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Tea and Mint Extracts Modulate the HSP70 Expression in Preeclamptic Placental Explant

E. Padmini, D. Lavanya, J.Tharani and S. Lavanya

ABSTRACT

Heat shock protein 70 is a molecular chaperone which expressed during oxidative stress to protect the cell from damage. Preeclampsia is a hypertensive disorder with an oxidative stress imbalance. Antioxidant defenses appear to be depleted during preeclampsia resulting in increased oxidative stress. Various alternative medicines are employed to increase the endogenous antioxidant level during preeclampsia. The aim of the present study was to determine the modulatory effect of medicinal plants, *Camellia sinensis* and *Mentha spicata* on oxidative stress, antioxidant and thiol status using placental explant as the model system. Placental explants were cultured from the placental tissues of both normotensive and preeclamptic subjects. The lipid peroxide, total antioxidant capacity, glutathione redox ratio, HSP70 levels were measured in the placental explants with and without incubation with tea, mint and mint-tea. The addition of these three extracts increased the TAC and GRR in both placental explants with decrease in the LPO level. The expression of HSP70 also decreased more significantly in preeclamptic explants on addition of tea and mint extracts due to the restoration of cell homeostasis via maintaining the antioxidant status. In view of the above results, mint-tea may emerge as an effective antioxidant, preventing cell damage during stress condition.

Keywords: Preeclampsia (PE), Lipid peroxide (LPO), Total antioxidant capacity (TAC), Glutathione redox ratio (GRR), Heat shock protein 70 (HSP70).

E. Padmini, D. Lavanya,
 J.Tharani and S. Lavanya
 Department of Biochemistry,
 Bharathi Women's College,
 Chennai-600 108, India.

INTRODUCTION

Molecular chaperones, including the heat-shock proteins (HSPs), are an ubiquitous feature of cells in which these proteins cope with stress-induced denaturation of other proteins (Feder & Hofmann, 1999; Fink, 1999). HSP acts as an antioxidant in maintaining the cellular redox homeostasis by enhanced peptide binding ability and peptide complex stability under oxidative stress conditions (Bukau & Horwich, 1998). HSP by interacting with a number of cellular systems and signaling proteins exerts an efficient cytoprotective mechanism. Thus over expression of HSP is an important means of cellular protection during physiological stress (Feder & Hofmann, 1999). HSP70 proteins are conserved molecular chaperones, found in the cytosol and in other compartments of the cell, that play an essential role in the life cycle of many proteins under both normal and stressful conditions.

For Correspondence
 Dr. E. Padmini,
 Associate Professor,
 Department of Biochemistry,
 Bharathi Women's College,
 Chennai-600 108, Tamilnadu, India.
 Tel.: + 91-9884207570/ 9884088577

The house-keeping functions of HSP70 include degradation of unstable and misfolded proteins, prevention and dissolution of protein aggregates, transport of proteins between cellular compartments, folding and refolding of proteins, uncoating of clathrin-coated vesicles and control of regulatory proteins. HSP70 chaperones regulate important physiological processes such as cell cycle, cell differentiation and programmed cell death (Mayer & Bukau, 2005; Cobreros *et al.* 2008). The over expression of HSP70 in relation to the oxidative stress generated in preeclamptic condition suggest their protective role in stress management prolonging the cell survival in the placental endothelial cells during preeclampsia (Padmini *et al.* 2009b; Padmini & Lavanya, 2011).

