta as observed by Takehara *et al.* (7) was due to the increased activity of catalase and superoxide dismutase in the placental tissue since both the enzymes were induced by exogenous radicals in the placenta (25). However, in our study, the placental extract inhibited the activities of glutathione peroxidase and catalase in rat liver.

Glutathione S-transferase is a multifunctional enzyme and it plays an important role in the detoxification of xenobiotic compounds with the help of GSH. The human placental extract-induced increase in the activity of this enzyme with a concomitant decrease in GSH level indicates an increased utilization of GSH in the removal of toxic radical species that may eventually deplete the intracellular GSH pool.

The elevated levels of serum enzymes SGOT, SGPT and LDH together with elevated methemoglobin concentration in blood upon the administration of human placental extract may confirm the agent as hepatotoxic in rats, although the degrees of serum enzyme elevations were much less than those induced by carbon tetrachloride.

The active compounds present in the human placental extract could be 19-hydroxyprogesterone (26) and corticotropin-releasing factor (CRF) (11). The possible explanation related to the contributing effect of these components may be either increase of serum iron and iron binding capacity effecting the liver synthesis of transferrin (27) or the inhibition of hepatic G-6-PDH activity and/or to a mechanism that results in a drastic deminution of GSH level and thus leading to enhanced lipid peroxidation and subsequent hepatic dysfunction. Resolution of these questions requires considerable further investigation. Active research is underway in our laboratory to achieve a full explanation of these effects of human placental extract.

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