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Full Length Research Paper

Nicotine-induced modulation of blastocyst implantation and fetal outcome: Reversal by concurrent supplementation with gamma-tocotrienol

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Abstract

Present study investigated estrogen (E2), progesterone (P4), malondialdehyde (MDA), glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) profiles in pregnant mice treated with nicotine concurrently with γ-tocotrienol (γ-TCT), a non-enzymatic antioxidant. Ninety-six (6 - 8 weeks old) day 1 pc mice (Mus musculus) were injected(sc) with either 0.9% saline or nicotine of 3.0 mg/kg/day; or gavaged with γ-TCT alone of 60 mg/kg/day or treated with nicotine concurrently with γ-TCT from day 1 through day 7 pc. Nicotine did not affect implantation but reduced the count of intrauterine embryo and fetal survival rate. Concomitant treatment of nicotine concurrently with γ-TCT reversed the altered fetal parameters back to normal. High levels of MDA with corresponding low levels of GPx, CAT and SOD in nicotine-induced plasma were also reversed. Nicotine-induced elevated levels of E2 with a corresponding decrease in P4 were also reverted. Gamma-TCT supplementation concurrently with nicotine seems to overcome the effects of nicotine on fetal development, fetal outcome as well as the survival rate of the newborn. The results of the present study show that foetal development and foetal outcome, also survival rate of the newborn in nicotine-treated mice are maintained by γ-TCT, a protective antioxidant, through combating free radicals generation in nicotine-induced oxidative stress.

Keywords: Nicotine, gamma-tocotrienol, implantation, fetus, embryo development.

INTRODUCTION

Free radicals may be produced normally as part of cellular mechanism and caused no harm if produced under controlled conditions. The problem starts when the free radical formation is overwhelmed. Reactive oxygen species (ROS), a major type of free radical, leads to oxidative stress (OS). OS affects sperms (Ball and Vo 2001; Mat et al., 2006), oocytes (Tamura et al., 2008; Rajikin et al., 2009), fertilization process (Saleh et al., 2003; Tamura et al., 2008), developing embryos (Mokhtar et al., 2008; Kamsani et al., 2012), blastocysts implantation (Wang et al., 2002; Kamsani et al., 2011, unpublished observation), pregnancy (Van der Schans et al., 2000) and pregnancy outcome (Mokhtar et al., 2008) through one of its various mechanisms, the lipid peroxidation (Lefevre et al., 1998). Other oxidative stress mechanisms include the inhibition of protein synthesis and depletion of adenosine triphosphate (ATP) (Agarwal and Gupta, 2006).

Nicotine is a weak base which is less ionized and penetrates membranes more easily in alkaline medium. It has been reported that nicotine, a component of tobacco smoke induces oxidative stress both *in vivo* and *in vitro* (Suleyman et al., 2002; Kamsani et al., 2010). In reproduction, potential adverse health consequences of nicotine include reproductive or perinatal disorders such as low birth weight, prematurity and spontaneous abortion (Benowitz et al., 1988). In the maternal circulation, nicotine crosses the placenta and enters the

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foetal circulation, amniotic fluid and can be absorbed by means of the skin of the foetus (Onuki et al., 2003).

Antioxidative enzymes are known to offer protection against oxidative damage, therefore a decrease in the activities of these enzymes may expose tissues to the free radical damage. Superoxide dismutase (SOD) destroys superoxide radical by converting it to peroxide that in turn can be destroyed by glutathione peroxidase (GPX) or catalase (Kamal et al., 1992). If antioxidant systems become depleted as a result of oxidative stress, cells and tissues become more susceptible to reactive oxygen species (ROS) damage (Chiaradia et al., 1988).

It has been revealed that maternal tobacco smoking is linked with increased levels of oxidative stress markers in the mother and offspring. *In vivo* and *in vitro* evidence suggested that exposure to nicotine results in oxidative stress in foetal, neonatal and adult tissues (Orhon et al., 2009). Mitochondria and mitochondrial DNA are shown to be more sensitive to the harmful effects of ROS than the nuclear DNA (Droge, 2002). In addition to inducing overproduction of oxidants, nicotine exposure results in a decreased activity of SOD, GPx and catalase. The increase in ROS concentration together with a decrease in the activities of enzymes with antioxidant functions results in an imbalance in the oxidant/antioxidant capacity (Ornoy, 2007; Oliveira et al., 2009).