Generation of reactive oxygen species and development of oxidative stress is mainly attributed to the pathophysiology of preeclampsia. During pregnancy, lipid peroxidation is induced in placenta and the rate of lipid peroxidation in placenta is very high in preeclampsia (Walsh & Wang, 1993). The free radicals are generated from the poorly perfused placenta that initiates the synthesis of lipid peroxide. Antioxidant is a substance which significantly inhibits the oxidation of the substrate. The involvement of various placental cells in preeclamptic pathogenesis suggests that placental explant analysis is superior to individual placental cell analysis during preeclampsia. In preeclampsia, the antioxidant defense is further compromised due to an excessive increase in the oxidative stress. This oxidative imbalance results in, endothelial injury and dysfunction, increased trophoblast cell death, fetal growth restriction, platelet and neutrophil activation ultimately results in abnormal placentation and placental dysfunction that causes preterm delivery (Soleymanlou *et al.* 2005). The placenta appears to be central in the aetio-pathogenesis of preeclampsia (Hladunewich *et al.* 2007). Various alternative medicines are employed to increase the endogenous antioxidant levels in preeclampsia (Allaire *et al.* 2000). Since preeclampsia is a pregnancy specific disorder, treatment with synthetic drugs can be deleterious to both fetus and mother. Tea (*Camellia sinensis*) and mint (*Mentha spicata*) leaves are extensively used as herbal medicines all over the world for their richness in micronutrients, antioxidant properties, and their role in the protection of human cells from adverse effects of reactive oxygen species (Padmini *et al.* 2008a; Padmini *et al.* 2008b; Padmini & Lavanya, 2009a). *C. sinensis* and *M. spicata* were authenticated by the Siddha Central Research Institute, Chennai (Central Council for Research in Ayurvedha and Siddha, New Delhi, Under the Ministry of Health & Family Welfare, Govt. of India). The present study was performed to determine the modulatory effect of tea, mint and mint-tea on the level of oxidative stress, antioxidant and thiol status exhibited in the placental explant through incubation studies. The level of MDA, TAC, GRR ratio and HSP70 expression were measured with and without tea, mint and mint-tea incubations to evaluate the status of oxidative stress changes expressed in preeclamptic explants for the first time.

MATERIALS AND METHODS

Selection of subjects

The study was carried out for a period of six month. The placental samples were obtained from a private hospital. Informed consent was obtained from the subjects and the study has been approved by college ethical committee and intimated to Indian Council of Medical Research (IEC/A/BWC/001102/2010). Placenta was collected from both normal (n=10) and preeclamptic (n=10) pregnancy women in the age group of 20-40 years, post delivery.

Patients with preeclampsia were defined on the basis of the following laboratory criteria: blood pressure >140/90 mmHg but <160/110 mmHg, proteinuria >300 mg/L and xanthine oxidase activity of approximately 2.6 units/ mg protein (Brown *et al.* 2001). Patients with severe preeclampsia and other severe maternal complications were excluded from the study.

Preparation of explant

The collected placenta was washed with ice cold PBS buffer and was stored at 4°C in HEPES buffer physiological salt solution (pH 7.4) having the following composition (in mmol/L): HEPES 10, NaCl 139, KCl 5, CaCl₂ 1, MgCl₂ 1, glucose 4.2 and 0.5% (w/v) dialyzed albumin until use. The explants were cultured as described by Yacobi *et al.* 2002 with slight modifications.

The placental tissue (villi) was dissected from the fetal membranes from both normotensive and preeclamptic subjects and about 10 mg of placenta were cut into 4 pieces, transferred to the Millicell-CM separate culture dish inserts which was layered with polymerized Matrigel. Medium (Dulbecco's Modified Eagle's Medium) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), antibiotics/antimycotic (10000 U penicillin, 10mg streptomycin, 25µg amphotericin B/ mL in 0.9% saline) supplied by Himedia (Mumbai, India), fetal bovine serum (10%) were added to all the culture dish. Culture plates were incubated overnight in 5% CO₂. The medium from all the culture dishes were changed after every 24 hrs following the beginning of the experiment, and the collected media were stored at -20°C until processing.

Preparation of black tea extract

About 2 grams of commercially available South Indian black tea leaves were brewed and extracted with 100 mL of PBS by heating for 10 minutes. The extract was filtered using Whatmann filter paper (No.2). The resulting filtrate was diluted (1:100) with PBS and was diluted to get the necessary concentration of 2%.

