

Antioxidant Reviving Role of Mint-Tea on Placenta During Gestational Diabetes Mellitus

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Abstract: *In the global scenario, about 1-14% of pregnancies are associated with increased perinatal morbidity, mortality due to increased risk of pregnancy-induced disorders. Gestational diabetes mellitus (GDM) is one of the severe and serious condition that occur due to high blood glucose levels during pregnancy, which leads to variety of structural and functional changes in placenta. Placental injury due to free radical induced oxidative stress (OS) plays a crucial role in the pathogenesis of complications during diabetic gestation. To ameliorate OS induction during diabetic pregnancy, herbs containing potential antioxidant constituents are required. Mint (*Mentha spicata*) and Tea (*Camellia sinensis*) are the renowned aromatic herbs rich in effective antioxidants. Hence the present study aims to monitor the possible beneficial effect of mint-tea on OS markers such as LPO, NO₂⁻, HNE, ADMA, antioxidants such as SOD, TAC, GRR along with the analysis of HSP70 expression. Oxidant-antioxidant imbalance along with increased expression of HSP70 was observed in the GDM placental homogenate without mint-tea incubation. Mint-tea incubation diminished the oxidants and increased the antioxidants which subsequently results in decreased HSP70 expression in GDM placental homogenate. The results revealed that mint-tea extract renders more cell protection and implying their effect in modulating GDM associated stress is more pronounced in combined form than their sole use. Hence the mint-tea extracts can be employed as an alternative herbal remedy to prevent the OS mediated complications during GDM.*

Keywords: Gestational diabetes mellitus (GDM), Mint, Tea, Heat shock protein 70 (HSP70) Oxidative stress (OS)

1. Introduction

Pregnancy is a diabetogenic state manifested by insulin resistance and hyperglycaemia. Gestational diabetes mellitus (GDM) is a complex, chronic illness requiring continuous medical care with multifactorial risk reduction strategies beyond glycemic control. It is a glucose intolerance of varying severity with onset during pregnancy and occurs in 1–14% of patients (1). GDM as a disease entity adversely affects maternal and fetal outcomes. The condition has been implicated as a risk factor for future diabetes and obesity in women as well as in their offspring by impaired carbohydrate metabolism (2). The major morbidities of GDM in maternal system include hypertensive disorders and in infants include respiratory distress, growth restriction, polycythemia, hypoglycemia, hypocalcemia, hypomagnesemia and congenital malformations (3). Therefore, it is imperative for early detection and management of the disease to ensure better maternal and fetal outcomes. Hence, the present study aims to assess the effect of mint tea on the oxidant-antioxidants status in GDM placenta for the therapeutic intervention to manage GDM.

In GDM, the placenta undergoes a variety of structural and functional changes. Placenta, a mirror of materno-fetal status, is a membranous vascular organ which mediates materno-foetal exchange (4). The diabetic environment may have profound effects on placental development and functions. The placenta of diabetic women have attracted much interest, primarily because placental damage may be responsible for the high incidence of fetal complications in pregnancies complicated by diabetes mellitus (5).

Placental perfusion becomes poor and promotes the generation of free radicals which further induce OS. OS refers to a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defences (6). It damages cellular lipids, proteins or DNA and inhibits their normal function. During GDM pregnancy, OS may alter embryo development, implantation, placental and fetal development. MDA and HNE are active lipid peroxide products which cause cytotoxicity under various oxidative insults. Hence it can be used as a biomarker for the extent of lipid peroxidation. Increased ROS products lead to elevation of reactive nitrogen species (RNS) such as nitrous oxide, nitric oxide and peroxy nitrite, ultimately resulting in nitrative stress. Nitric oxide (NO) is a vasodilator, is involved in the regulation of feto-placental vascular reactivity and placental bed vascular resistance (7). Placental vascular development is a crucial process required for adequate fetal development Placenta insufficiency leads to abnormal regulation of vascular tone that conduit to vascular endothelial dysfunction. Asymmetric dimethylarginine (ADMA), an important modulator of vascular function was determined in the present work. It is an endogenous inhibitor of the NO synthase pathway which inturn leads to vascular dysfunction (8,9). Hence it was monitored in the present work to assess the role of ADMA in vascular function of placenta during GDM induced pregnancy stress.