The embryo's journey through the fallopian tube requires healthy microenvironments, which are influenced by ovarian steroid hormones and other factors (Jansen, 1984). In the female, oxidative stress is also associated with a decreased effect of antioxidants possessed by pregnancy hormone oestrogen (MacMahon et al., 1982). A subsequent rise in ROS concentration under stressed condition then interferes with oocyte maturation (Onuki et al., 2003), induces embryo fragmentation (Blondin et al., 1997), implantation failure or abortion (Mokhtar et al., 2008; Soares and Melo, 2008). In rats, treatment with nicotine increases circulating 17β-estrodial levels and the ratio of oestrogen to progesterone during the pre-implantation period (Yoshinaga et al., 1979).

Gamma-tocotrienol (γ –TCT) (Raederstorff et al., 2002), a vitamin E derivative has recently been regarded as a potent lipid antioxidant (Aggarwal et al., 2010). The additional 3 double bonds in its side chain allow tocotrienols to have better mobility through the cell membrane (Suzuki et al., 1993), and make it much more potent antioxidant compared to the other isomers (Serbinova et al., 1991). The beneficial efficacy of γ –TCT in human diseases (Sen et al., 2007), cardiovascular diseases (Rasool et al., 2008), hypercholesterolomia (Qureshi et al., 1995), osteoporosis (Maniam et al., 2008) and in mammalian fertility (Geva et al., 1996) is well documented. We have also reported that γ –TCT could repair nicotine-treated ultrastructural damage of the preovulatory oocyte by retaining its shape and smooth boundary of zona pellucida with tight perivitelline space (Rajikin et al., 2009). Other studies have shown that γ –TCT supplementation increased the number of retrieved 2-, 4-cell and 8-cell stage embryos (Mokhtar et al., 2008; Kamsani et al., 2012) and reversed the nicotine-induced retarded embryogenesis in mice (Kamsani et al., 2012) and pregnancy loss in rats (Mokhtar et al., 2008). We have also noted that TCT-rich vitamin E supplementation in the nicotine-treated rats have lower plasma malondialdehyde (MDA), a biomarker for oxidative stress, as compared to its nicotine-treated counterpart (Mokhtar et al., 2008; Kamsani et al., 2012). In addition, supplementation of γ-TCT significantly reduces foetal loss in the nicotine-treated mice (Kamsani et al., unpublished observation). These findings clearly indicate that γ-TCT, a potent antioxidant, has a huge potential in the regulation of reproductive processes although the molecular mechanism of γ-TCT in reproduction has yet to be explored. Our study therefore, intends to evaluate the adverse effects of nicotine, a producer of reactive oxygen species (ROS) on blastocyst implantation, and foetal outcome including the effects of concomitant treatment of γ-TCT concurrently with nicotine on GPx, CAT and SOD enzyme activity, as well as levels of progesterone and oestrogen in pregnant mice.

MATERIALS AND METHODS

Ninety-six (6 – 8 weeks old) female mice (*Mus musculus*) weighed 30-35 g were housed in polyurethane cages with temperature maintained at 25°C under 12:12h light/dark cycle. Animals were fed with food pellets and water *ad libitum*, divided into 12 groups of 8 animals in each group and cohabited with fertile males at a ratio 1:1. The presence of vaginal plug was considered as day 1 of pregnancy (pc). Animals were subjected to subcutaneous (sc) injection of either 0.9% saline (Groups 1, 5 and 9) or nicotine (ICN,USA) of 3.0 mg/kg/day from day 1 through day 7 pc (Groups 2, 6 and 10); gavaged with γ-TCT alone (Golden Hope, Malaysia) of 60 mg/kg/day (Groups 3, 7 and 11) or treated with nicotine of 3.0 mg/kg/day concurrently with 60 mg/kg/day of γ-TCT (Groups 4, 8 and 12) from day 1 through day 7 pc. Animals of groups 1-4 were sacrificed on day 10 pc, blood samples were collected and the number of embryonic swellings recorded. Animals of groups 5-8 were laparotomized under anesthesia implantation sites were counted on day 8 pc, sacrificed on day 13 pc, blood samples were collected and number of embryonic swellings was recounted. Animals of groups 9-12 were laparotomized under anesthesia on day 8 pc, implantation sites were counted; pregnancy outcomes as well as the survival rate of the newborn were evaluated until the age of 14 days. All procedures on the animals have been approved by UiTM Committee on Animal Research and Ethics (CARE).

Table 1. Effects of nicotine and nicotine supplemented with γ-TCT on blastocyst implantation, foetal outcome and neonatal survival rate.

