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EFFECTS OF TOCOTRIENOL SUPPLEMENTATION ON PREGNANCY OUTCOME IN MICE SUBJECTED TO MATERNAL CORTICOSTERONE ADMINISTRATION

NASIBAH, A*; RAJIKIN, M H*; NOR-ASHIKIN, M N K* and NURALIZA, A S*

ABSTRACT

*The present study was designed to observe the beneficial effects of tocotrienol (TCT) supplementation on pregnancy outcome in corticosterone (CORT)-treated mice. CORT is reported to adversely affect pregnancy outcomes in mice. Seven- to eight-week-old female mice (*Mus musculus*) were divided into five groups and subjected to their respective treatment for the first seven days of pregnancy. Group 1 (control group) had 0.1 ml corn oil intraperitoneal (ip) and orally per day. Group 2 had CORT (10 mg kg^{-1} in 0.1 ml corn oil) ip and 0.1 ml corn oil orally per day. Animals of Group 3, 4 and 5 received CORT concurrently with TCT at the dose of 60, 90 and 120 mg kg^{-1} in 0.1 ml corn oil orally per day. On Day 7 of pregnancy, laparotomy was done to determine the number of implantation sites and to observe any resorption signs. Litter sizes were measured at birth and compared with the number of implantations to determine the resorption rate in various groups. The results of this study showed that the number of implantation sites in the group treated with CORT and 120 mg kg^{-1} TCT (Group 5) was significantly higher ($p<0.05$) as compared to CORT-treated mice (Group 2). On the other hand, the resorption rate in CORT-treated mice (Group 2) was significantly higher ($p<0.001$) as compared to control (Group 1). Conversely, TCT at the dose of 120 mg kg^{-1} given to CORT-treated mice (Group 5) reduced the resorption rate towards control. Therefore, the optimum dose of TCT that is able to overcome the effect of CORT on the implantation numbers and resorption rate is 120 mg kg^{-1} body weight (BW). This finding suggests that TCT administration is able to reverse the adverse effects of CORT on pregnancy outcome.*

Keywords: corticosterone, tocotrienol, pregnancy, implantation, resorption.

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INTRODUCTION

Exposure to stress during pregnancy has been shown to have adverse effects on pregnancy outcomes, such as less number of implantations and increased fetal resorptions, in human (Nepomnaschy *et al*., 2006) as well as in experimental animals (Euker and Riegle, 1973). Cortisol in human and corticosterone (CORT) in rodents are glucocorticoid hormones that are released under stress condition (Tamashiro *et al*., 2005). Stress results in the enhanced release of glucocorticoid due to the activation of sympathoadrenals and hypothalamic-pituitary-adrenal axis (Joëls *et al*., 2007). CORT administration has been reported to directly inhibit the male reproductive system in rats (Lerman *et al*., 1997). Our previous study (Nasibah *et al*., 2011a, b) on pregnant mice verified that CORT administration for the first seven days of pregnancy at a dose of 10 mg kg^{-1} body weight (BW) decreased the number of implantations and increased the rate of resorption.

* Faculty of Medicine,
Universiti Teknologi MARA,
40450 Shah Alam, Selangor, Malaysia.
E-mail: nuraliza064@salam.uitm.edu.my

However, the mechanism by which CORT affects the male (Lerman *et al*., 1997) and female (Nasibah *et al*., 2011b) reproductive systems in those studies remain unclear. It is suggested that the mechanism in which CORT induces its effect is through a stress-induced oxidative stress condition (Zafir and Banu, 2009). Oxidative stress is attributed to induce cell-damage that is, via lipid peroxidation, DNA and protein damage (Agarwal *et al*., 2006; Sies, 1997).