Cells produce antioxidants which significantly inhibit the oxidation of the substrate, scavenge and eliminate ROS & RNS to counterbalance stress. If the balance between oxidative stress and antioxidant defense deteriorates, pathological processes develops (10). Antioxidant defense

mechanisms prevent ROS induced damage of lipid, protein and DNA. SOD, CAT and GRR are important components of the antioxidant defense system which scavenges the free radicals in cells. Assessment of scavenging enzyme activities reflects the antioxidant defense status and hence the present study analyzed the antioxidant status of GDM placenta.

Molecular chaperones, including the heat-shock proteins (HSPs), form one ubiquitous feature of cells in which these proteins cope with stress-induced denaturation of other proteins (11). HSP by interacting with a number of cellular systems and signaling proteins exerts an efficient cytoprotective mechanism. Its expression is an important means of cellular protection during physiological stress. One major group of HSP is the HSP70 family, which is a stress-inducible protein that plays important role in defense mechanism against agents that promote oxidative injury, thereby preventing stress induced protein aggregation and restoring normal protein folding (12). The coordinated activities of the signaling proteins modulate multiple events within apoptotic pathways to help sustain cell survival following damaging stimuli (13).

The importance of nutrient balance in pregnant women is increasingly being recognized. A balanced diet constituting a natural source of antioxidants able to protect the cells from several diseases attributed to the reactions of the free radicals is most preferred (14). Tea (*Camellia sinensis*) leaves are extensively used as herbal medicines all over the world as they are rich in catechins, flavonols and flavonols. It is a powerful antioxidant capable of rapid reduction of superoxide radical and alkyl peroxy radicals. Mint (*Mentha spicata*) is used and valued as an aromatic herb for thousands of years (15) and it is considered as stimulant, carminative, antispasmodic, stomachic and diuretic. 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. The presences of free hydroxyl group in phenolic compounds of mint are mainly responsible for antioxidant activity (16).

Hence the purpose of the study is to monitor the beneficial effect of mint, tea and mint-tea on the oxidants-antioxidants and HSP70 expression in GDM placental tissue homogenate. This may emphasize the potent target for therapeutic intervention to manage the complications during GDM.

2. Materials And Methods:

Selection of subjects

The study was carried out for a period of six months. 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/S/BWC/611/2014). The placenta was collected from both normal (n=10) and GDM (n=10) pregnancy women in the age group of 20-40 years, post delivery. Patients with GDM were defined on the basis of criteria given in

American Diabetes Association(17): fasting blood glucose level >125 mg/dl or occasional plasma glucose >200 mg/dl. Patients with severe gestational diabetes mellitus and other severe maternal complications were excluded from the study.

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 placental homogenate of normal and GDM subjects 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 protein concentration was estimated by the method of Bradford (18) with the use of bovine serum albumin as the standard.

Estimation of lipid peroxide (LPO):

The level of LPO was determined by the method of Ohkawa et al., (19). The lipid peroxide content was expressed as nanomoles of MDA/mg of protein.

Quantification of 4-Hydroxynonenal (4-HNE):

The inducible form of 4-HNE in the placental homogenate was quantified using 4-HNE (MBS161454, Biosource, USA) according to the manufacturer's instruction.

Estimation of nitrite (NO₂⁻):

The level of nitrite was estimated by the method of Yokoi et al. (20) with slight modifications using Griess reagent. The nitrite content was expressed as nanomoles of nitrite/mg of protein.

Assay of superoxide dismutase (SOD):

The activity of SOD was estimated by monitoring the oxidation of epinephrine according to the procedure of Misra and Fridovich,(21). The activity was expressed as Units/minute/mg protein.

Determination of total antioxidant capacity (TAC):

TAC analysis was performed by the method of Prieto *et al.* (22) and 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, (23).

Quantification of HSP70 by ELISA

The cytoprotective expression of HSP70 is quantified using HSP70 (EKS-700B, Stressgen, Canada) ELISA kit according to the manufacturer’s instruction.

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 placental mitochondria of GDM with the normal groups using Student ‘t’ test. Differences were taken to be statistically significant for values of p<0.05, p<0.01, p<0.001.