Preparation of mint extract

About 2 grams of fresh mint leaves were washed refluxed with 100 mL of PBS and filtered using Whatmann filter paper (No.2). The resulting filtrate was diluted (1:100) with PBS and was diluted to get the necessary concentration of 2%.

Preparation of mint-tea extract

Mint-tea extract was prepared by mixing the individual extracts of tea and mint in appropriate proportions.

Incubation studies

The cultured placental explants from both normotensive and preeclamptic groups were incubated with 0.02% of the plant extracts such as tea, mint and tea fortified with mint in 5% CO₂ atmosphere at 37°C, for a maximum of 48 hrs.

Estimation of protein

The cultured normotensive and preeclamptic explants were pooled and recovered by lysing with lysis buffer (0.1 M Tris, 38 mM glycine, 2 mM EDTA, 2 mM N-ethylmaleimide, 2 mM iodoacetic acid, and 0.4 mM phenylmethylsulfonylfluoride pH 8.7). The cell suspension was incubated for 30 minutes at 4°C, with occasional shaking and centrifuged at 15,000 Xg for 15 minutes to remove cellular debris.

The supernatant was the cell lysate, whose protein concentration was determined by the classical Bradford method with Coomassie brilliant blue G-250, using bovine serum albumin as the standard (Bradford, 1976). The protein concentration was expressed as mg protein/g placental explant. The lysate was used for the estimation of the following parameters.

Quantification of HSP70 by ELISA technique

The inducible form of HSP70 in the placental explant was quantified using HSP70 ELISA kit (EKS-700B, Stressgen, Canada) according to the manufacturer's instruction.

Estimation of lipid peroxide

Lipid peroxide analysis was determined by TBA reaction by the method of Ohkawa *et al.* (1970). The lipid peroxide content was expressed as nanomoles of MDA/ mg of protein.

Determination of total antioxidant capacity

Total antioxidant capacity (TAC) analysis was performed by the method of Prieto *et al.* (1999). The total antioxidant activity was expressed as Trolox equivalent in mmol/ L.

Determination of glutathione redox ratio (GSH/GSSG)

Thiol status was assessed spectrofluorimetrically using the method of Hissin & Hilf, (1976). The fluorescence and excitation were determined at 420 nm and 350 nm, respectively. The values were expressed as nanomoles/ mg protein.

Statistical analysis

The results were expressed as mean value \pm standard deviation. Statistical analysis of the data was carried out using SPSS 7.5 version package. Statistical significance was arrived by comparing the results of preeclamptic placental explants with the normotensive explants using Student 't' test. Differences were taken to be statistically significant for values of $p < 0.05$, $p < 0.01$, $p < 0.001$.

RESULTS

HSP70 expression

To evaluate the stress response in the preeclamptic patients, the level of cytoprotective HSP70 was analyzed in the placental explants of both normotensive and preeclamptic pregnant women (Figure 1). The results revealed that HSP70 levels were significantly increased by 40% in preeclamptic placental explants ($p < 0.001$) than normotensive placental explants. Addition of tea, mint and mint-tea decreased HSP70 significantly in preeclamptic placental explant. The level of HSP70 was decreased by 28%, 21% and 27% in preeclamptic placental explant and by 5%, 2% and 9% in normotensive placental explant by tea, mint and mint-tea extract respectively.