According to Zafir and Banu (2009), the effect of CORT administration is comparable to the effect of restraint stress in inducing oxidative stress condition. A decline in endogenous antioxidant status and an increase in oxidative stress markers by CORT administration are comparable to the effects of restraint stress. Restraint stress was known to cause oxidative stress by inducing both psychological and physical stress in rats (Kashif *et al*., 2003). Many previous studies on the influence of CORT in affecting the brain and nervous system managed to correlate between the detrimental effects of CORT with an increase level of oxidative stress (Behl *et al*., 1997; Laura and Robert, 1996; Ahlbom *et al*., 2000; Zafir and Banu, 2009). Lin *et al*. (2004) proved that there was a clear positive relationship between CORT levels and plasma lipid peroxidation in plasma of broiler chickens. Pre-treatment of hippocampal neurons with glucocorticoids enhanced the oxidative-stress induced cell death *in vitro* (Behl *et al*., 1997). Ahlbom *et al*. (2000) verified that pre-natal rats exposed to high level of glucocorticoids are susceptible to oxidative stress-induced cell death in hippocampal neurons. Lou and Chen (1998) found that high CORT level rapidly inhibit the increment of intracellular calcium on the plasma membrane which in turn generate the calcium dyshomeostasis followed by the generation of oxidative stress condition (Laura and Robert, 1996).

Tocotrienol (TCT), as a constituent of natural vitamin E, on the other hand has been reported to play a great role as an antioxidant. The molecular structure of TCT composed of a polar chromanol ring linked to unsaturated isoprenyl side chain allows for more efficient penetration into tissues that have saturated fatty layers which leads to powerful disordering of membrane lipids and scavenging peroxyl radicals (Serbinova *et al*., 1991). According to Mutalib *et al*. (2003), TCT as compared to tocopherol, exerts a more potent antioxidant activity *in vitro* and is more efficient in suppressing reactive oxygen species (ROS) levels. Kamat *et al*. (1997) suggested that palm oil derived TCT-rich fraction has shown its antioxidant activity through the inhibition of lipid peroxidation and protein oxidation in rat liver microsomes. Thus, it is believed that TCT biological activities as antioxidant is exerted through its lipid peroxidation inhibiton action in biological membranes (Zielinski, 2008). The role of TCT as antioxidant in female reproductive system was documented by Mokhtar *et al*. (2008). The authors showed that TCT supplementation at a dose of 60 mg kg^{-1} throughout the gestation period was able to reverse the effect of nicotine; one of pro-oxidant agent on pregnancy outcome in rats. In addition, the authors also proved that TCT supplementation for 30 consecutive days was able to reverse the effect of nicotine on retarded embryogenesis. However, more data are required to determine the beneficial effects of antioxidant, especially TCT in female reproduction.

The present study thus aims to explore the beneficial effects of TCT on pregnancy outcomes in CORT-treated mice. This study was designed to ascertain whether TCT supplementation could overcome the effect of stress due to increased glucocorticoid during pregnancy. It is hoped that the outcome of this study would highlight a new dimension in preventing early pregnancy loss in women subjected to stress.

MATERIALS AND METHODS

Materials

The following chemicals were obtained from the indicated source: tocopherol-stripped corn oil (MP Biomedicals, USA), progesterone II test kit (RocheDiagnostics, Mannheim, Germany) and TCT-rich fraction palm oil (Sime Darby Bioganic Sdn Bhd, Malaysia). TCT-rich fraction palm oil per 100 g contained: α-tocotrienol (19.04%), β-tocotrienol (3.60%), γ-tocotrienol (21.12%), δ-tocotrienol (15.04%), α-tocopherol (17.11%) and olein (24.09%). TCT was diluted with tocopherol-stripped corn oil (Mokhtar *et al*., 2008) to obtain the desired concentration; 60, 90 mg, and 120 mg kg^{-1} body weight concentrations. TCT was given to mice by force feeding technique using straight feeding needle, 20 gauge, 3.8 cm and smooth ball on tip (Kent Scientific Corporation, USA) and the dispensing volume was 0.1 ml. CORT (Sigma Chemical, USA) was first dissolved in small volume of absolute ethanol (1% of total volume) and then further diluted with tocopherol-stripped corn oil (Takahashi and Rubin, 1993) to obtain the desired concentration of 10 mg kg^{-1} BW concentration. CORT was given to mice by intraperitoneal (ip) injection and the dispensing volume was 0.1 ml.