3. Results

Lipid peroxidation

The level of MDA was significantly higher in the GDM placental tissue (33%) than the normal placental tissue (Fig. 1). An insignificant decrease in MDA levels was observed in normal placental tissue (7%) and GDM placental tissue (9%) after incubation with tea. The mint extract significantly reduced the level of MDA by 11% in normal placental tissue and by 16% in GDM placental tissue. When incubated with mint-tea extracts the level of MDA was significantly

decreased by 29% in GDM placental tissue, where as in normal placental tissue 13% decrease was observed.

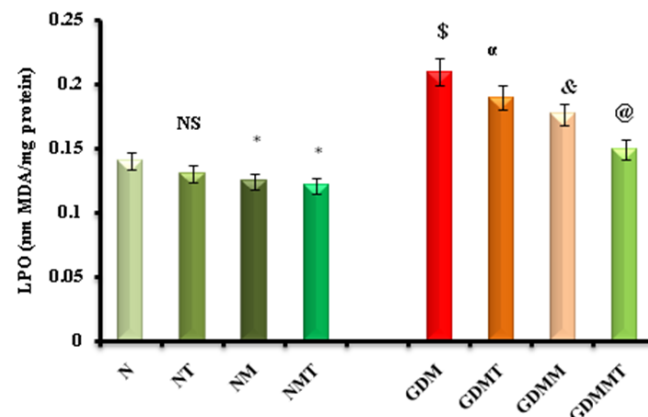


Figure 1: Lipid peroxide level in the normal and GDM placental tissue homogenate before and after incubation with tea, mint and mint-tea extracts. Values are expressed as mean ± SD (for 10 samples in each group).

Normal (N) placental tissue homogenate	GDM placental tissue homogenate
N- without any incubation	GDM- without any incubation
NT- with tea	GDMT- with tea
NM- with mint	GDMM- with mint
NMT- with mint-tea	GDMMT- with mint-tea

\$p<0.001, *p<0.05, NS not significant; when compared with normal placental tissue homogenate without any incubation
 αnot significant, &p<0.05, @p<0.01 when compared with GDM placental tissue homogenate without any incubation

NO₂⁻

NO₂⁻ was significantly increased in the GDM placental tissue (28%) than the normal placental tissue (Fig. 2). After incubation with tea, an insignificant decrease in NO₂⁻ levels in normal placental tissue (8%) and GDM placental tissue (11%) was observed. Mint extract significantly decreased the level of NO₂⁻ by 9% in normal placental tissue and by 19% in GDM placental tissue. Upon mint-tea incubation, the level of NO₂⁻ was significantly decreased by 30% in GDM placental tissue and 11% in normal placental tissue .

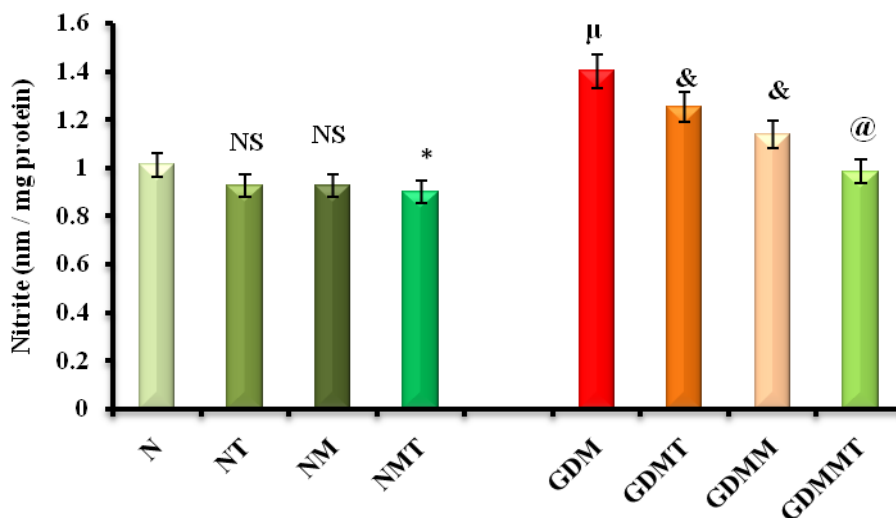


Figure 2: Level of nitrite in the normal and GDM placental tissue homogenate before and after incubation with tea, mint and mint-tea extracts. Values are expressed as mean ± SD (for 10 samples in each group).

[#]p<0.01, *p<0.05, ^{NS} not significant; when compared with normal placental tissue homogenate without any incubation &p<0.05, @p<0.01 when compared with GDM placental tissue homogenate without any incubation.

