THE EFFECT OF TOCOTRIENOL SUPPLEMENTATION ON PLASMA ESTRADIOL LEVELS AND QUALITY OF EMBRYOS IN AGING MICE

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Accumulation of reactive oxygen species leads to oxidative stress condition that can accelerate ovarian aging. Ovarian aging caused a reduction in plasma estradiol levels, quality of embryo and eventually will lead to infertility. Tocotrienol has been proven to possess antioxidant properties by protecting the cellular membrane from free radicals damage. Therefore, the aim of this study was to determine the effect of tocotrienol supplementation on the plasma estradiol levels, quality and development of embryos in aging mice. Female mice (Mus musculus) used in this study were divided into six groups. Six weeks old mice (young group) were used as negative control while eight months old mice (aging group) were used as age-matched (positive control) group. Group 1 (6 months old mice) were given corn oil as control, group 2, 3 and 4 (6 months old mice) were supplemented orally for two months with tocotrienol (TCT) at the dose of 90, 120 and 150 mg/kg body weight (BW), respectively. Subsequently, after two months the mice were superovulated, euthanized and 2-cell stage embryos were harvested and cultured in vitro to monitor the embryonic development. Plasma was analysed using enzyme-like immunosorbent assay. The results of this study showed that there was no significant correlation between plasma estradiol levels and the quality of embryo between young and aging group. Similarly, no significant change on plasma estradiol levels were noted in all TCT supplemented groups as compared to its vehicle control. On the other hand, there was a significant reduction on the percentage of normal embryo in all aging groups including TCT supplemented groups as compared to young group. Conversely, TCT supplementation at the dose of 150 mg/kg BW was able to increase the percentage of embryos that developed to blastocyst stage as compared to control. This finding proposed that TCT supplementations for two months are not able to cause a significant change in plasma estradiol levels and quality of embryo but it can delay the consequence of aging in embryonic development.

Keywords: embryo, estradiol, tocotrienol, aging mice

INTRODUCTION

Reproductive aging in female mammals leads to a progressive decline in fertility credited to the loss of follicles in the ovary, reduction in the quality of oocytes, age related defect in the uterus and changes in neuroendocrine axis. These events are noted both in women and rodents [1]. Ovarian aging is characterized by a reduction in the number of ovarian follicles and quality of the remaining oocyte. Normally, the oocyte quality decline due to build up of DNA

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damage in oocyte as a woman aged. As a result, some of the oocytes are incompetent for further development to become embryo following fertilization [2]. It was proven that maternal aging is associated with elevated rates of infertility, miscarriage and trisomic conceptions [3].

Estrogens are secreted by the ovarian follicles and the aging ovarian follicle will produce significantly reduce amount of estrogens [4]. According to Bayne et al. [5], that demonstrated the effect of estrogen regulation in telomere remodelling, the insufficiency of estrogens may cause aging of the ovarian tissue.

Presently, one of the most reasonable explanations of aging process is the involvement of reactive nature of free radicals and related forms of activated oxygen that is identified as reactive oxygen species (ROS). Reactive oxygen species are generated during normal metabolism whereby in access may lead to oxidative stress condition [6]. Free Radical Theory of Aging suggests that aging and its associated diseases are the result of free radical-induced damage to cellular macromolecules and the inability to counterbalance these changes by endogenous antioxidant defences [7].

Oxidative stress impacts various cellular pathways that can direct to the beginning and a sequence of diverse disorders throughout the body. The consequence of oxidative stress would probably speed up the ovarian aging. Follicular atresia along with a decline in oocyte quality and quantity may be contributed by an increased in oxidative stress during ovarian aging. Multiple physiological processes in aging ovary i.e. from oocyte maturation to fertilization, embryo development, fertility and pregnancy have been negatively affected by accumulation of ROS [8].

Reducing oxidative stress by antioxidant supplementation could potentially reduce ROS-induced damage, therefore preserving the quality and quantity of oocytes in women. There have been studies proven that tocotrienol, an antioxidant can improve the quality of impaired embryo in stress-induced mouse [9] and reversed the adverse effect of nicotine on embryonic development [10]. Nevertheless, little is known about the effect of tocotrienol supplement on female reproductive system in aging mouse.

Thus, the present study was designed to determine whether tocotrienol supplementation could overcome the effect of aging on the plasma estradiol levels, quality and development of embryos derived from aging mouse. The effectiveness of tocotrienol supplementation is proposed via its properties in reducing the level of ROS and delaying the negative effect of ovarian aging in mouse.