Animal Treatment

Seven- to eight-week old female mice (*Mus musculus*) with an average BW between 26 to 30 g were housed at 27°C in 12 hr light-dark cycles. Animals were given food pellets and water *ad libitum*. Vaginal smears were taken daily and those

animals with regular cycles were divided randomly into five different groups (six mice per group). Animals were then mated with experienced males after a proestrous smear. The presence of vaginal plug was defined as Day 1 of pregnancy (Nagy *et al*., 2003). Pregnant females were treated daily for seven days from Day 1 to Day 7 of pregnancy. Animals in Group 1 (control) were given 0.1 ml tocopherol-stripped corn oil ip and also received 0.1 ml tocopherol-stripped corn oil orally by force feeding technique. On the other hand, animals in Group 2 received CORT [10 mg kg^{-1} BW (ip)] concurrently with 0.1 ml tocopherol-stripped corn oil orally. Groups 3, 4 5 (TCT-supplemented groups) each received ip injection of 0.1 ml CORT at a dose of 10 mg kg^{-1} BW and followed by oral TCT at a dose of 60 mg kg^{-1}, 90 mg kg^{-1} and 120 mg kg^{-1} BW, respectively. The experimental protocol was in strict accordance with regulations and prescribed animal ethical procedures outlined by the Medical Research and Ethics Committee of the Faculty of Medicine, UiTM [Code No: ACUC/CA/06 (02) Nuraliza].

Implantation Sites and Resorption Rate as Pregnancy Outcome Parameters

On Day 7 of pregnancy, all healthy embryos and resorbed embryos were indicated by gross observation of the uterus during laparotomy under anesthesia. The resorbed embryos were identified by their small size and necrotic hemorrhagic appearance, compared with that of healthy embryos (Holinka *et al*., 1979). Healthy embryos showed normal decidual swelling alongside the right and left uterus horn. Therefore, the number of implantation sites was determined by counting the number of normal and resorbed embryos in the uterus (Hoar, 1969).

The wounds were then sutured and the animals continued their pregnancy to term. All animals were inspected daily during the gestational period for any signs of blood or expulsion from the vagina to determine whether a pregnancy loss had occured according to vaginal examination and maternal weight. The litter size were counted at birth and compared with the number of implantations sites; to determine the resorption rate in treatment and control group (Euker and Riegle, 1973). The resorption rate was defined as the number of implantation sites (normal embryos and resorbed embryos) minus live fetuses × 100/number of implantation sites (Piffer and Pereira, 2004).

Sample Collection and Hormonal Measurement

Blood samples were collected at Day 7 of pregnancy via retro-orbital sinus puncture after the mice were anaesthetised using Ketamine/Xylazine solution (Nagy *et al*., 2003). Plasma was separated by centrifugation (3000 rpm, 4ºC for 15 min) and frozen at -70ºC until progesterone and malondialdehydes (MDA) analysis. Plasma progesterone was measured using the automated Elecsys immunoanalyser (Roche Diagnostics, Mannheim, Germany).

MDA: Lipid Peroxidation as a Biomarker of Oxidative Stress

Plasma was processed for evaluation of MDA levels using the thiobarbituric acid reactive substances (TBARS) method (Ledwozyw *et al*., 1986). The absorbance was measured photometrically at 632 nm and the concentration was expressed as nanomoles MDA per gramme protein ($nmolg^{-1}$).

