

# Effects of Supplementation with Annatto (*Bixa orellana*)-Derived $\delta$ -Tocotrienol on the Nicotine-Induced Reduction in Body Weight and 8-Cell Preimplantation Embryonic Development in Mice

M. H. Rajikin, S. M. M. Syairah, A. R. Sharaniza

**Abstract**—Effects of nicotine on pre-partum body weight and preimplantation embryonic development has been reported previously. Present study was conducted to determine the effects of annatto (*Bixa orellana*)-derived delta-tocotrienol (TCT) (with presence of 10% gamma-TCT isomer) on the nicotine-induced reduction in body weight and 8-cell embryonic growth in mice. Twenty-four 6-8 weeks old (23-25g) female balb/c mice were randomly divided into four groups (G1-G4; n=6). Those groups were subjected to the following treatments for 7 consecutive days: G1 (control) were gavaged with 0.1 ml tocopherol stripped corn oil. G2 was subcutaneously (s.c.) injected with 3 mg/kg/day of nicotine. G3 received concurrent treatment of nicotine (3 mg/kg/day) and 60 mg/kg/day of  $\delta$ -TCT mixture (contains 90% delta & 10% gamma isomers) and G4 was given 60 mg/kg/day of  $\delta$ -TCT mixture alone. Body weights were recorded daily during the treatment. On Day 8, females were superovulated with 5 IU Pregnant Mare's Serum Gonadotropin (PMSG) for 48 hours followed with 5 IU human Chorionic Gonadotropin (hCG) before mated with males at the ratio of 1:1. Females were sacrificed by cervical dislocation for embryo collection 48 hours post-coitum. Collected embryos were cultured *in vitro*. Results showed that throughout Day 1 to Day 7, the body weight of nicotine treated group (G2) was significantly lower ( $p < 0.05$ ) than that of G1, G3 and G4. Intervention with  $\delta$ -TCT mixture (G3) managed to increase the body weight close to the control group. This is also observed in the group treated with  $\delta$ -TCT mixture alone (G4). The development of 8-cell embryos following *in vitro* culture (IVC) was totally inhibited in G2. Intervention with  $\delta$ -TCT mixture (G3) resulted in the production of 8-cell embryos, although it was not up to that of the control group. Treatment with  $\delta$ -TCT mixture alone (G4) caused significant increase in the average number of produced 8-cell embryo compared to G1. Present data indicated that  $\delta$ -TCT mixture was able to reverse the body weight loss in nicotine treated mice and the development of 8-cell embryos was also improved. Further analysis on the quality of embryos need to be done to confirm the effects of  $\delta$ -TCT mixture on preimplantation embryos.

**Keywords**— $\delta$ -tocotrienol, body weight, nicotine, preimplantation embryonic development.

Rajikin M.H and Sharaniza A-R are with the Faculty of Medicine, Universiti Teknologi MARA (UiTM) Sg. Buloh Campus, Selangor, Malaysia, (e-mail: hamim400@salam.uitm.edu.my).

Syairah, S. M. M. is with the Faculty of Medicine, Universiti Teknologi MARA (UiTM) Sg. Buloh Campus, Selangor, Malaysia, on leave from Faculty of Pharmacy, UiTM, Puncak Alam Campus, Selangor, Malaysia (e-mail: syairah@puncakalam.uitm.edu.my).

## I. INTRODUCTION

BEING listed as one of the reproductive toxicant, nicotine has been well studied for its harmful effects on pregnancy and embryonic development; amongst which are the increase risks of gestational toxicity, foetal resorption, intrauterine growth retardation and reduced birth weight [1]. Besides, nicotine also reduced the rate of embryonic cleavage, number of retrieved embryos, number of hatched blastocysts and rate of implantation [2]-[5]. On the other hand, Vitamin E, which comprises of both tocopherol and tocotrienol is well documented for its function to maintain body mass due to its fat-soluble vitamin activity. A few studies has reported that rats fed with a vitamin E-deficient diet had declining body weight as compared to the control rats [6] and vitamin E supplementation is able to reduce corticosterone-induced oxidative stress (OS) in rats' plasma [7].

Specific studies on tocotrienols (TCTs) have reported on the benefits of TCTs as an essential nutrient in the diet [8]. Tocotrienol-rich fractions (TRFs) have been shown to reverse the effects of nicotine-induced body weight loss in rats [9]. This is followed by our recent study, which has shown that palm  $\gamma$ -TCT possesses the ability to improve the rate of implantation in mice [10]. However, the effects of  $\delta$ -TCT on body weight change and embryonic growth in mice remain unknown. Therefore, this study was designed to determine the effects of annatto (*Bixa orellana*)-derived delta-tocotrienol (TCT) (with presence of 10% gamma-TCT isomer) on the nicotine-induced reduction in body weight and 8-cell stage embryonic growth in mice.