MATERIALS AND METHODS

Materials

Chemicals used in this study were purchased from the indicated sources: Tocopherol-stripped corn oil (MP Biomedicals, USA) and Tocotrienol-rich fraction palm oil (Sime Darby Bioganic Sdn. Bhd., Malaysia). Tocotrienol-rich fraction palm oil per 100g contained: α-tocotrienol (27.3%), β-tocotrienol (3.3%), γ-tocotrienol (35.5%), δ-tocotrienol (10.4%) and α-tocopherol (23.4%). Tocotrienol (TCT) was diluted with tocopherol-stripped corn oil [11] to obtain the desired concentration of 90, 120 and 150 mg/kg body weight.

Experimental Animals

The experimental procedures were in strict accordance with regulations prescribed by the Research Committee on the Ethical Use of Animals (UiTM Care: 98/2015). Female Mus Musculus mice at the age of 6 weeks and 6 months were used for experiments and were housed at 27^0C in 12 hours light-dark cycles. Animals were given food pellets and water ad libitum daily. For two months duration the mice were supplemented with TCT with the doses of 90, 120 and 150 mg/kg body weight (BW). Dispensing volume of

0.1ml tocotrienol were given by force-feeding technique using oral gavage, 20 gauge straight feeding needle with smooth ball on the tip (Kent Scientific Corporation, USA) [12]. After supplementation for 2 months, the animals were superovulated by given intraperitoneal (ip) injection of 0.1 ml 5 IU pregnant mare serum gonadotropin (PMSG) (Sigma, Aldrich), followed by 0.1 ml 5 IU human chorionic gonadotropin (hCG) (Sigma, Aldrich), 48 hours later and mated with fertile male mice at ratio 1:1 [13].

Blood collection and hormonal measurement

Blood samples were collected via cardiac puncture after the mice were anaesthetised with 0.1 ml per mice using ketamine, zoletil and xylazine solution mixed with distilled water [14]. Plasma were separated by centrifugation (3000 rpm, 4^0C for 15 minutes) and frozen at -80^0C until analysis. Concentrations of plasma estradiol are quantified using commercially available enzyme immunoassay kit (BioVision, USA) following the manufacturer recommended procedures. The absorbances of estradiol level were measured by Victor X Multilable Plate Reader (Perkin Elmer, USA).

Collection of embryo and in vitro development of embryo

Superovulated female mice that were successfully mated were euthanized 48 hours after hCG injection and embryos were collected by flushing out using flushing needle. Numbers of normal and abnormal embryos were counted and recorded [15]. Fig. 1 shows normal (i) and abnormal embryo (ii) to (v) at 2-cell stage. The normal embryos were cultured in a 35 mm culture dish (100 µl droplets of Whitten's medium covered with mineral oil). The cultures were kept in a humidified atmosphere incubator (at 37^0C, 90% air and 5% CO2) for 6 days. Every 20-24 hours, in vitro development of the embryos and number of blastocysts were observed and counted [15] by inverted microscope (Leica DM IRB) and photos were taken using PixeLINK Megapixel FireWire Camera (Canada).

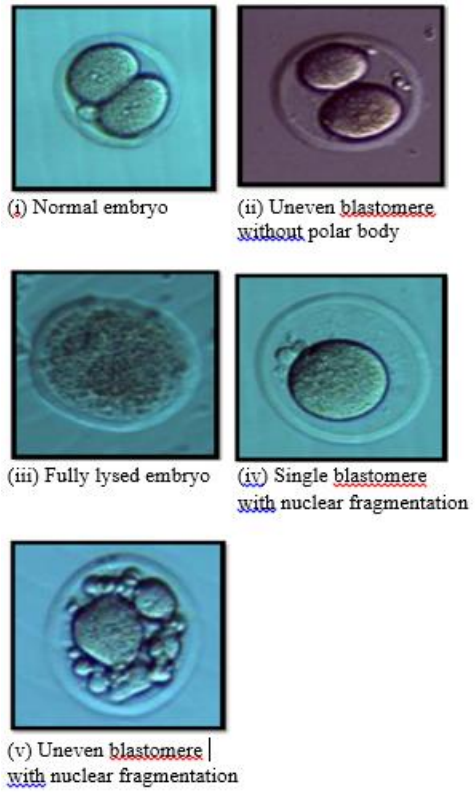

Fig. 1: *The light micrograph of normal and abnormal embryo at 2-cell stage x 100 magnifications.*

Sample and statistical analysis

Data were analyzed using Statistical Package for Social Science (SPSS). Data on the quality and quantity of embryos between control and treatment groups were analysed using Chi-square test and ANOVA for the measurement of estradiol level. Differences were considered significant at $p< 0.05$. The relationship between estradiol level and quality of embryo were analysed using Spearmen correlation.

RESULTS AND DISCUSSION

The process of aging will cause the deterioration progression in ovaries and this will lead to a significant decline in oocyte reserve and ovarian follicle [16], the embryo quantity, quality as well as its development [17]. Aging also caused a decline in plasma estrogen levels [4], a hormone that is important in implantation of embryo for a successful pregnancy [18]. However, in this finding no significant correlation (r=0.231) between the plasma estradiol level and quality of embryo in young and aging group were noted (Figure 2).