Hydroxynonenal

Significantly higher HNE level was noted in the GDM placental tissue (50%) than the normal placental tissue (Fig. 3).

3). Decrease in HNE levels was observed in normal placental tissue (8%) and GDM placental tissue (17%) after incubation with tea. Upon mint incubation, the level of HNE was decreased by 13% in normal placental tissue and by 14% in GDM placental tissue. Addition of mint-tea extracts, significantly decreased the level of HNE significantly in GDM and normal placental tissue by 29% and 18% respectively.

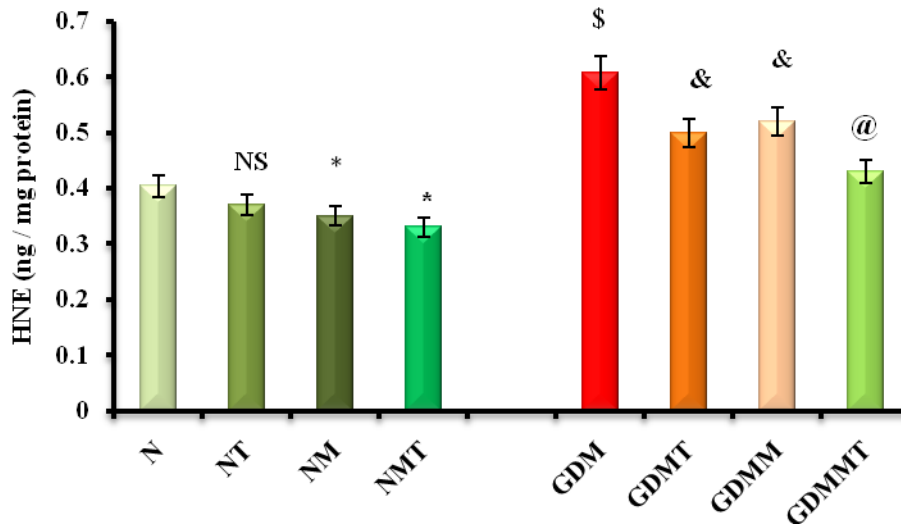


Figure 3: Level of HNE in the normal and GDM placental tissue homogenate before and after incubation with tea, mint and mint-tea extracts. Values are expressed as mean ± SD (for 10 samples in each group).

^{\$}p<0.001, *p<0.05, ^{NS} not significant; when compared with normal placental tissue homogenate without any incubation &p<0.05, @p<0.01 when compared with GDM placental tissue homogenate without any incubation

ADMA

In GDM placental tissue, ADMA level was significantly elevated by 59% than the normal placental tissue (Fig. 4).

Tea extracts decrease the ADMA levels in both normal placental tissue (6%) and GDM placental tissue (19%). The mint extract reduced the level of ADMA by 14% in normal placental tissue and by 22% in GDM placental tissue. When incubated with mint-tea extracts, the level of ADMA was significantly decreased by 26% in GDM placental tissue where as in normal placental tissue 18% decrease was observed.

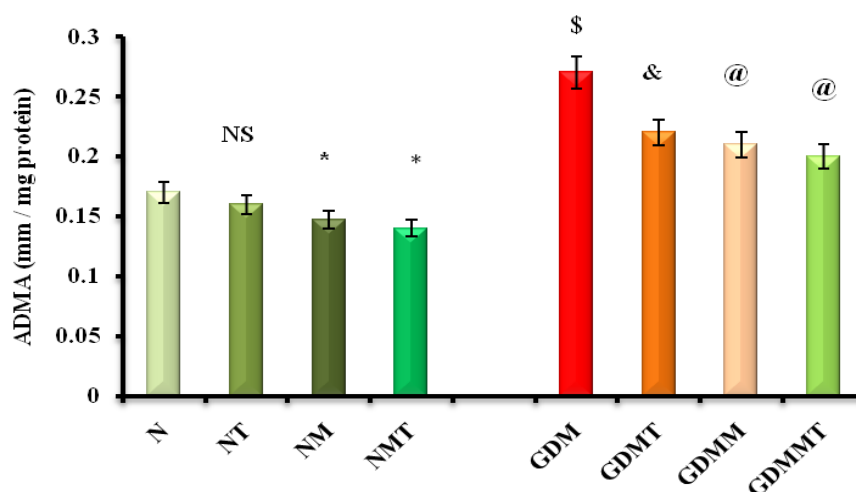


Figure 4: Level of ADMA in the normal and GDM placental tissue homogenate before and after incubation with tea, mint and mint-tea extracts. Values are expressed as mean ± SD (for 10 samples in each group).