Statistical Analysis

Data were analysed using the SPSS package program (SPSS 16.0, Chicago, IL, USA). A Kolmogorov-Smirnov test was used to test the normality of data distribution. Implantation and progesterone data were determined by ANOVA test. The significance of difference in the resorption rates was tested by a χ^2 test (Chi-Square Test). The *P* values of < 0.05 were considered statistically significant. Box plots were used to show the median value of plasma MDA levels in all five groups.

RESULTS AND DISCUSSION

Effect of Tocotrienol on the Number of Implantation Sites in Corticosterone-treated Mice

All pregnant mice were evaluated for implantation sites during laparatomy on Day 7 of pregnancy. *Table 1* shows that the implantation sites in group treated with CORT and 120 mg kg^{-1} TCT (Group 5) was significantly higher ($p<0.05$) as compared to CORT-treated mice (Group 2). This finding indicates that TCT supplementation at the dose of 120 mg kg^{-1} for the first seven days of pregnancy is able to increase the number of implantations in CORT-treated mice.

Effect of Tocotrienol on Fetal Resorption in Corticosterone-treated Mice

Table 1 shows the effect of various doses of TCT on the resorption rate in CORT-treated mice. This table shows that the resorption percentages were significantly higher in groups receiving; CORT (Group 2), CORT and TCT 60 mg kg^{-1} BW (Group 3) and CORT and TCT 90 mg kg^{-1} BW (Group 4), as compared to control (Group 1). On the

TABLE 1. NUMBER OF IMPLANTED SITES AND RESORPTION PERCENTAGE IN MICE FOLLOWING DIFFERENT DOSES OF TOCOTRIENOL (TCT) IN CORTICOSTERONE (CORT)-TREATED MICE

Experimental groups	No. of implanted sites	No. of live fetuses	No. of resorbed embryos	Resorption percentage
Control	12.8±0.63	70	7	9.0
CORT	11.0±0.68	39	27	40.9 **
CORT + TCT 60 mg kg^{-1} BW	12.7±0.92	46	30	39.5 **
CORT + TCT 90 mg kg^{-1} BW	11.7±1.31	53	22	29.3 *
CORT + TCT 120 mg kg^{-1} BW	13.8±0.95°	79	4	4.8

Note: χ^2 test; (Chi-Square Test), *p<0.05, **p<0.001, significantly different from the control group.
One-way ANOVA, °p<0.05, significantly different from the CORT-treated group. Data were expressed as mean ± SEM.

contrary, no significant difference on the percentage of resorption was detected in the group which received treatment of CORT and TCT 120 mg kg^{-1} BW (Group 5), as compared to control.

Therefore, the findings in *Table 1* suggest that there is a restoration of resorption percentage in CORT-treated mice towards normal control values by increasing the dose of TCT to 120 mg kg^{-1} BW. *Figures 1* and *2* depict the normal implantation in TCT-supplemented mice and resorption in CORT-treated mice, respectively.

Effect of Tocotrienol on Plasma Progesterone Levels

Progesterone levels in all groups were analysed from plasma samples taken on Day 7 of pregnancy using the enzyme immunoassay method. *Figure 3* shows the effect of different doses of TCT supplementation on plasma progesterone levels in mice. This results show that progesterone levels were significantly lower ($p<0.05$) in groups treated with CORT at a dose of 10 mg kg^{-1} BW (Group 2) as compared to control (Group 1). Similarly, progesterone levels ($p<0.01$) were found to be lower in groups receiving CORT and TCT 60 mg kg^{-1} BW (Group 3) as compared to control (Group 1). However, in groups which received CORT and TCT of 90 (Group 4) and 120 mg kg^{-1} BW (Group 5), progesterone levels were not significantly different from that of control (Group 1). This finding suggests that TCT supplementation at a dose of 90 to 120 mg kg^{-1} BW is able to maintain adequately high progesterone levels in order to maintain pregnancy.

Figure 1. Normal implantation in tocotrienol (TCT)-supplemented mouse on Day 7 of pregnancy.