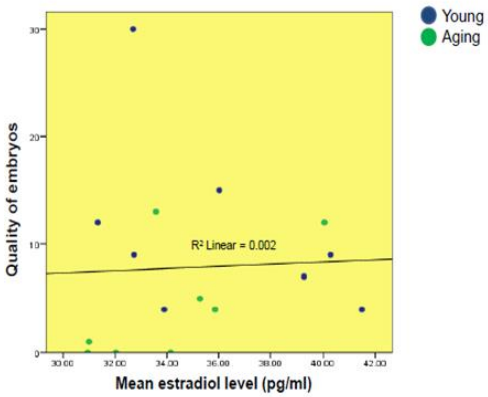

Fig. 2: *Relationship between plasma estradiol levels and quality of embryos in young and aging mice.*

Likewise, no significant difference on plasma estradiol levels were noted in all TCT supplemented groups as compared to vehicle control group (Figure 3). It is proposed that there is no significant reduction yet on the plasma estradiol levels in 8 months old mice.

Reactive oxygen species-induced ovarian aging will lead to oxidative stress condition that eventually contributes to increase in embryo fragmentation. As a result the arrested embryos cannot further developed to blastocyst and this will prevent the successful implantation process. In order to reduce the oxidative stress effect, supplementation of antioxidant could potentially reduce the ROS damage in cell by improving the quality of embryos and embryonic development due to aging. The antioxidants, namely tocotrienol reduced the oxidative stress and subsequently reduced the DNA damage in embryos [19]. Similarly, Nasibah et al. [9] and Kamsani et al. [10] reported that tocotrienol was able to reverse the effect of oxidative stress in CORT-induced and nicotine-induced retardation of embryogenesis, thus permit the success of implantation process.

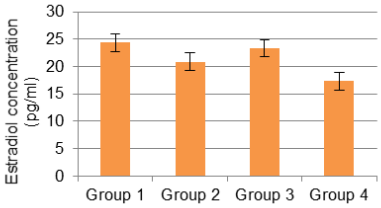

Fig. 3: *The effect of TCT supplementation on plasma estradiol levels in aging mice. Group 1: Vehicle control, Group 2: TCT 90mg/kg BW, Group 3: TCT 120 mg/kg BW and Group 4: TCT 150mg/kg BW. Data were analysed using ANOVA test. No significant difference on plasma estradiol levels between all TCT-supplemented groups (Group 2, 3 &4) as compared to control group (Group 1).*

In this study, the quality of embryos was assessed based on the morphology of normal and abnormal embryos at 2-cell stage that was observed under an inverted microscope [15]. Fig. 4 demonstrated that tocotrienol supplementation was not able to increase the percentage of normal embryos in aging mice, where the percentage of normal embryos in all aging mice supplemented with TCT were significantly lower as compared to young mice. The embryonic development were evaluated based on the observation of embryo from 2-cell until it reaches blastocyst stage (Figure 5). The percentage of embryos that reached blastocyst stage in group supplemented with 150 mg/kg of tocotrienol was improved and restored towards normal (Table 1). This finding is comparable to a recent study reported by Saidatul et al. [20].

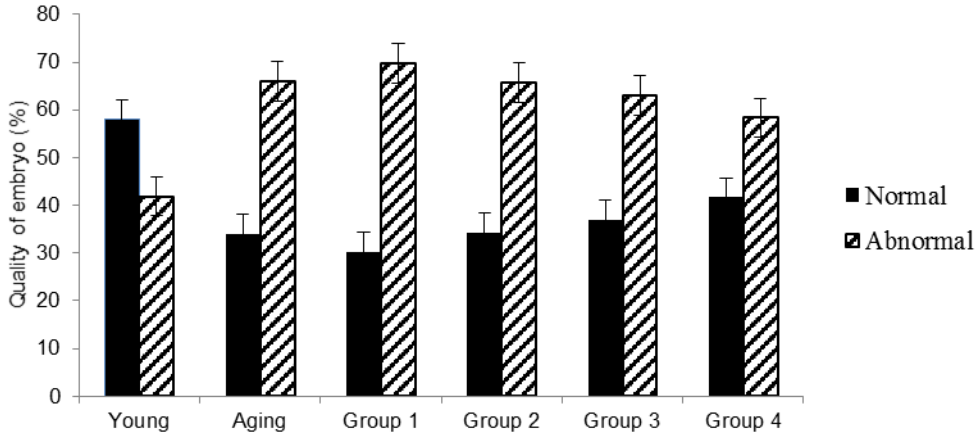

Fig. 4 *The effect of tocotrienol supplementation on the quality of embryo. Young (6 weeks), Aging (8 months), Group 1: Vehicle control, Group 2: TCT 90mg/kg BW, Group 3: TCT 120 mg/kg BW and Group 4: TCT 150mg/kg BW. Data were analysed using Chi-Square test: The percentage of normal embryos in aging (***p<0.001) and TCT-supplemented groups (*p<0.05, **p<0.01, ***p<0.001) were significantly lower as compared to young group. No significant difference on the percentage of normal embryos between all TCT-supplemented groups (Group 2, 3 &4) as compared to control group (Group 1).*

