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ASK-1 Expression Down-regulated in Preeclamptic Placental Explants Incubated with Mint-Tea

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ABSTRACT: Apoptosis, the programmed cell death, is an essential feature of normal placental development but is exaggerated in association with placental disease. Preeclampsia is associated with an increase in placental apoptosis and differential expression of mediators of apoptosis. Since treatment of the disease like preeclampsia with modern medicine is often associated with serious maternal and foetal side-effects, a search for natural drug that would eventually reduce the placental cell damage in preeclampsia will be beneficial. The aim of the present study is to demonstrate the apoptosis mediated protein ASK-1 with modulating oxidant–antioxidant status during preeclampsia using mint-tea infusion. The possible positive effects of mint-tea administration in modulating oxidant–antioxidant status and the expression of ASK-1, using preeclamptic placental explants in *in-vitro* condition were assessed using spectrophotometry and ELISA method, respectively. The study suggests that mint tea mediates an increase the level of CAT and decreases the level of LHP and expression ASK-1 in preeclamptic placental explant. Therefore, we speculate that the mint-tea mediated explant survival is through the control of ASK-1 expression. Thus, mint-tea extracts with natural medical properties can be recommended as an alternative herbal remedy for preeclamptic pregnancy to reduce oxidative stress-mediated apoptosis and enhance live foetal delivery.

KEYWORDS: *Preeclampsia; Oxidative stress; Apoptosis; Antioxidant; Mint-tea*

Introduction

Preeclampsia is a pregnancy specific disease with worldwide significance to mothers and infants.[1] Its greatest impact is in developing countries, where it accounts for 20–80% of the strikingly increased maternal mortality.[2] In preeclampsia, incomplete conversion of the spiral arteries results in retention of smooth muscle cells within their walls and some vasoreactivity persists in 30–50% of the placental vascular bed. This may lead not only to diminished perfusion of the intervillous space but more importantly to intermittent perfusion. Because the placenta and fetus continually extract O_2, it is expected that transient hypoxia will result and increased oxidative stress in the placenta of women with preeclampsia is well documented.[3,4] As a result of defective remodeling of spiral arteries in the preeclampsia, the reduction in uterine blood flow leads to fluctuating oxygen concentration within the placenta.[5] Oxidative damage of vascular endothelium is a common event in the pathophysiology of preeclampsia.[4,6]

Preeclampsia is characterized by the increased generation of pro-oxidants in the placenta. Poor antioxidant reserves can also lift the balance in favour of pro-oxidants. The disturbance in the oxidant–antioxidant balance renders the tissue more vulnerable to oxygen free radical (OFR) injury. Pregnancy, mostly because of the mitochondria-rich placenta, is a condition that favours oxidative stress. Placental cells are highly sensitive to oxidative stress due to their proliferative phenotype with the syncytiotrophoblast being most expressed due to its surface localization and its low content of antioxidant enzymes.[7] As the gestation progresses, subsequent development of localized placental hypoxia is encountered. Depending up on the severity of hypoxia and the ability of the scavenging system in the placental tissue, oxidative stress may result in severe tissue damage that may lead to infarction.[8] Placental explants contain syncytiotrophoblast, trophoblast, cytotrophoblast and extra villous trophoblast. Explant culture is the process of tissue maintenance in artificial condition mimicking the *in-vivo* model. Monitoring the changes in explants will reflect the status of stress in entire placenta *in-utero* and aid in identifying the key mediator for the live foetal delivery despite the existing complications. This may provides a potent target for further therapeutic interventions.[9]

Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein 3 kinase that plays an important role in oxidative stress-induced apoptosis.[10] ASK1 is preferentially activated in response to various types of stress, such as ROS, TNF-α, lipopolysaccharide (LPS), and ER stress, and has pivotal roles in a wide variety

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of cellular responses, including apoptosis, differentiation, and inflammation.[11,12] The excessive activation and dysregulation of ASK1 are closely linked to various diseases.[13] Here, we focus on the molecular mechanisms of ASK1 activation and the involvement of ASK1 in oxidative stress-induced pregnancy diseases like preeclampsia.