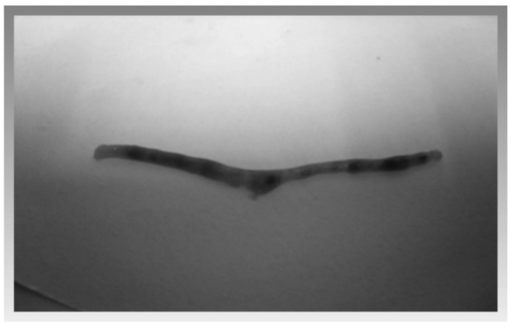


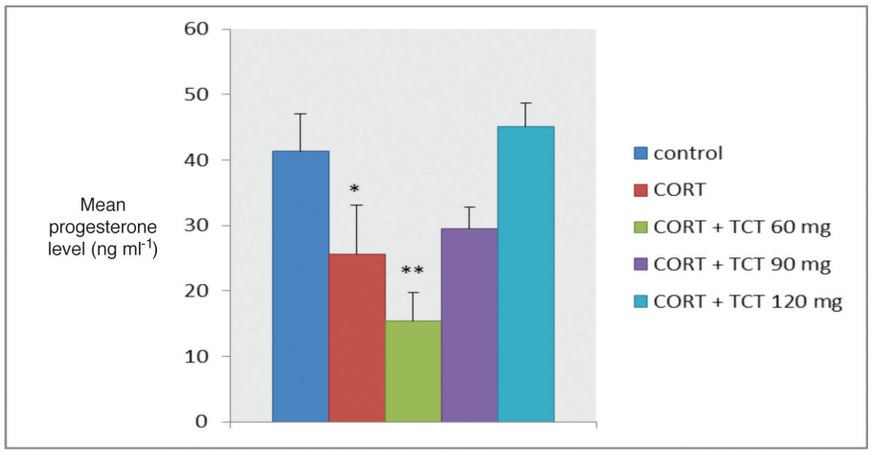
Figure 2. Resorption in corticosterone (CORT)-treated mouse on Day 7 of pregnancy.

Tocotrienol Supplementation and Malondialdehydes Concentration in Plasma

Figure 4 shows that plasma MDA in all TCT supplemented groups and in CORT group were not significantly different as compared to control. Supplementation with TCT did not caused any change on plasma MDA level. It is possible that the seven-day duration of CORT and TCT were inadequate to cause any changes on plasma MDA. Conversely, a study on the effect of CORT on nervous system showed that MDA levels were affected after 14 and 21 days of treatment (Lin *et al.*, 2004; Zafir and Banu, 2009). However, seven-day of CORT treatment in this study was chosen to avoid totally resorbed litters (Hackman and Brown, 1972) and to mimick physiological stress during early pregnancy (Wiebold *et al.*, 1986).

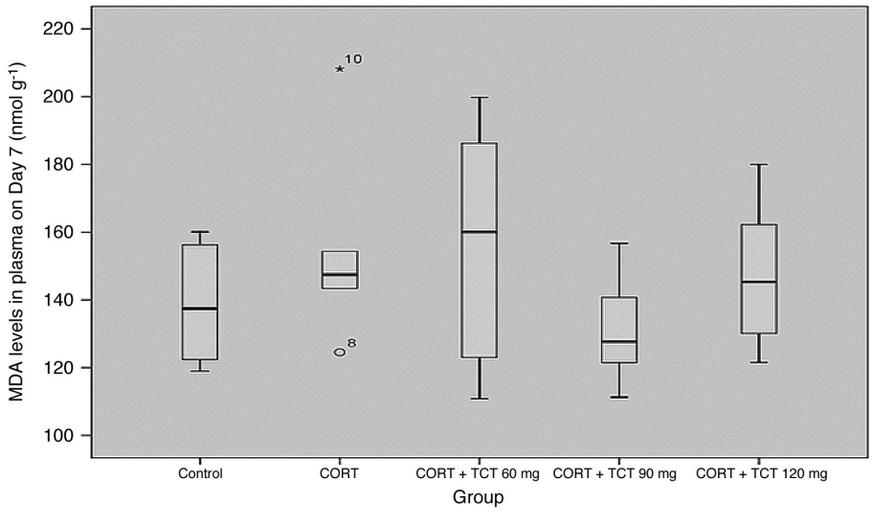
Protective Role of Tocotrienol Supplementation on Pregnancy Outcome