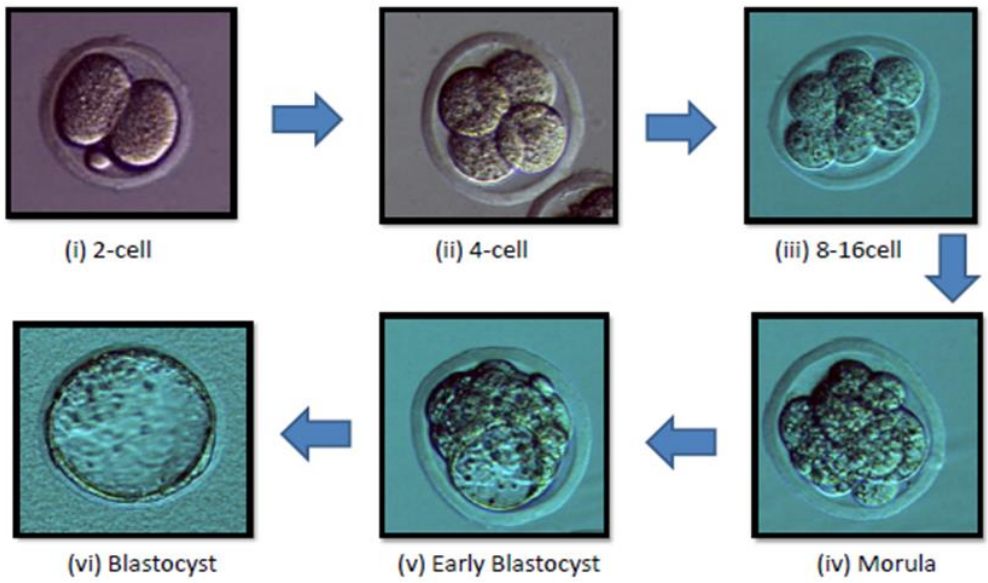

Fig. 5: *The light micrograph of normal developmental stage of embryos in vitro x 100 x magnifications.*

Table 1 *The effect of tocotrienol supplementation on the embryo development in aging mice. Young (6 weeks), Aging (8 months), Group 1: Vehicle control, Group 2: TCT 90mg/kg BW, Group 3: TCT 120 mg/kg BW and Group 4: TCT 150mg/kg BW. Data were analysed using Chi-Square test: **p<0.01, *p<0.05 significantly different from control group (Young, Group1).*

Development stages		**Embryonic Development (%)**					
		Young	**Aging**	**Group 1**	**Group 2**	**Group 3**	**Group 4**
2-cell	Normal	100.00	100.00	100.00	100.00	100.00	100.00
	Arrested	0.00	0.00	0.00	0.00	0.00	0.00
4-cell	Normal	97.80	100.00	100.00	92.00	91.20	91.10
	Arrested	2.20	0.00	0.00	8.00	8.80	8.90
8-cell	Normal	96.70	91.40	100.00	84.00	91.20	91.10
	Arrested	3.30	8.60	0.00	16.00*	8.80	8.90
16-cell	Normal	94.40	91.40	73.10	36.00	91.20	71.10
	Arrested	5.60	8.60	26.90	64.00**	8.80	28.90
Morula	Normal	83.30	62.90	57.70	20.00	61.80	66.70
	Arrested	16.70	37.10**	42.30	80.00**	38.20	33.30
Blastocyst	Normal	56.70	37.10	26.90	16.00	17.60	42.20
	Arrested	43.30	62.90*	73.10	84.00	82.40	57.80

Thus, it is suggested that the effect of TCT in delaying the consequences of aging on embryonic development is by protecting the developing embryos from further DNA damage following aging process, thus allow it to continue to develop to blastocyst stage.

Even though our study shown the beneficial effect of tocotrienol in protecting the embryonic development against free radical, tocotrienol supplementation does not able to improve the quality of embryo and estradiol levels in aging mice. Further study on the mechanism of action of tocotrienol is needed to verify our findings.

CONCLUSION

In conclusion, tocotrienol supplementation at the dose of 150 mg/kg for two months exerts its antioxidant effect on the development of embryos derived from aging mice. However, the understanding of tocotrienol and its mechanism of action remain unclear and need further investigation.

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