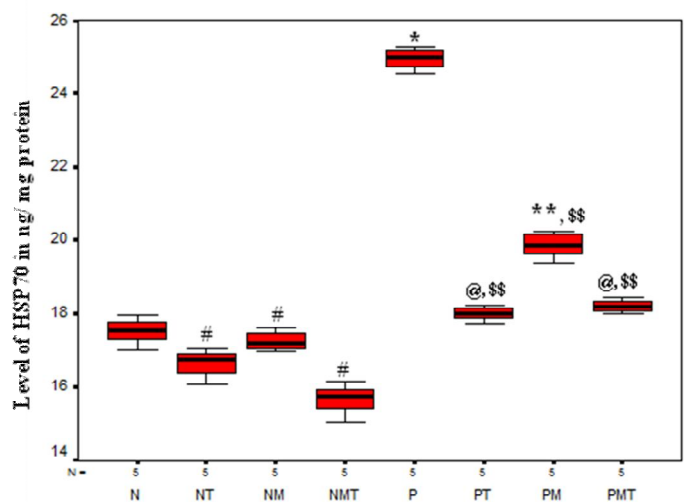


Fig. 1: HSP70 expression normotensive and preeclamptic placental explant before and after incubation with tea, mint and mint-tea extracts. Values are expressed as mean \pm SD (for 10 samples in each group).

Normotensive (N) placental explant	Preeclamptic (P) placental explant
N- without any incubation	P- without any incubation
NT- with tea	PT- with tea
NM- with mint	PM- with mint
NMT- with mint-tea	PMT- with mint-tea

* $p < 0.001$, ** $p < 0.01$, #not significant, @ $p < 0.05$, \$ $p < 0.01$ when compared with normotensive placental explant without any incubation. @ $p < 0.05$, \$ $p < 0.001$, NSnot significant when compared with preeclamptic placental explant without any incubation

Lipid peroxidation

The level of MDA was significantly higher in the preeclamptic placental explants 59% ($p < 0.001$) than the normotensive placental explants (Table 1). An insignificant decrease in MDA levels was observed in preeclamptic (9%) and normotensive placental explants (7%) after incubation with tea. The mint extract significantly reduced the level of MDA by 11% in preeclamptic placental explants ($p < 0.05$) and by 13% in normotensive placental explants ($p < 0.05$). When incubated with mint-tea extracts, the level of MDA was significantly decreased by 19% in preeclamptic placental explants ($p < 0.05$) where as in normotensive placental explants a 27% ($p < 0.01$) decrease was observed than tea and mint incubation.

Table 1: Level of malondialdehyde (MDA) and total antioxidant in the normotensive and preeclamptic placental explant before and after incubation with tea, mint and mint-tea extracts.

S.No	Parameters	Normotensive Placental Explant				P	Preeclamptic Placental Explant			
		N	NT	NM	NMT		PT	PM	PMT	
1.	Lipid peroxide (nmol/mg protein)	164 ± 23	153 ± 26 [#]	143 ± 19 [@]	128 ± 13 [§]	260 ± 25 [*]	237 ± 18 ^{*,NS}	234 ± 20 ^{*,NS}	211 ± 11 ^{*,α}	
2.	TAC (Trolox equivalent in mmol/L)	7.2 ± 0.01	12 ± 0.7 [*]	11.8 ± 0.7 [*]	12.5 ± 0.5 [*]	4.8 ± 0.04 [*]	6.5 ± 0.2 ^{#, &}	6.8 ± 0.1 ^{#, &}	7.1 ± 0.8 ^{#, &}	

Values are expressed as mean ± SD (for 10 samples in each group).

Normotensive (N) placental explant				Preeclamptic (P) placental explant			
N- without any incubation				P- without any incubation			
NT- with tea				PT- with tea			
NM- with mint				PM- with mint			
NMT- with mint-tea				PMT- with mint-tea			

*p<0.001, **p<0.01, # not significant, @p<0.05 when compared with normotensive placental explant without any incubation

°p<0.05, §p<0.01, &p<0.001, NS not significant when compared with preeclamptic placental explant without any incubation

Antioxidant and thiol status

The total antioxidant capacity and glutathione redox ratio were determined in the placental explants with and without mint-tea extracts. The level of TAC was significantly decreased by 33% in preeclamptic placental explants (p<0.001) than the normotensive placental explants (Table 1). After incubation with tea, the level of TAC was significantly higher in preeclampsia 35% and by 67% in normotensive placental explants (p<0.001). The mint extracts significantly increased the level of TAC normotensive placental explants (p<0.001). The mint extracts significantly increased the level of TAC by 42% (p<0.001) in preeclamptic placental explants and in normotensive placental explants by 64% (p<0.001). When incubated with mint-tea extracts, the level of TAC significantly increased by 48% in preeclamptic placental explants (p<0.001) as compared to normotensive placental explants by 74%.