Endogenous protective mechanisms may not be enough to limit ROS and their harmful effects as a result of disproportionate production of ROS.[14,15] Many artificial and natural agents possessing antioxidant and radical scavenging properties, such as dietary antioxidants, may be of great importance as additional protective measures and have been proposed to prevent and/or treat oxidative damage induced by ROS.[16,17] Most popular amongst the dietary antioxidants are different types of Teas and herbal Teas widely used as non-alcoholic health beverages.[18,19] The therapeutic and medicinal value of tea (*Camellia sinensis*) is the subject of many studies and several researchers have described their functional health benefits.[20–23] The popularity of tea is due to their advantages such as low or no toxicity, as well as containing rich antioxidants which cover dismutation and trapping of almost all types of ROS.

Tea (*Camellia sinensis*) is unique in its diverse medicinal properties.[24] Tea leaves are effective in preventing and resisting a variety of ailments. It has anti-stress and anti-ageing properties. Studies concentrating on individual diseases show the beneficial effects of tea extracts. Catechin, a key component of tea extract is a powerful antioxidant, directly acts against or neutralizes the disease causing agents at the cellular level.[25] Tea possesses high antioxidant properties and protects human cells from the adverse effect of ROS. Mint (*Mentha spicata*) is used and valued as an aromatic herb for thousands of years.[26] It is considered as stimulant, carminative, antispasmodic, stomachic, and diuretic.[27] Mint extracts also possess polyphenols that act as scavengers to prevent cellular damage. Mint leaves have powerful anti-angiogenetic effect, further supporting its use in treatment of preeclampsia.[28] Combination of mint and tea extract was observed to be more beneficial than using alone in preeclamptic cells.[9]

In this study, we have shown for the first time that mint-tea extract suppressed oxidative stress induced apoptosis signal regulating kinase-1 protein expression in preeclamptic placental explants. The mechanisms by which mint-tea extract inhibited ASK-1 protein expression seemed to involve in maintaining the cell homeostasis through increasing the antioxidant level. These findings provided further understanding of the molecular mechanisms underlying the anti-apoptotic effects of mint tea extract. This helps to delineate further target of therapeutic intervention and post delivery management during preeclampsia.

Materials and Methods

Selection of Subjects

Patient registered in a public sector hospital in Chennai were enrolled in this study. Clearance was obtained from Institute Ethical Committee prior to the commencement of study and the informed consent was received from all the subjects. Placenta was collected from both normal (n = 10) and preeclamptic (n = 10) pregnant 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.[29] Patients with severe preeclampsia and other severe maternal complications were excluded from the study.

Preparation of Explants

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, $CaCl_2$ 1, $MgCl_2$ 1, glucose 4.2 and 0.5% (w/v) dialyzed albumin until use. The explants were cultured as described by Yacobi *et al.*[30] with slight modifications. The placental tissue (villi) was dissected from the foetal 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 (10,000 U penicillin, 10 mg streptomycin, 25 µg amphotericin B/ mL in 0.9% saline) supplied by Himedia (Mumbai, India), foetal bovine serum (10%) were added to all the culture dish. Culture plates were incubated overnight in 5% CO_2. The medium from all the culture dishes were changed after every 24 hr following the beginning of the experiment, and the collected media were stored at –20°C until processing.

Preparation of Black Tea Extract

Commercially available South Indian black tea leaves

(~2 g) were brewed and extracted with 100 mL of PBS by heating for 10 min. The extract was filtered using the 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

Fresh mint leaves (~2 g) were washed refluxed with 100 mL of PBS and filtered using the Whatmann filter paper (No. 2). The resulting filtrate was diluted (1:100) with PBS and was diluted to get the necessary concentration of 2%. Fresh mint leaves (~2 g) were washed refluxed with 100 mL of PBS and filtered using the 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% CO_2 atmosphere at 37°C, for a maximum of 48 hr.[9]

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 min at 4°C, with occasional shaking and centrifuged at 15,000×*g* for 15 min 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.[31] The protein concentration was expressed as mg protein/g placental explant. The lysate was used for the estimation of the following parameters.

