An Experimental Examination on ohe Effects of Supplementations with Palm Tocotrienol-Rich-Fraction (TRF) and Annatto Δ-Tocotrienol on Body Weight and Pre-Implantation Embryonic Development in Nicotine-Treated Mice

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Abstract: ***Supplementation of vitamin E to pre-pregnant mice reduces the hazardous impact of nicotine on pregnancy outcome. There are emerging evidences on vitamin E, particularly tocotrienol (TCT), exerting some roles in pre-pregnancy body weight management and pre-implantation embryonic development. This study investigated the effects of supplementations with palm tocotrienol-rich fraction (palm-TRF) and annatto δ-TCT (> 98% purity) on the pre-partum body weight and embryonic development following nicotine treatment in mice. Thirty-six (4–6 weeks old) female mice (Mus musculus) were divided into 6 groups (G1-G6). All groups were subjected to treatments either with 3 mg/kg bw/day nicotine, 60 mg/kg bw/day palm-TRF, 60 mg/kg bw/day annatto δ-TCT or; combination of nicotine concurrently with palm-TRF or annatto δ-TCT for 7 consecutive days. Body weights were recorded daily throughout the treatment period. Superovulation was conducted on Day 7 and 9, followed with cohabitation with fertile males. Animals were euthanized 48 hours post-coitum and embryos were retrieved through uterine flushing. Selected embryos were incubated in M16 medium and observed daily. Results showed that nicotine (G2) decreased the pre-partum body weight (22.2 ± 1.1g vs 29.8 ± 0.6g) (p<0.05) and the number of cleaving embryos at all stages in G2 were significantly decreased (p<0.05) compared to control. Intervention with annatto δ-TCT attenuated the embryonic development, unlike the intervention with palm-TRF. Supplementations with palm-TRF and annatto δ-TCT alone resulted in unchanged body weight and increased in the number of retrieved hatched blastocysts. Present results suggest that future efforts in determining the regulating signaling pathways are important, and the mechanisms of actions by both nicotine and TCT could be elucidated further.***

Keywords: : δ-tocotrienol, body weight, nicotine, palm-TRF, pre-implantation embryonic development

I. INTRODUCTION

II. Vitamin E is one of the well-recognized lipid-soluble vitamins. It is widely known as a powerful antioxidant and must be taken orally from dietary since it is not produced by the body. The sources of vitamin E are numerous, including edible oils, rice bran, coconut, palm and annatto [1,2]. Besides, vitamin E derivatives have also been detected in human milk [3] and palm date (Phoenix canariensis) [4].

III. Vitamin E contains two major substances; tocotrienols (TCTs) and tocopherols (TOCs). Both are present in four different homologs, known as α-tocotrienol, β-tocotrienol, γ-tocotrienol, δ-tocotrienol, α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol [5].

IV. Among the sources of vitamin E, palm oil and rice bran contain approximately between 25–50% of the α-TOC homolog in their total vitamin E content. α-TOC has been reported to interfere with the benefits of TCTs [6,7]. In contrast, TCTs derived from annatto (Bixa orellana) seeds were discovered to not containing any tocopherol (TOC) homologs [8], making it highly valuable as it is the first and only true source of naturally derived vitamin E that supplies only TCTs (contains only γ- and δ-homologs) to-date [8]. The additional discovery of annatto-TCT also makes it the only known source of tocotrienol that provides the highest content of the powerful δ-tocotrienol [8].

V. The health benefits of vitamin E have been widely reported [9-18], however studies on its effects on reproductive health, particularly on fertility, sterility and preimplantation embryonic development remains largely unknown, with several attempts have been made recently [19-29].

VI. Nicotine is a known reproductive disruptor in tobacco smoke and it remains the major alkaloid of cigarette responsible for its addiction and dependence. In pregnancy, various findings have reported that tobacco smoking and second-hand smoke exposure are linked with negative outcomes including intrauterine growth restriction [30,31]. In both human and animal, nicotine treatment has reduced the rate of embryonic cleavage, number of retrieved embryos, number of hatched blastocysts and rate of implantation [21, 32].

VII. Taking together the adverse effects of nicotine and the potential benefits of vitamin E, this study was conducted to determine the differences between the effects of palm-TRF (contains α-, β-, γ-, δ-TCT and α-TOC) and

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annatto δ-TCT (purity >98%) against the nicotine-induced adverse effects.

VIII. MATERIALS & METHODS

A. Ethics Approval

Ethics approval to conduct this experiment was granted by the university's Committee on Animal Research and Ethics (CARE) (ACUC-7/13). All procedures were carried out in accordance with the approved guidelines.