## II. MATERIALS AND METHODS

### A. Ethics Approval

All procedures were carried out in accordance with the guidelines approved by Laboratory Animal Care Unit (LACU) as per the recommendations of the university's Committee on Animal Research and Ethics (CARE) and Animal Care and Use Committee (ACUC-7/13).

### B. Animal Treatment

Twenty-four of 6-8 weeks old (23-25g) male and female balb/c mice (purchased from Chenur Supplier, Kajang, Malaysia) were acclimatized in controlled temperature and humidity (24°C, 12-h light/dark cycle) with vitamin E-free

pellets and water given *ad libitum* for one week. Following this, all females were randomly grouped into four groups (G1-G4) of six mice each. The groups were treated according to the treatments shown in Table I for 7 consecutive days.

TABLE I  
TREATMENTS GIVEN TO THE EXPERIMENTAL GROUPS

Groups	Treatment	Route of Intake
G1 (control)	Corn oil tocopherol stripped (Acros Brand, Premier Diagnostics Sdn. Bhd., Malaysia)	Oral-gavage
G2	Nicotine (3 mg/kg/day)	Subcutaneous (s.c.) injection
G3	Nicotine (3 mg/kg/day) and $\delta$ - TCT mixture (90% delta:10% gamma) (60 mg/kg/day)	S.c. and oral-gavage ( $\delta$ -TCT mixture was dissolved in tocopherol- stripped corn oil prior to force-feeding)
G4	$\delta$ -TCT mixture (60 mg/kg/day) alone	Oral-gavage

### C. Mating

Prior to mating, all females were super ovulated with subcutaneous (s.c.) injection of 5 IU Pregnant Mare's Serum Gonadotropin (PMSG), followed with 5 IU of human Chorionic Gonadotropin (hCG) (s.c.) 48 hours later. All mice were individually housed in a cage for mating (48 hours) in the ratio of 1 male to 1 female. Mating was confirmed by the presence of a vaginal plug. Animals were then sacrificed by cervical dislocation.

### D. Embryo Collection and Culture

Sacrificed females were immediately dissected to retrieve the Fallopian tubes for embryo collection. Uterus were flushed with M2 medium (Sigma Aldrich, USA) under a dissecting microscope (Leica Zoom 2000, Japan). Collected embryos were washed in M2 medium before cultured *in vitro* in 100  $\mu$ l M16 medium (Sigma Aldrich, USA) and overlaid with mineral oil (Sigma Aldrich, USA). Culture mediums were prepared overnight for homogenization prior to use. Embryos were incubated at 37°C (5% CO<sub>2</sub>). Embryonic development until 8-cell stage was observed daily under the inverted microscope (Olympus 1X81 SF-3, Japan). Present data were statistically analyzed using independent sample t-test to identify the significant ( $p < 0.05$ ) change in the body weight and 8-cell embryo development between the experimental groups.

## III. RESULTS

### A. Body Weight

Results showed that throughout Day 1 to Day 7, the body weight of nicotine treated group (G2) is significantly lower ( $p < 0.05$ ) than that of G1, G3 and G4. Intervention with  $\delta$ -TCT mixture (G3) managed to increase the body weight close to the control group. Meanwhile, the body weight in-group treated with  $\delta$ -TCT mixture alone (G4) showed a similar values as to the control group (G1). Results on the body weight change are shown in Fig. 3.

### B. Number of 8-Cell Embryos

The 8-cell embryos following the respective treatment in G1 – G4 are shown in Figs. 1 and 2. Normal development of 8-cell embryos was observed in G1, G3 and G4 (Fig. 1). However, observations in G2 showed failure in the embryonic cleavage, resulting from the high rate of blastomere fragmentation and unequal blastomere divisions following maternal nicotine treatment (Fig. 2).

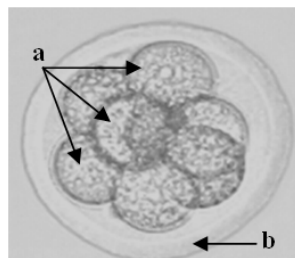


Fig. 1 The normal 8-cell embryos from G1 (control), G3 and G4 (X20). Normal appearances are characterized by the (a) equal blastomere size and (b) normal *zona pellucida* (ZP) lining

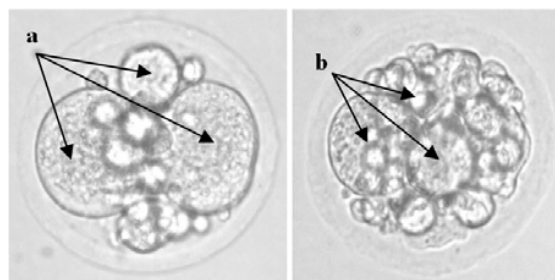


Fig. 2 Arrested development in embryos following maternal nicotine treatment in G2. Abnormal appearances are characterized by the (a) unequal blastomere size and (b) blastomere fragmentations

Data summarized in Table II indicated that the development of preimplantation embryos to the 8-cell stage in *in vitro* culture (IVC) was totally inhibited in G2. Intervention with  $\delta$ -TCT mixture (G3) results in production of 8-cell embryos, although the number of 8-cell embryos is lower than the control group (G1). Treatment with  $\delta$ -TCT mixture alone (G4) results in significant increase ( $p < 0.05$ ) in the average number of produced 8-cell embryo compared to G1.