Group	Number of implantated sites (Day 8 pc.)	Number of foetuses (Day 10 pc.)	Number of foetuses (Day 13 pc.)	Fetal outcome (at parturition)	Number of litters survived (Day 7 pn.)	Number of litters survived (Day 14 pn.)
0.9 % NaCl	15.4±1.61	15.4±0.74	15.2±3.02	15.1±0.21	14.9±2.1	14.6±1.89
Nicotine (3.0 mg/kg/day)	13.2±0.49	4.6±0.75**	4.3±0.34*	2.2±1.7**	2.2±0.7**	1.98±1.16**
γ-TCT (60 mg/kg/day)	14.5±2.1	14.5±3.9	14.5±2.1	14.4±1.3	14.3±1.08	13.8±2.04
Nicotine (3.0 mg/kg/day) + γ-TCT (60 mg/kg/day)	13.3±2.06	13.2±1.74	13.2±0.99	13.1±2.75	13.1±2.2	13.1±1.48

*p < 0.05, ** p < 0.001,
pc = day of pregnancy, pn = postnatal age

Sample collection

Blood samples were collected via cardiac puncture, centrifuged (2500 rpm, at 4 °C for 15 minutes) and plasma samples were frozen at -70 °C until analyzed.

Chemicals

Glutathione peroxidase **(GPx),** catalase **(CAT),** superoxide dismutase **(SOD)** kits (Cayman Chemical, USA), progesterone **(P_4)** and oestrogen **(E_2)** kits (Roche Diagnostics, USA) were used. All other chemicals used were of analytical grade.

Determination of malondialdehyde (MDA) in plasma

Plasma samples were analyzed for MDA using the thiobarbituric acid reactive substances (TBARS) method (Lefevre et al., 1998). The absorbance was measured photometrically at 532 nm and the concentrations were expressed as nanomoles MDA per gram protein (nmol/g).

Determination of GPx, CAT and SOD in plasma

By using the commercial kits (Cayman Chemical, USA), GPx activity was measured at 340 nm, CAT activity at 540 nm and SOD activity at 450 nm.

Determination of progesterone (P_4) and estradiol (E_2) in plasma

The analyses were carried out by using electrochemiluminescence immunoassay (ECLIA) for P_4 and E_2 (Roche Diagnostics, USA).

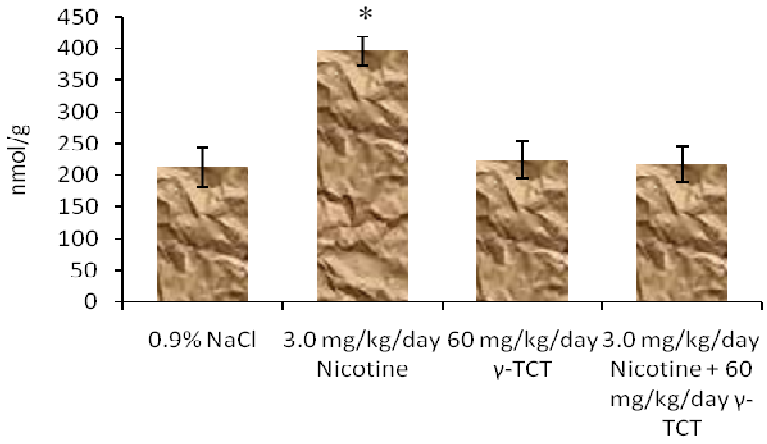
Statistical analysis

Data were analyzed using the SPSS package program (SPSS 17.0, Chicago, IL, USA). Statistical methods included a two-way ANOVA. All continuous variables were expressed as mean±SEM. A p value of <0.05 was considered statistically significant.

RESULTS

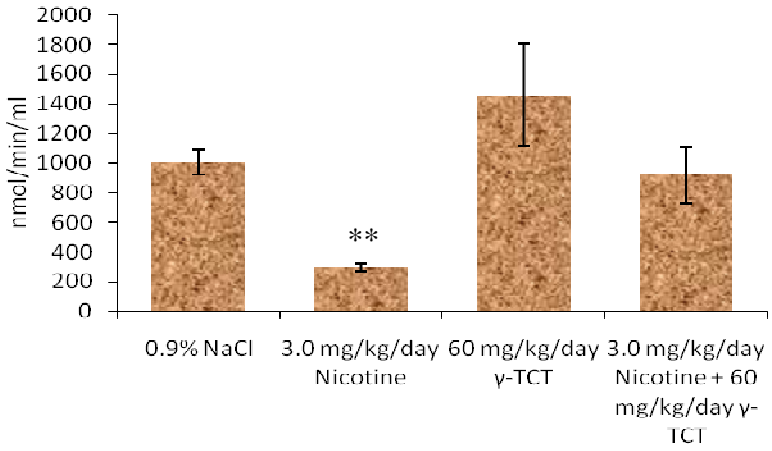
Table 1 shows the number of implanted blastocysts, foetal outcome and the survived neonates in nicotine-treated groups which were significantly reduced compared to controls. However, γ-TCT supplementation reversed most of the nicotine-induced altered parameters back to control levels.