Oxidative Stress Studies

The conjugated diene and lipid hydroperoxide analysis, and determination of catalase are the stress-specific parameters assessed to evaluate the oxidative stress status of the placental explants. Lipid hydro peroxide (LHP) was estimated by the method of Jiang *et al.*[32] The formation of conjugated diene (CD) in the processed samples was measured by method of Recknagel and Glende.[33] The antioxidant status was assessed by determining the Catalase (CAT) by the method of Beers and Seizer.[34]

Qunatification of ASK-1 using ELISA Kit

The inducible form of ASK-1 in the placental explant was quantified using ASK-1 ELISA kit (E91358Hu 96 T, Uscn Life Science Inc, USA). The protein was diluted using buffer and plated on the 96 well plates along with the diluted recombinant ASK-1 standard. After incubation for 2 hr at room temperature, the contents should not be removed, incubated with detection reagent A for 1 hr and washed with wash buffer for three times, followed by incubation with detection reagent B for 30 min. The washing procedure was repeated for five times. The assay was developed with tetramethylbenzidine (TMB) substrate and the blue colour developed was in proportion to the amount of captured ASK-1. The colour development was stopped with acid stop solution, which converted the endpoint colour to yellow, and the colour intensity was measured in a micro plate reader at 450 nm.

Statistical Analysis

The results were expressed as mean value ± 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

Lipid Hydroperoxide

The level of LHP was significantly higher in the preeclamptic placental explants 83% ($P<0.001$) than the normotensive placental explants (Table 1). When incubated with tea, the level of LHP was decreased moderately in preeclamptic placental explant 16% than normotensive placental explants 8%. The mint extract significantly reduced the level of LHP by 18% in preeclamptic placental explants ($P<0.05$) when compared with normotensive placental explants (8%). The level of LHP was significantly decreased by 25% in preeclamptic placental explants ($P<0.01$) whereas 10% decrease was observed in normotensive placental explants after incubation with mint-tea.

Table 1: Levels of Lipid Hydroperoxide in the Placental Explant of Normotensive and Preeclamptic Women Before and After Incubation with Tea, Mint, and Mint-Tea Extracts

	Lipid Hydroperoxide (μ moles of H_2O_2/ mg Protein)	
	Normotensive Placental Explant	Preeclamptic Placental Explant
Before incubation	138 ± 6.50	253 ± 9.92***
After incubation with		
Tea	130 ± 6.48^{NS}	213 ± 9.90*
Mint	127 ± 6.51^{NS}	207 ± 9.94*
Mint-Tea	124 ± 6.49^{NS}	190 ± 9.89**

Values are expressed as mean ± SD (for 10 samples in each group). ^{NS} Not significant; ***P<0.001 when compared with normotensive placental explant without any incubation.
*P<0.05; **P<0.01 when compared with preeclamptic placental explant without any incubation.

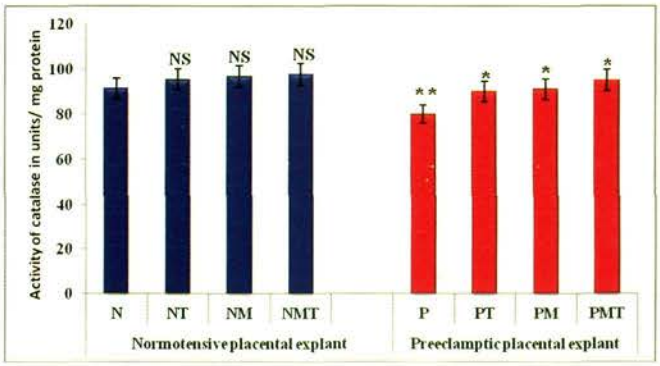

Fig. 1: Activity of catalase in the placental explant of normotensive and preeclamptic women before and after incubation with tea, mint and mint-tea extracts.

Note: Each bar represents mean ± SD (for 10 samples in each group). ^{NS}Not significant; **P<0.05 when compared with normotensive placental explant without any incubation. *P<0.05 when compared with preeclamptic placental explant without any incubation.