^{\$}p<0.001, *p<0.05, ^{NS} not significant; when compared with normal placental tissue homogenate without any incubation.

&p<0.05, @p<0.01 when compared with GDM placental tissue homogenate without any incubation.

Antioxidant status

Antioxidant status was assessed by analyzing SOD, TAC and GRR in both normal and GDM placental homogenate (Table 1). The level of SOD, TAC and GRR were significantly decreased by 34%, 26% and 26% in GDM placental tissue than the normal placental tissue. After incubation with tea, the levels of SOD, TAC and GRR were higher in normal placental tissue by 9%, 10%, 8% and by

10%, 12%, 13% in GDM placental tissue. The mint extracts significantly increased the level of SOD, TAC and GRR in normal placental tissue by 12%, 11%, 12% and by 17%, 16%, 17% in GDM placental tissue. When incubated with mint-tea extracts, the level of SOD, TAC and GRR increased significantly by 38%, 38%, 30% in GDM placental tissue ($p < 0.001$) as compared to normal placental tissue by 13%, 18%, 14%.

Table 1: Level of SOD TAC and GRR in the normal and GDM placental tissue homogenate before and after incubation with tea, mint and mint-tea extracts

Parameters	N	NT	NM	NMT	GDM	GDMT	GDMM	GDMMT
SOD (units / mg protein)	2.892	3.165 ^{NS}	3.283 [*]	3.317 [*]	1.890 [§]	2.093 ^α	2.271 ^{α&}	2.615 [#]
TAC (Trolox equivalents in mmol / L)	0.445	0.495 ^{NS}	0.507 [*]	0.549 [*]	0.230 ^μ	0.261 ^{α&}	0.273 ^{α&}	0.370 [#]
GRR (GSH:GSSG)	0.682	0.745 ^{NS}	0.769 [*]	0.792 [*]	0.503 ^μ	0.437 ^{α&}	0.417 ^{α&}	0.718 [#]

SOD (Units/mg protein), TAC (Trolox equivalents in mmol/L), GRR (GSH:GSSG)

and mint-tea extracts. Values are expressed as mean ± SD (for 10 samples in each group).

Normal (N) placental tissue homogenate	GDM placental tissue homogenate
N- without any incubation	GDM- without any incubation
NT- with tea	GDMT- with tea
NM- with mint	GDMM- with mint
NMT- with mint-tea	GDMMT- with mint-tea

[§] $p < 0.001$, ^{*} $p < 0.05$, ^{NS} not significant; when compared with normal placental tissue homogenate without any incubation. ^α $p < 0.05$, [@] $p < 0.01$, [#] $p < 0.01$ when compared with GDM placental tissue homogenate without any incubation.

[§] $p < 0.001$, ^μ $p < 0.01$, ^{*} $p < 0.05$, ^{NS} not significant; when compared with normal placental tissue homogenate without any incubation
^α $p < 0.05$, [@] $p < 0.01$, [#] $p < 0.01$ when compared with GDM placental tissue homogenate without any incubation

4. Discussion

One of the most prevalent complications of pregnancy is gestational diabetes mellitus, a heterogeneous disorder which is associated with both neonatal morbidity and obstetric complications (24,25). Oxidants mediated damage plays a prime role in the induction of later life complications for both mother and fetus. During oxidative stress there is an imbalance of prooxidant and antioxidant factors which can result in severe placental cell dysfunction (26). Various conventional methods were followed in the treatment of GDM. However the treatment itself turns out to be a major peril for the GDM patients. Hence the requirement of natural medicine from herbs is more, which should render the efficient antioxidant properties. Mint and tea are well known herbs with potential antioxidants and they were utilized in the present study to develop alternative antioxidant therapy for GDM pregnancy.