Figures 1 through 6 show plasma levels of MDA (Figure 1), GPx (Figure 2), CAT (Figure 3), SOD (Figure 4), P_4 (Figure 5) and E_2 (Figure 6), respectively on day 10 pc following nicotine, but before being supplemented with



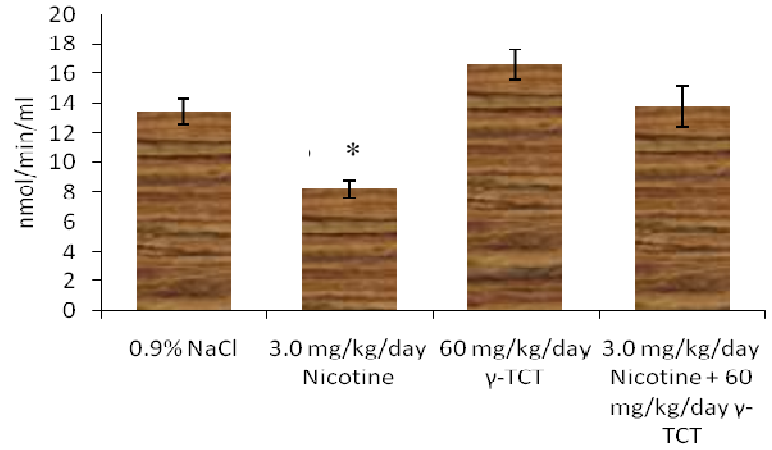
* $p < 0.05$

Figure 1. Levels of plasma MDA in nicotine-treated mice on Day 10 pc supplemented with γ-TCT.



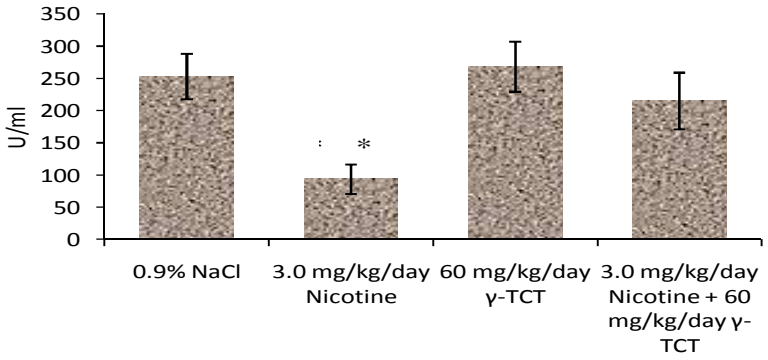
** $p < 0.001$

Figure 2. Levels of plasma GPx in nicotine-treated mice on Day 10 pc supplemented with γ-TCT



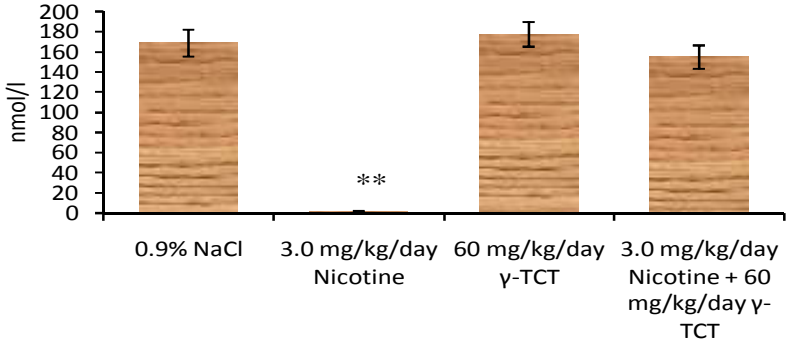
* $p < 0.05$

Figure 3. Levels of plasma CAT in nicotine-treated mice on Day 10 pc supplemented with γ-TCT



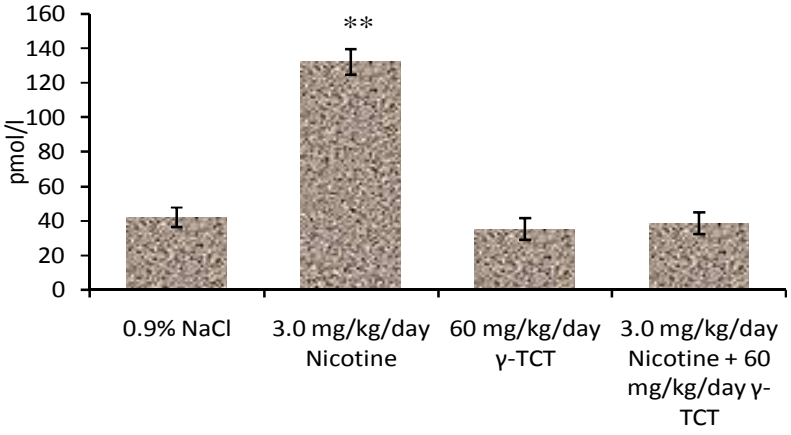
* $p < 0.05$

Figure 4. Levels of plasma SOD in nicotine-treated mice on Day 10 pc supplemented with γ-TCT