Conjugated Diene

To evaluate the stress response in the preeclamptic patients, the level of conjugated diene was analyzed in the placental explants of both normotensive and preeclamptic pregnant women (Table 2). The results revealed that CD levels were significantly increased by onefold in preeclamptic placental explants (P<0.001) than normotensive placental explants. Addition of tea, mint and mint-tea decreased CD significantly in preeclamptic placental explant. The level of CD was decreased by 15%, 17% and 27% in preeclamptic placental explant and by 7%, 9% and 10% in normotensive placental explant by tea, mint and mint-tea extract, respectively.

Table 2: Levels of Conjugated Diene in the Placental Explant of Normotensive and Preeclamptic Women Before and After Incubation with Tea, Mint, and Mint-Tea Extracts

	Conjugated Diene (Δ 233 / mg Protein)	
	Normotensive Placental Explant	Preeclamptic Placental Explant
Before incubation	0.072 ± 0.007	0.168 ± 0.009***
After incubation with		
Tea	0.067 ± 0.005^{NS}	0.143 ± 0.006*
Mint	0.066 ± 0.004^{NS}	0.139 ± 0.008*
Mint-Tea	0.065 ± 0.003^{NS}	0.123 ± 0.007**

Values are expressed as mean ± SD (for 10 samples in each group). ^{NS} Not significant; ***P<0.001 when compared with normotensive placental explant without any incubation.
*P<0.05; **P<0.01 when compared with preeclamptic placental explant without any incubation.

Catalase

The catalase was determined in the placental explants with and without mint-tea extracts. The level of CAT was significantly decreased by 13% in preeclamptic placental explants (P<0.05) than the normotensive placental explants (Fig. 1). After incubation with tea, the level of CAT was significantly higher (12%) in preeclampsia (P<0.05) than normotensive placental explants (3%). The mint extracts significantly increased the level of CAT by 14% (P<0.05) in preeclamptic placental explants and in normotensive placental explants by 5%. When incubated with mint-tea extracts, the level of CAT significantly increased by 19% in preeclamptic placental explants (P<0.05) as compared to 7% in normotensive placental explants.

Quantification of ASK-1

ASK-1 an apoptosis mediator protein was monitored in both explants incubated with and without plant extracts. The result reflected that the level of ASK-1 was significantly decreased by 18% in preeclamptic placental explants (P<0.05) than normotensive placental explants (Fig. 2). Addition of tea significantly reduced in preeclamptic placental explants by 23% (P<0.01) and in normotensive by 6%. The level of ASK-1 was sig-

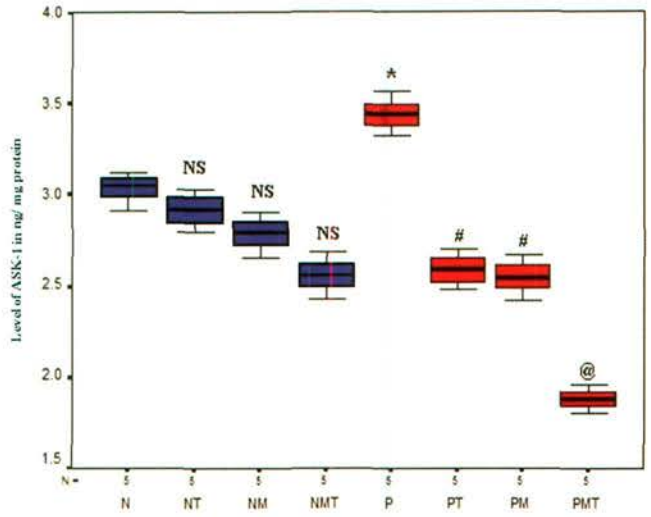

Fig. 2: ASK-1 expression in normotensive and preeclamptic placental explants with and without incubation with tea, mint and mint tea extracts.

Note: N: without any incubation; NT: with tea; NM: with mint; NMT: with mint-tea; P: without any incubation; PT: with tea; PM: with mint; PMT: with mint-tea.

Values represent mean ± SD (for 10 samples in each group).