HSP70 Expression

The expression of HSP70 was analyzed in the placental tissue homogenate of both normal and GDM pregnant women (Fig. 5). The results revealed that HSP70 level was significantly increased by 45% in GDM placental tissue than normal placental tissue. Addition of tea, mint and mint-tea decreased HSP70 levels in GDM placental tissue. The level of HSP70 was decreased by incubation with tea, mint and mint-tea extract in normal placental tissue by 3%, 5% and in GDM placental tissue by 9% and by 22%, 25% and 29% respectively.

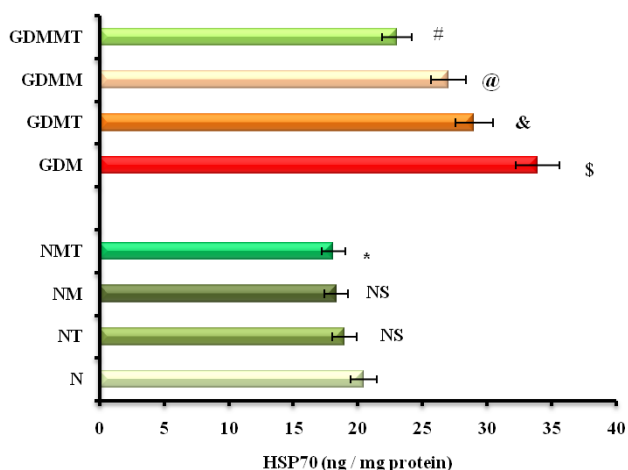


Figure 5: Level of HSP70 in the normal and GDM placental tissue homogenate before and after incubation with tea, mint

Free radical mediated oxidative stress has been implicated in the pathogenesis of diabetes mellitus and its complications (27,28). Elevated glucose levels and low insulin sensitivity has been suggested to be the cause of oxidative stress in diabetes which eventually leads to free radical generation ((29). It results in the production of stress markers which will further induce the intracellular peroxide production, leading to exacerbated oxidative stress. Studies suggested that increased OS products such as LPO and ADMA concentrations were induced and positively correlated with the glucose levels (30). Similarly in the present study elevated LPO, NO₂⁻, HNE and ADMA were observed in placental homogenate of GDM patients. Their increased expression is believed to be largely responsible for the presence of complexities during GDM condition. Increased ADMA concentration also reflects the abnormal vascular development of GDM placenta (31). However aqueous extract of tea, mint and mint-tea reduced their level suggesting their crucial requirement during pregnancy

complications like GDM. It also depicts that it may be due to the antioxidant potency of tea, mint and mint-tea (32).

Antioxidant is a substance which significantly inhibits the oxidation of the substrate and has the potential to re-enforce our body's natural defense against free radicals and minimize damage to the body. Oxidative imbalance results in abnormal placentation and placental dysfunction. Hence the present study analyzed the antioxidant defense mechanism in GDM placenta through the assay of SOD, TAC and GRR. A significant decrease in the levels of SOD, TAC and GRR was observed in GDM placental tissue when compared to normal placental tissue which is similar to result observed by Carone (33). It is suggesting that the decreased activity may be due to its role as an antioxidant enzyme for scavenging free radicals produced in this condition. However mint, tea and mint-tea incubation shift the endogenous antioxidant to be maintain in the GDM placenta. It might be due to the antioxidant efficiency of the mentioned extracts which proficiently increase the antioxidants and diminish the stress markers.

HSP70 was quantified in the present work as it is a crucial molecular chaperones act as secondary line of defense. It is altered in response to oxidative stress to maintenance of the cell homeostasis (34). The first line of defense for oxidative stress is not sufficient to combat with the generated stress. Hence the second line defense mechanism will be activated which was reflected by the propagation of HSP70 expression, an important means of cell protection during physiological stress (35,36). Coherently, HSP70 expression was significantly increased in the GDM placenta which suggests its antiapoptotic role in GDM condition. However mint, tea and mint-tea decrease the HSP70 in GDM placental homogenate which may be due to the subsequent effect of the extracts in diminishing oxidants and restoring inbuilt antioxidants. It is depicting that HSP70 is crucial for rendering cytoprotective during pregnancy complications like GDM.

The present study investigated the antioxidant effects of mint, tea and mint-tea in GDM placenta. Both mint and tea exert the potent antioxidant activity, however mint-tea extract combination exhibits more than their sole use. It is concluded that mint, tea and mint-tea can be employed as an alternate medical care for oxidative damage during GDM condition.

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