Despite the mechanism of TCT in reversing the deleterious effect of CORT on pregnancy outcomes in our study remains unclear, it is still strongly suggested that CORT induces its deleterious effect in reproductive system via the oxidative stress.



Note: Data were expressed as mean ± SEM and analysed by one-way Anova, *p<0.05, **p<0.01 are significantly different from the control group.

Figure 3. Plasma progesterone level in mice on Day 7 of pregnancy following supplementation with different doses of tocotrienol (TCT).



Note: Data were expressed as median.

Figure 4. Plasma malondialdehydes (MDA) levels in mice following different doses of corticosternone (CORT) treatment.

A recent finding (Shahidee *et al*., 2012) reported that CORT exposure at the dose of 10 mg kg^{-1} BW for seven and 14 days induced oxidative DNA damage in mouse embryos. This finding reveals that CORT administration as early as seven days is able to induce DNA damage in the developing mouse embryos where it will have a negative impact on embryos viability. This will disturb the implantation process and the success of the pregnancy.

Corticosteroid receptors were identified in ovarian granulosa cells (Schreiber *et al*., 1982) and in placental tissues (Speeg *et al*., 1979). Thus, CORT may exert its oxidative stress effect in reproductive functions via these receptors; where via these receptors, reactive oxygen species play a role in oocyte development, maturation, follicular atresia, corpus luteum function and luteolysis (Agarwal *et al*., 2005; Al-Gubory *et al*., 2010; Garrel *et al*., 2010; Guerin *et al*., 2000). Subsequently, oxidative stress lead to defective pre-implantation embryo development, retardation of embryo growth and decreased progesterone production leading to failure of implantation and embryonic loss. In cows under breeding period, stress causes a lower level of progesterone together with overproduction of free radicals; in particular, ROS, which is probably responsible for failure to conceive (Rizzo *et al*., 2007). Consistent with the aforementioned findings, our recent results (Nasibah *et al*., 2011a) shows that CORT starting at the dose of 10 mg kg^{-1} and higher, has a deleterious effect on female reproductive function, where it decreases the embryo quality in terms of increased abnormal embryos and retards the pre-implantation embryonic development. Corticosterone administration in our finding (Nasibah *et al*., 2011a, b) also able to decrease

the number of implantation sites and decrease progesterone levels in pregnant mice thus increase the rate of resorption.

Reducing oxidative stress by supplementation of antioxidants could potentially reduce ROS-induced damage thus increasing and maintaining the number of embryos implanted. Similarly, antioxidant supplementation able to maintain adequately high progesterone levels to retain pregnancy throughout gestational period which in turn improving the reproductive outcomes. Based on our findings in *Figures 1* and *2* and in *Table 1*, it is seen that TCT supplementation at a dose of 120 mg kg^{-1} has a role in improving the number of implantations and pregnancy outcomes in CORT-treated mice, by decreasing the rate of resorption. This concurs with the findings of Mokhtar *et al*. (2008) which showed that TCT is able to reverse the effect of nicotine as prooxidant on pre-implantation embryonic development and pregnancy outcome in rats. Findings in that study confirmed that TCT was able to reverse the nicotine induced-retardation of embryogenesis and pregnancy loss in rats via the antioxidant activity as shown by decreased MDA plasma level, as one of lipid peroxidation indicator.