** $p < 0.001$

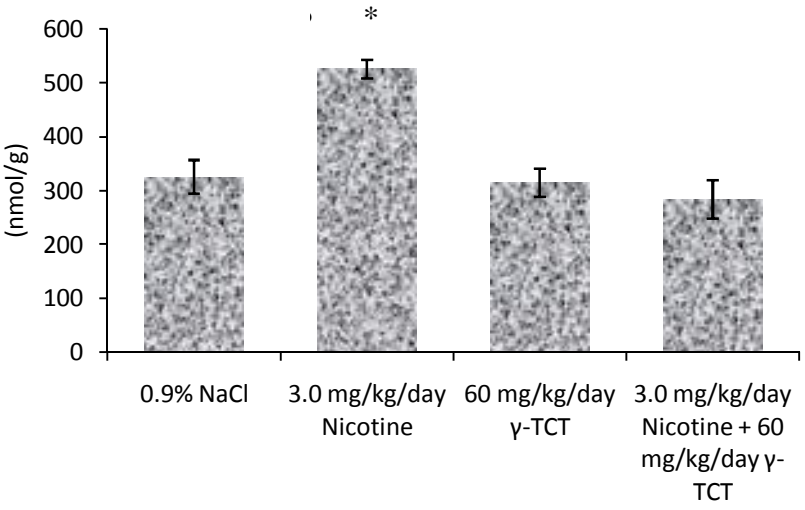
Figure 5. Levels of plasma P_4 in nicotine-treated mice on Day 10 pc supplemented with γ-TCT



** $p < 0.001$

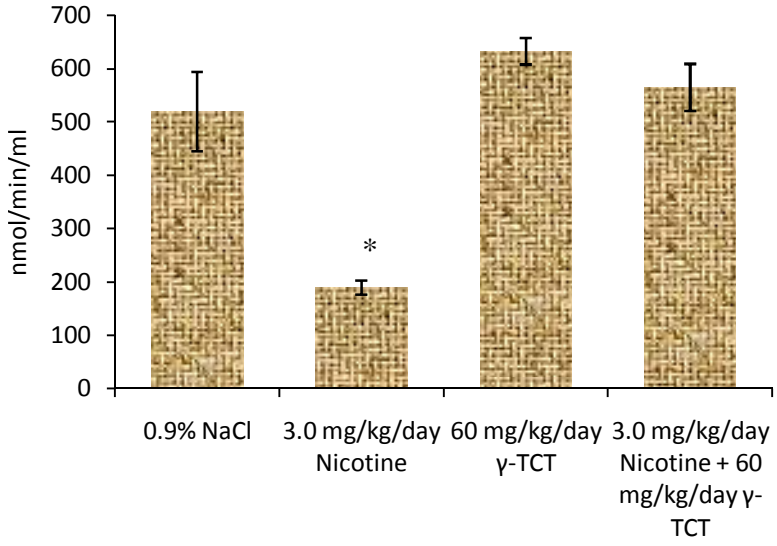
Figure 6. Levels of plasma E_2 in nicotine-treated mice on Day 10 pc supplemented with γ-TCT

γ-TCT. Compared with controls, nicotine of 3.0 mg/kg/day from day 1 through day 7 pc showed a significant increase in plasma concentration of MDA (212.03 ± 31.22 nmol/g vs 395.74 ± 23.02 nmol/g; $p<0.05$), with a corresponding decreases in plasma levels of GPx (1010.02 ± 85.12 nmol/min/ml vs 295.9 ± 21.21 nmol/min/ml; $p<0.001$), CAT (13.43 ± 0.86 nmol/min/ml vs 8.2 ± 1.02 nmol/min/ml; $p<0.05$) and SOD (252.5 ±



* $p < 0.05$

Figure 7. Plasma levels of MDA on Day 13 pc in nicotine-treated mice supplemented with γ-TCT