^{NS}Not significant; $^{**}P<0.05$ when compared with normotensive placental explant without any incubation. $^{\#}P<0.01$ and $^{@}P<0.001$ when compared with preeclampsia placental explant without any incubation.

nificantly decreased by 26% in preeclamptic placental explants ($P<0.01$) as compared to 8% in normotensive placental explants after incubation with mint extracts. The mint-tea suppressed the apoptosis by decreasing the level of ASK-1 in preeclamptic placental explants by 34% ($P<0.001$). In normotensive placental explants, 10% decreases in the expressions of ASK-1 were recorded.

Discussion

Preeclampsia results from generated inflammatory response, which causes maternal endothelial dysfunction and trophoblast cell death.[35] The causes of preeclampsia remain largely unknown, but poor placentation is an important predisposing factor. The risk of preeclampsia markedly increases in women associated with vascular diseases and various post-partum complications. Preeclampsia increases oxygen-free radical production, and the resulting oxidative stress impairs placental blood flow. This may be due to the overproduction of free radicals.[36] Tea has been found to effectively down regulate induction of reactive oxygen radicals in the stress condition and tea can also act as a natural quencher of superoxides.

The beneficial effect of dietary antioxidants to human health has been purported, the evidence that antioxidant supplementation decreases oxidative stress in human has not been validated completely. Unfortunately, the antioxidant properties of tea have not been demonstrated during pregnancy and *in-vitro* models. Earlier study reported that the long term incubation studies on the explants will mimic the exact scenario and aids in analyzing the protective role of black tea and mint tea on the placenta under conditions of preeclampsia.[9] The present study is performed using multiple biomarkers to investigate the antioxidant effects of tea in placenta. The present results showed that consumption of mint-tea reduced oxidative damage induced by free radicals. This was evidenced by decreased oxidative damage in placental explant of preeclampsia and normotensive. The decrease in oxidative damage was correlated to decreased levels of LHP and CD significantly when incubated with tea, mint and mint-tea effectively was shown in the Tables 1 and 2.

The decreased oxidative damage found in this study was consistent with increased total antioxidant capacity previously reported in humans who consumed tea.[37] The antioxidant effects of mint-tea were determined in placenta in this study and verify previous studies showing that mint-tea is an effective antioxidant. Mint-tea, which contains high amounts of tea polyphenols, showed very strong antioxidant capacity *in-vitro* and effectively decreased oxidative stress.[9, 38] The findings are reported that tea, mint and mint-tea increased the level of catalase in preeclamptic placental explants when compared with normotensive placental explants (Fig. 1).

From the preceding descriptions, it can be seen that oxidative stress may induce a range of cellular responses depending upon the severity of the insult and the compartment in which the ROS are generated. Some of the more major signaling pathways involved and potential outcomes are presented in Figure 2. Oxidative stress significantly induced the expression of ASK-1 in preeclamptic placental explant as compared to normotensive placental explant. More severe attack by ROS may lead to more extensive and irreparable cell damage, resulting ultimately in cell apoptosis by activating ASK-1. These more pathological effects are mediated by opening of ion channels, lipid peroxidation, protein modifications and DNA oxidation.[39] The placental explants incubated with tea, mint and mint-tea decreased the expression of ASK-1 significantly in both the groups. This deciphers that mint-tea enhances the cell to survive and may promote live foetal delivery.

In conclusion, oxidative stress influences the entire

reproductive lifespan of a woman. Pregnancy is an important phase in every woman's life. In general, antioxidant defenses appear to be poor during preeclampsia. Condition like preeclampsia modulates oxidative status and severely alters the antioxidant systems. Increase in the expression of ASK and its related molecules act as the only means for inducing the oxidative stress mediated apoptosis. Many antioxidant therapies and alternative medicines are administered to increase the antioxidant deficiencies. As administration of drugs may be deleterious to mother and fetus, natural antioxidants rich herbs like tea and mint can be used to treat preeclampsia or taken as general antioxidant therapy. Mint-tea acts as potent antioxidant by its inbuilt antioxidant capacity which is attributed by the high phenolic, flavinoids, polyphenols and minerals like Na, Mg, K, Cr, Fe, Co, Cu, Zn, and Se present in tea and mint extracts which possess OH radical scavenging activity, reducing power. The usage of these mint-tea extracts as an antioxidant supplement may provide some beneficiary effect to the oxidative stress related conditions like preeclampsia during pregnancy.

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