Consistent with resorption data, our results showed that TCT supplementation at a dose of 90 to 120 mg kg^{-1} could maintain the required progesterone level during pregnancy as it negate the effect of CORT on pregnancy outcome. Results from this study showed that by increasing the dose of TCT supplementation from 60- to 120 mg kg^{-1} in CORT-treated mice, it caused progesterone levels to be restored towards normal, thus reducing the resorption rate to normal values. Singh and Kamboj (1992) supported this finding by suggesting that a decrease in circulating progesterone levels was associated with the resorption of all implantation. There is also an inverse relationship between the degrees of resorption with the level of progesterone. We believe that high dose of TCT negate the CORT-induced luteolysis effect (Agarwal *et al*., 2005; Al-Gubory *et al*., 2010; Garrel *et al*., 2010; Guerin *et al*., 2001) which lead to decreased progesterone production leading to failure of implantation and fetal resorption. Progesterone is a principle hormone required to facilitate the embryonic implantation and maintain the pregnancy (Sokol and Brindley, 1990) produced by corpus luteum. Corpus luteum is essential throughout the whole pregnancy in mice but in humans it is only important in the first trimester of pregnancy (Ryan, 1969).

Our findings on antioxidant supplementation corroborate with a few other findings (Mokhtar *et al*., 2008; Nur Azlina *et al*., 2005; Rozzana *et al*., 2005) that used antioxidant supplementation to reverse the deleterious effects of oxidative stress on various body systems. Rozzana *et al*. (2005) showed that alpha lipoic acid; which is known as a universal antioxidant has a protective role in female reproductive systems by protecting embryos against free radical activites. Alpha lipoic acid administration in that study managed to reverse the nicotine effect on the *in vitro* development of embryos. In the digestive system, TCT managed to inhibit the formation of gastritis in rats exposed to restraint stress, in which the oxygen radicals mediate the lesion (Nur Azlina *et al*., 2005). These studies suggest that the use of antioxidants reverses the deleterious effect of oxidative stress.

This study therefore, explores the role of TCT in the female reproductive system with the use of CORT-treated mice as experimental models. The mechanism of TCT action as an antioxidant has been established in many studies (Kamat *et al*., 1997; Yu *et al*., 2008; Zielinski, 2008). The strong antioxidant action of TCT is due to its uniform distribution in the tissue membrane, which in turn leads to powerful disordering of membrane lipids and the scavenging of peroxyl radicals. It was found that in rat liver microsomes, TCT showed its ability to interfere the oxidative damage by inhibiting the role of ferrous sulphate which acts as a prooxidant in the body (Serbinova *et al*., 1991).

TCT as a component of vitamin E plays a protective role during pregnancy. A study by Linder (1985) found that vitamin E is essential in the formation of the vascular system. The lack of vitamin E in women's reproductive system results in the failure of the adequate vascular system formation in the uterus. When this happens, the embryo cannot embed itself into the uterus during implantation and fetal resorption can occur, due to the lack of adequate blood supply. Vitamin E is required to prevent fetal resorption in rodents (Evans and Bishop, 1922; Urner, 1931) as shown by deprived-vitamin E rats. Jishage *et al.* (2005) showed that vitamin E is essential for the development and maturation of placenta in mice. Based on our findings, it shows that maternal supplementation of TCT has a beneficial role in ensuring the success of pregnancy outcomes by increasing the number of implantations, decreasing the rate of resorption and maintaining the required level of progesterone hormone during pregnancy.

CONCLUSION

Stress burdens on pregnancy outcomes call upon the intake of specific nutrients in the diet and supplements which produce beneficial effects with daily consumption. The current study provides a new insight of prevention strategy by maternal supplementation of TCT to pregnant females, which can even be started from the pre-conception period, in order to ensure positive outcomes in pregnancy. The use of TCT in this study resulted

a positive pregnancy outcome when given as maternal supplementation to pregnant mice. The mechanism by which TCT exerts its effect remains unclear and alludes further exploration.

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