B. Experimental Design and Animal Treatment

A total of thirty-six (36) female mice (Mus musculus) (4-6 weeks old, 20-30g) were obtained from Chenur Supplier, Selangor, Malaysia. All animals were acclimatized for a week and housed at 24°C with 12-h light/dark cycle. Animals were fed with vitamin E-free pellets (Gold Coin Holdings, Kuala Lumpur, Malaysia), and water was given ad libitum. Sample of palm-TRF was obtained from Malaysian Palm Oil Board (MPOB), Malaysia and sample of pure annatto (Bixa orellana)-derived δ-TCT was provided by American River Nutrition Inc. (ARN), Hadley, MA, United States of America (USA).

All females were randomly divided into six groups (Grp. 1 – Grp. 6) with six mice each (n = 6). Those groups were assigned according to the following treatments (Table 1) for seven consecutive days. Body weight of each mouse was recorded daily prior to the commencement of treatments. Palm-TRF and annatto δ-TCT were dissolved in tocopherol-stripped corn oil prior to force-feeding
.

Table (1): Treatments given to the experimental groups (n=6).

Groups	Treatment	Route of Administration
Grp.1	0.9% (NaCl)	Subcutaneous
Grp.2	Nicotine (3 mg/kg/day)	(s. c.) injection
Grp.3	Palm-TRF alone (60 mg/kg/day)	Oral gavage
Grp.4	Nicotine (3 mg/kg/day) and palm-TRF (60 mg/kg/day)	s. c. & oral gavage
Grp.5	Annatto δ-TCT alone (60 mg/kg/day)	Oral gavage
Grp.6	Nicotine (3 mg/kg/day) and Annatto δ-TCT alone (60 mg/kg/day)	s. c. & oral gavage

C. Mating

IX. Male mice (5-6 weeks old, 20-30g) were obtained from Chenur Supplier, Kajang, Malaysia. They were acclimatized for a week and maintained at controlled temperature and humidity (24ºC, 12h light/12-h dark cycle) with vitamin E-free pellet and water available ad libitum. Prior to mating, female mice were subjected to 5IU pregnant mare's serum gonadotropin (PMSG) injections (s.c.) to mature the ovarian follicles, followed by injections with 5IU human chorionic gonadotropin (hCG) (s.c.) (48 hours after PMSG injections) to induce superovulation. Animals were then mated at the ratio of 1:1. The presence of copulation plug was considered as successful mating. Successfully mated females were euthanized 48 hours post-coitum (p.c.) through cervical dislocation.

A. Embryo Retrieval and In Vitro Culture

Following euthanization, the fallopian tubes were excised, and embryos were flushed through uterine flushing using M2 medium (Sigma Aldrich, USA), under a dissecting microscope (Leica Zoom 2000, Japan). Obtained embryos were graded according to the criteria set by Ziebe et al. (1997) [33]. Only normal with good quality 2-cell pre-implantation embryos were selected for subsequent in vitro culture. Selected embryos were washed in M2 medium before being cultured in a 35-mm culture dish (Nunclon, Roskilde, Denmark) filled with a 100µl droplets of M16 medium (Sigma Aldrich, USA), overlaid with mineral oil (Sigma Aldrich, USA). Culture droplets were incubated overnight prior to use for homogenization. The cultures were maintained in a humidified atmosphere containing 5% CO2 at 37oC for 5 consecutive days. Observations on the embryonic development were made daily under inverted microscope (Olympus 1X81 SF-3, Japan) until the embryos reached the hatched blastocysts stage.

A. Statistical Analysis

Data were analyzed using the SPSS program (version 24.0). All continuous variables were expressed as mean ± SEM. A p-value of <0.05 was considered statistically significant.

II. RESULT AND DISCUSSION

A. Body Weight

Body weight in the nicotine treated group (G2) were significantly decreased beginning on Day 4 ($p<0.05$) until Day 7 ($p<0.001$), compared to the control group (G1), and intervention with palm-TRF (G4) increased the body weight to the levels similar to its control group (G3). However, mice treated concurrently with nicotine and annatto δ-TCT (G6) shows a fluctuating pattern between body weight loss and gain with no significant changes in the overall body weight. Non-significant changes in the body weight of mice supplemented with annatto δ-TCT alone (G5) were also recorded. Results are summarized in Fig. 1.

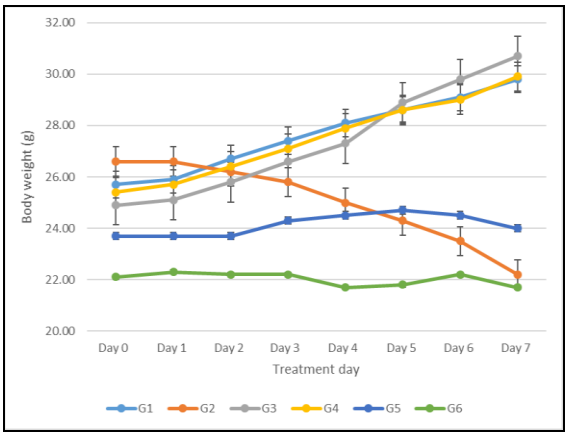

Fig. 1: Results on the daily recorded body weights (note that axis label starts from 20.0g).