## IV. DISCUSSIONS

### A. Body Weight

Present study provided a new data on the effects of  $\delta$ -TCT mixture on nicotine-induced body weight reduction (Fig. 3) and the decrease in the production of 8-cell embryos (Table II). Body weight reduction following nicotine treatment in present study is in line with previous studies [11], [12]. Report by [11] has shown that nicotine results in decreased food intake, body mass index and body weight in mice by activating the hypothalamic pro-opiomelanocortin (POMC) neuron pathway through central activation of  $\alpha 3\beta 4$  nicotinic acetylcholine receptors.

TABLE II  
AVERAGE NUMBER OF 8-CELL EMBRYOS FOLLOWING *IN VITRO* CULTURE (IVC)

Groups (Treatment)	Number of 8-cell embryos (n=6, Mean±SEM)
G1-Corn oil tocopherol stripped (Control)	18.33 ± 0.82
G2-Nicotine (3 mg/kg/day)	None
G3-Nicotine (3 mg/kg/day) and 60 mg/kg/day $\delta$ -TCT mixture (90% $\delta$ :10% $\gamma$ )	12.17±0.75
G4- $\delta$ -TCT mixture alone	28.83±1.47 <sup>a</sup>

<sup>a</sup> Statistically significant compared with G1 ( $p < 0.05$ )

Another possibility is through the modulation of hypothalamic AMP-activated protein kinase, which results in decreased feeding and increased brown adipose tissue thermogenesis through the sympathetic nervous system, as reported by [12].

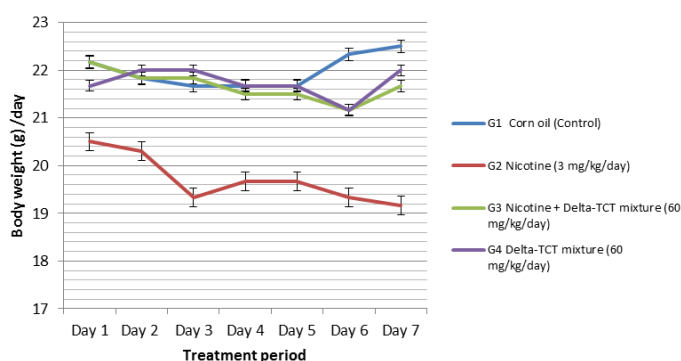


Fig. 3 Body weight change following 7 days of respective treatments (n=6)

### B. Number of 8-Cell Embryos

Following IVC, the development of 8-cell embryos in the nicotine-treated group (G2) was totally inhibited. This finding supports the reports on the decline in the number of nicotine-induced retrieved embryos [4], [15], [16]. This decline might be explained by our previous results, which show that nicotine-induced oxidative stress caused defects on the ultrastructure of oocytes [17]. In addition, the low embryonic development following nicotine induction might be attributed to the poor ovarian reserve, which subsequently reduces fertilization rate [2].

Intervention with  $\delta$ -TCT mixture (G3) results in production of 8-cell embryos, although it is lower than control group.

Treatment with  $\delta$ -TCT mixture alone (G4) results in significant increase ( $p < 0.05$ ) in the average number of produced 8-cell embryo compared to G1. This finding indicates the ability of  $\delta$ -TCT mixture to improve 8-cell embryonic development in nicotine pre-exposed mice. However, further analysis on the DNA integrity and genome stability need to be done to confirm on the beneficial effects of  $\delta$ -TCT mixture throughout the embryonic development stages.

### ACKNOWLEDGMENT

This study was financially supported by the Ministry of Education (MOE), Malaysia through Fundamental Research Grant Scheme (FRGS Grant) (600-RMI/FRGS 5/3/ (60/2012).

Intervention with  $\delta$ -TCT mixture in nicotine-induced mice managed to improve body weight similar to controls (Fig. 3). This is also parallel with findings from previous studies on TCT. TCT was reported to reverse body weight loss in diabetic-induced rats [13]. Reference [7] reported that vitamin E blocked the increasing corticosterone-induced OS levels. Corticotropin-Releasing-Factor (CRF) increased as a response towards stress and it is regulated in various locations in the brain including those involved in the regulation of food intake [14]. Thus, it might be possible that  $\delta$ -TCT mixture used in this study has the ability to block the effect of nicotine-induced OS and subsequently inhibit the release of CRF, resulting in improved body weight. However, further studies need to be done to confirm this effect.

We would like to express our deepest appreciation to Ministry of Education, Malaysia and Research Management Institute (RMI), UiTM Shah Alam, Selangor, Malaysia. In addition, a great thanks to American River Nutrition, USA for providing the  $\delta$ -TCT samples.

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