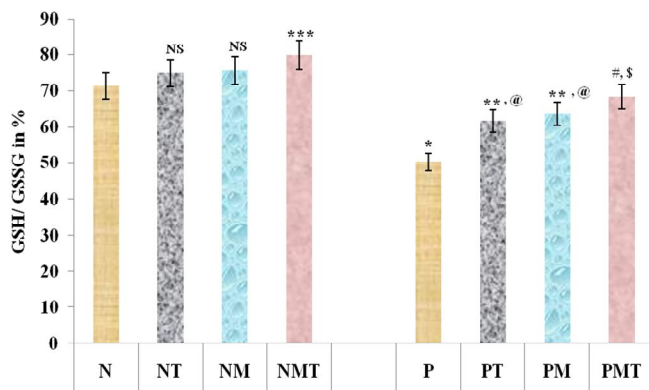


Fig. 2: Glutathione redox ratio in the normotensive and preeclamptic placental explant before and after incubation with tea, mint and mint-tea extracts. Values are expressed as mean ± SD (for 10 samples in each group).

Normotensive (N) placental explant	Preeclamptic (P) placental explant
N- without any incubation	P- without any incubation
NT- with tea	PT- with tea
NM- with mint	PM- with mint
NMT- with mint-tea	PMT- with mint-tea

*p<0.001, **p<0.05, ***p<0.05, # not significant, NS not significant when compared with normotensive placental explant without any incubation

@p<0.01, §p<0.001 when compared with preeclamptic placental explant without any incubation

The glutathione redox ratio GSH/GSSG (Figure 2) was significantly decreased by 30% in placental explants of preeclamptic women (p<0.001) than normotensive pregnant women. Tea extract significantly increased the level of GRR by

23% in preeclamptic placental explants (p<0.01) and in normotensive placental explants by 5%. When incubated with mint extract, the level of GRR was significantly increased (27%) in preeclamptic placental explants (p<0.01) where as it was increased in normotensive placental explants by only 6%. The mint-tea extracts significantly increased the GRR level by 36% in preeclampsia and by 12% in normotensive placental explants respectively.

DISCUSSION

Preeclampsia still remains one of the most serious complications of pregnancy (Stegers *et al.* 2010). Controlling the oxidative stress in preeclampsia with antioxidant therapy can prevent from the complications caused by disease. Antioxidants may have the potential to re-enforce our body's natural defense against free radicals and thus may minimize damage to the body (Valko *et al.* 2007).

Studies have demonstrated that oxidative stress is one of the key factor in complicating preeclampsia which results in preterm birth (Padmini *et al.* 2009b; Padmini & Lavanya, 2011; Padmini & Lavanya, 2009a). The first line of defense for oxidative stress is not sufficient enough to combat with the generated stress. Thus HSPs, a group of chaperones act as secondary line of defense whose induction is altered in response to oxidative stress as they are involved in maintenance of the cell homeostasis (Menoret *et al.* 2002). Over expression of HSP is an important means of cell protection during physiological stress (Wu *et al.* 1996; Minowada & Welch, 1995). This may be the reason for increase in the expression of HSP70, an antiapoptotic protein in preeclamptic placental explants than the normotensive placental explants. A significant decrease in the expression of HSP70 observed in the present study on addition of tea and mint extracts may be due to the restoration of cell homeostasis by maintaining the antioxidant status. This property is attributed to the flavonoids, polyphenols and minerals present in tea and mint extracts (Padmini *et al.* 2009b; Padmini *et al.* 2008a; Padmini *et al.* 2008b).