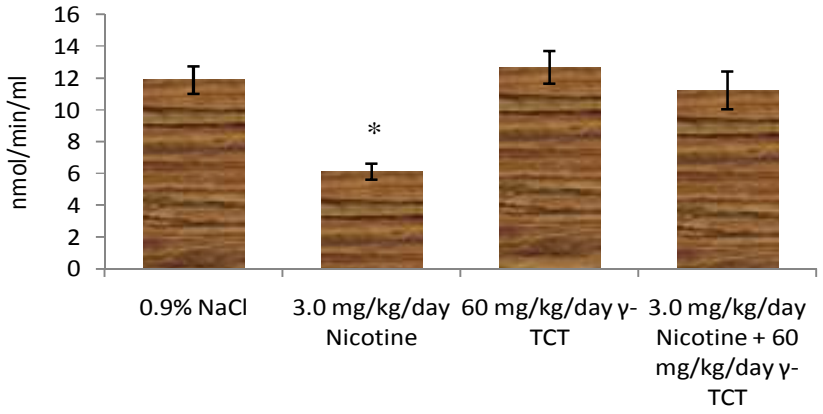
* $p < 0.05$

Figure 8. Plasma levels of GPx on Day 13 pc in nicotine-treated mice supplemented with γ-TCT

35.38 U/ml vs 93.22 ± 23.15 U/ml; $p<0.05$). P_4 level was decreased (168.37 ± 13.35 nmol/l vs 2.05 ± 0.29 nmol/l; $p<0.001$) with a corresponding rise in E_2 (42.04 ± 5.67 pmol/l vs 132.06 ± 7.43 pmol/l; $p<0.001$).

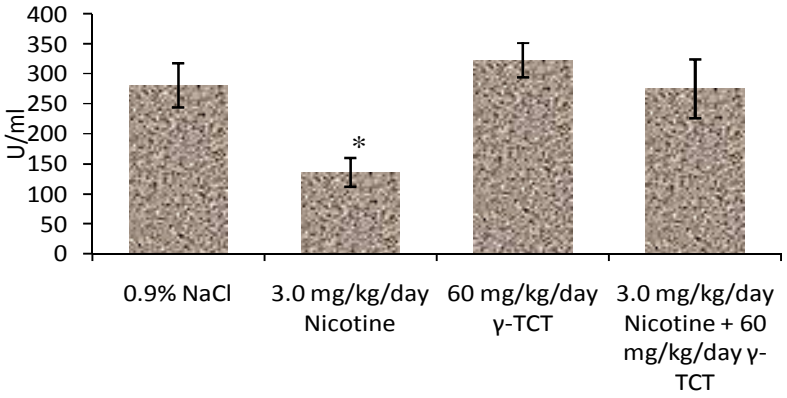
However, γ-TCT concurrently with nicotine from day 1 through day 7 pc showed tendency to normalize plasma concentrations of MDA (223.6 ± 30.04 nmol/g vs 217.02 ± 29.07 nmol/g), GPx (1459.1 ± 342.1 nmol/min/ml vs 896.01± 162.24 nmol/min/ml), CAT (16.61 ± 1.35 nmol/min/ml vs 13.76 ± 1.24 nmol/min/ml) and SOD (267.77 ± 38.91 U/ml vs 214.69 ± 43.91 U/ml) identical to control values. Similarly, the levels of P_4 (177.03 ± 12.18 nmol/l vs 154.39 ± 11.54 nmol/l) and E_2 (35.31 ± 6.24 pmol/l vs 38.58 ± 6.36 pmol/l) were found to be statistically identical to controls.

Figures 7 through 12 show levels of plasma MDA (Figure 7), GPx (Figure 8), CAT (Figure 9), SOD (Figure 10), P_4 (Figure 11) and E_2 (Figure 12) on Day 13 pc in mice treated with nicotine. Compared with the controls, nicotine treatment showed a significant increase in plasma MDA (325.09 ± 31.24 nmol/g vs 525.33 ± 16.99 nmol/g; $p<0.05$) with corresponding decrease in plasma GPx (518.98 ± 74.5 nmol/min/ml vs 189.15 ± 24.98 nmol/min/ml; $p<0.05$), CAT (11.86 ± 0.85 nmol/min/ml vs



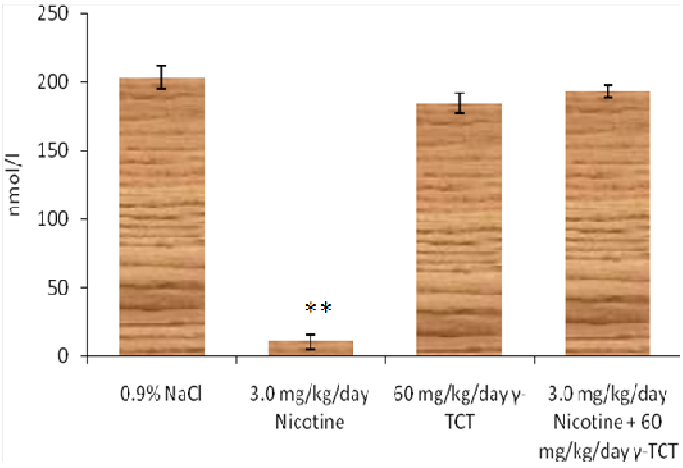
* $p < 0.05$

Figure 9. Plasma levels of CAT on Day 13 pc in nicotine-treated mice supplemented with γ-TCT