Earlier studies from our laboratory have shown a significant elevation in the levels of MDA in placental tissue (Padmini *et al.* 2008a) and placental endothelial cell during preeclampsia (Padmini & Lavanya, 2009a). Consistent with this, in the present study, the level of lipid peroxide have been increased

significantly in placental explant of the patients with preeclampsia. The elevation of MDA could be due to over production of ROS generated in preeclampsia (Sharma *et al.* 2006). The addition of tea, mint and mint-tea extracts to the placental explant of both groups significantly decreased the level of MDA, indicative of the up-regulation of antioxidant by the tea and mint extracts. Also this reduction in LPO level may be attributed to the inbuilt scavenging and reducing property of the plant extracts (Padmini *et al.* 2008a; Padmini *et al.* 2008b).

A significant decrease in the level of TAC was observed in preeclamptic placental explant when compared to normotensive placental explant. This may be due to the increased turnover of antioxidants, for preventing oxidative damage in preeclampsia (Chandra *et al.* 2000). The study showed that the addition of tea, mint and mint-tea extracts to the placental explant increased the TAC level significantly in both normotensive and preeclamptic pregnant women to a greater extent. The rise in the level of TAC could be due to its induction to counter the effect of increased oxidative stress. A significant decrease in GSH/GSSG redox ratio was associated with a significant increase in the oxidized GSSG disulfide form of glutathione (Nemeth & Boda, 1994). This is due to the reaction of GSH with free radicals catalyzed by glutathione peroxidase generating the GSSG. This is one of the most effective mechanisms against oxidative stress. Indeed, glutathione redox ratio (GSH-GSSG) gives us an indication of the redox state of the cells and thus indicates the actual level of oxidation in preeclamptic women. The results indicated that the addition of tea, mint and mint-tea extracts to the placental explant increased the GRR level significantly in both groups to a greater extent associated with significant increase in the reduced glutathione (GSH).

Tea has been considered a medicine and healthful beverages for ages (Yang *et al.* 2001). This beverage is consumed worldwide because of its unique aroma, low cost and wide availability (Yeh *et al.* 2003). Tea contains high level of polyphenols and flavonoids which acts as an antioxidant and protects the cell from the oxidative damage by scavenging free radicals (Dufresne & Farnworth, 2001). The polyphenols are known to reduce the formation of free radicals by scavenging or chelating iron and copper and preventing them from participating in the Fenton reaction (Van Acker *et al.* 1998). Flavonoids are potent water soluble antioxidants and free radical scavengers which prevent oxidative cell damage and have strong anticancer activity and anti-inflammatory activity (Okwu, 2004). Mint is used with spices to give the food a special flavor and fragrance. Mint extract has been found to have antioxidant and antiperoxidant properties due to the presence of eugenol, caffeic acid, rosmarinic acid, alpha tocopherol and it would enhance error free repair for DNA damage and hence could be antimutagenic (Gacis & Simic, 1993). The presences of free hydroxyl group in phenolic compounds of mint are mainly responsible for antioxidant activity (Padmini & Lavanya, 2009a; Werg & Wang, 2000). The catechins and tannins present in the tea interfere with iron absorption in the stomach, but mint extract contains provitamins enabling the iron to be available

in soluble state, thereby increasing its absorption (Padmini & Lavanya, 2009a; Bicudo *et al.* 2000). Thus, a combination of tea and mint extract will have more beneficial effect than administered alone.

CONCLUSION

This explant culture study from normotensive and preeclamptic women clearly indicates that the tea and mint extracts when combined may not necessarily exhibit effective protection against oxidative stress. However, the increased consumption of tea enriched with mint may contribute to the improvement in quality of healthy life by increasing the antioxidant defense and delaying the onset of various degenerative diseases during preeclampsia caused by oxidative stress.

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DISCLOSURE STATEMENT

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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