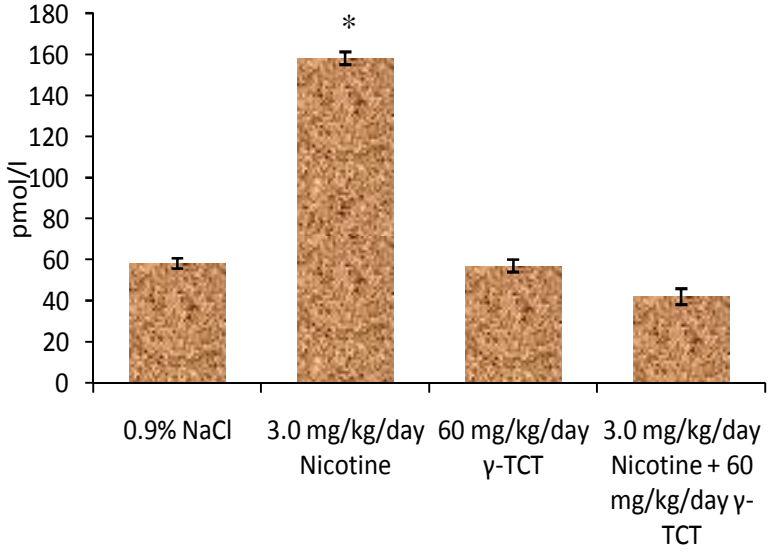
* $p < 0.05$

Figure 10. Plasma levels of SOD on Day 13 pc in nicotine-treated mice supplemented with γ-TCT



** $p < 0.001$

Figure 11. Plasma levels of P_4 on Day 13 pc in nicotine-treated mice supplemented with γ-TCT



* $p < 0.05$

Figure 12. Plasma levels of E_2 on Day 13 pc in nicotine-treated mice supplemented with γ-TCT

6.19 ± 1.03 nmol/min/ml; $p<0.05$) and SOD (280.64 ± 36.47 U/ml vs 135.27 ± 28.63 U/ml; $p<0.05$). Levels of P_4 were decreased (203.15 ± 8.33 nmol/l vs 10.73 ± 5.21 nmol/l; $p<0.001$) with a subsequent rise in E_2 (58.39 ± 2.54 pmol/l vs 158.31 ± 3.09 pmol/l; $p<0.05$).

γ-TCT concurrently with nicotine from day 1 through day 7 pc had tendency to normalise plasma concentrations of MDA (314.01 ± 26.35 nmol/g vs 283.03 ± 35.76 nmol/g), GPx (632.27 ± 43.9 nmol/min/ml vs 564.72 ± 12.41 nmol/min/ml), CAT (12.67 ± 1.18 nmol/min/ml vs 11.22 ± 1.94 nmol/min/ml) and SOD (322.46 ± 49.21 U/ml vs 274.51 ± 17.83 U/ml) back to control values. Likewise, the levels of P_4 (184.5 ± 7.18 nmol/l vs 193.19 ± 4.32 nmol/l) and E_2 (57.24 ± 3.89 pmol/l vs 42.17 ± 3.45 pmol/l) were found to be identical to controls.

DISCUSSION

Substantial evidence shows that nicotine causes damage to the membrane structure at the chromosomal level (Wang et al., 2002) and ultrastructural level (Rajikin et al., 2009) which subsequently altered oocyte maturation (Tamura et al., 2008; Rajikin et al., 2009), fertilization (Saleh et al., 2003; Tamura et al., 2008), embryonic development (Mokhtar et al., 2008; Kamsani et al., 2012) and pregnancy outcome (Mokhtar et al., 2008; Kamsani et al., unpublished observation). Nicotine dose of 2.0 mg/kg/day has been reported in previous studies to simulate moderate smoking (Murrin et al., 1987; Lichtensteiger et al., 1988). In another study, 3.0 mg/kg/day nicotine produced plasma levels typical of smokers (Slotkin et al., 2010). We were therefore tempted to investigate the effect of 3.0 mg/kg/day nicotine on plasma and tissue lipid peroxidation, enzymatic antioxidant and hormonal levels before supplementation with 60 mg/kg/day γ-tocotrienol (γ-TCT), the optimum γ-TCT concentration that effectively reversed nicotine-induced altered preimplantation embryo development *in vitro* (Kamsani et al., 2012).

Gamma-tocotrienol, sustains preimplantation embryonic development (Mokhtar *et. al.* 2008; Kamsani et al., 2012) and blastocyst implantation (Wang et al., 2002). However, changes in oestrogen and progesterone profile, embryonic and foetal development in pregnancy following nicotine or nicotine concurrently with γ-TCT have not yet been reported.

The antioxidant defense systems of the living body consist of enzymatic antioxidants such as glutathione peroxidise (GPx), catalase (CAT) and superoxide dismutase (SOD). The non-enzymatic antioxidants such as vitamin C and vitamin E mainly do exist as dietary supplements. Both types of antioxidants may be involved in combating free radical-induced OS (Zoppi et al., 2006; Ciocoiu et al., 2007). GPx enzyme, an important cellular reductant, offers protection against free radicals, peroxides and toxic compounds by reducing lipid hydroperoxide to prevent oxidation and promotes decomposition of free radicals (Niki et al., 2005). SOD, also known as the first line of defence against free radicals, destroys superoxide radical by converting it to peroxide which in turn can be destroyed by GPx or CAT (Lee et al., 2009). Depletion of activities of GPx, CAT and SOD in plasma of the nicotine-treated rats may possibly result from increased utilization of the endogenous antio-

xidants to counter lipid peroxidation (Kalpana and Menon, 2004).

Protein carbonyl and TBARS, oxidative products of protein and lipid, respectively, are well known as useful markers for assessing OS in vivo (Lee et al., 2009). TBARS as measured by production of MDA, increased in blood of adult smokers suggesting increased OS (Ciocoiu et al., 2007). In our study, enhanced lipid peroxidation associated with depletion of endogenous antioxidants in nicotine-treated pregnant mice is a characteristic feature of OS. An increased level of MDA, an indication of OS, following nicotine treatment has also been reported from our laboratory (Mokhtar et al., 2008; Kamsani et al., 2010).

Reduced GPx and SOD activities in smokers compared to non-smokers have also been noted (Zoppi et al., 2006; Ciocoiu et al., 2007). Conversely, others have found insignificant change in the tissue oxidant defense system (Gupta et al., 1998; Maniam et al., 2008) or increased activities of antioxidant systems (Abou-Seif MA 1996; Hilbert and Mohsenin 1996). An *in vitro* study has, however, shown an increase in GPx but not CAT or SOD enzyme activity in cardiomyocytes supplemented with α-tocopherol (Li et al., 1996). Although inconsistent findings do exist, significant decrease in GPx, CAT and SOD with a corresponding increase in plasma MDA in our nicotine-treated pregnant mice consistently agree with findings of others (Zoppi et al., 2006; Ciocoiu et al., 2007; Gannon et al., 2012).

In our study, supplementation of γ-TCT concurrently with nicotine during pregnancy significantly lowered the level of lipid peroxidation with a subsequent decrease in plasma MDA and a corresponding increase in GPx, CAT as well as SOD enzyme activities. Tocotrienol is suggested to possess specific distribution, higher antioxidant activity and recycling efficiency, which help interaction between tocotrienol molecules and lipid radicals more efficiently compared to its other isomers (Serbinova et al., 1991).

The decreased levels of plasma progesterone with corresponding increase in oestrogen in the nicotine-treated pregnant mice were found to be reversed back to control values following concomitant supplementation of γ-TCT concurrently with nicotine. Nutt (2001) has reported that nicotine alters plasma ratio of oestrogen to progesterone during preimplantation stage of pregnancy. We, moreover recorded that compared to controls, both the intrauterine fetal survival rate and neonatal survival rate were significantly reduced in the nicotine-treated animals.

Our study, however, showed that nicotine treatment during pregnancy developed oxidative stress which was evident by an increase in plasma MDA with a corresponding decrease in enzymes GPx, CAT and SOD activities. Moreover, attenuated levels of plasma progesterone with a corresponding elevation of oestrogen evidently resulted in fetal wastage (Pal et al., 1976; Chatterjee et al., 1977).

CONCLUSION

Results of present study suggest that by combating free radicals generated in nicotine-induced oxidative stress, γ-TCT possibly maintains the sequences of blastocyst implantation, foetal development and outcome and survival rate of the newborn. In addition, appropriate plasma levels of progesterone and oestrogen possibly favour the pregnancy to sustain until term. However, further studies are needed to elucidate the proposed mechanism.

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