Present findings showed that nicotine exerted significant detrimental effects on the body

weight. This is in line with previously published reports on the adverse effects of nicotine towards the body health [34-36]. Nicotine has been reported to decrease food intake, body mass index and body weight in mice [35]. Nicotine also has been suggested to decrease feeding and increase brown adipose tissue thermogenesis through the sympathetic nervous system, as reported by [36].

Intervention with palm-TRF in nicotine treated mice (G4) resulted in an increase in body weight throughout the seven (7) days of treatment (Fig. 1) to the level similar to its control group (G3), suggesting the improving effects introduced by palm-TRF against the nicotinic adverse effects. To-date, there is no similar study reported on the effects of nicotine and palm-TRF on the body weight, however the benefit of vitamin E supplementation in reversing body weight loss in diabetic-induced rats was previously described by [37]. Besides, an earlier study by [38] also has reported that vitamin E deficiency impaired weight gain in estrogen-depleted states.

On the other hand, the opposite results were obtained following concurrent treatment with annatto δ-TCT in nicotine-treated mice (G6). Results showed that the nicotine-induced body weight loss was not reversed following supplementation with annatto δ-TCT (Fig. 1). The values of body weight were fluctuating between weight loss and gain with no significant changes in the overall body weight. This suggest that annatto δ-TCT does not improve/reverse the nicotinic effects. This result is different from our previous study on the effects of annatto δ-TCT mixture (with presence of 10% gamma-TCT isomer) on the nicotine-induced reduction in body weight, which showed that intervention with δ-TCT mixture in nicotine-induced mice managed to improve body weight similar its controls [39]. Further studies are needed to understand the underlying mechanism of palm-TRF and annatto δ-TCT in regulating the nicotinic effects on body weight.

B. Pre-Implantation Embryonic Development

Fig. 2 shows the normal pre-implantation embryonic development following in vitro culture from 2-cell to hatched blastocysts stages. Normal developments were observed in G1, G3 and G5. The numbers of embryos were significantly reduced in G2, compared to G1 ($p<0.05$). Present study also shows that the number of cleaving embryos at each developmental stage was declined ($p<0.05$) in G2 (Fig. 3)

Treatment of nicotine concurrently with palm-TRF (G4) increased the number of embryos in all stages similar to its control group (G3) ($p<0.05$), suggesting that palm-TRF may have reversed the nicotine-induced damages on the embryos. However, it wasn't the case in the treatment of annatto δ-TCT with nicotine (G6), which resulted in cessation of embryonic development at morula stage. Supplementation with annatto δ-TCT alone (G5) supported embryonic development up to hatched blastocysts stage. The comparative numbers of embryos at each developmental stages of each group is shown in Fig. 3.

In terms of embryonic development, the decreased number of normal developing embryos in nicotine-treated group (G2) compared to the control

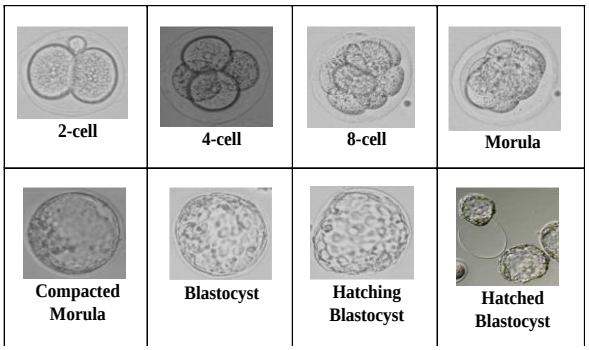

Fig. 2: The normal pre-implantation embryonic developmental stages from 2-cell stage to hatched blastocysts stage.

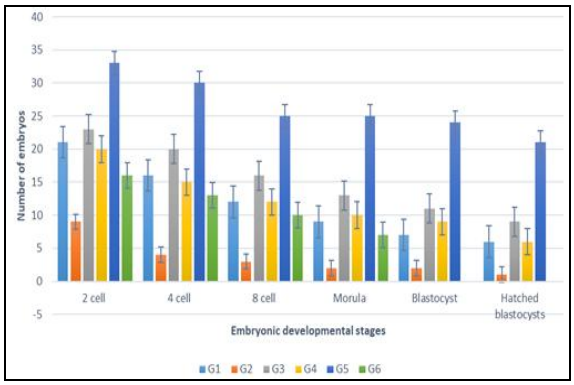

Fig. 3: Numbers of embryos at each developmental stages of each experimental group.

group (G1) ($p<0.05$) is in agreement with the previously reported data [29, 40]. This result is also in line with study reports on the effects of nicotine on oocytes and embryos which reported the adverse effects on the structures of oocytes [20], the rate of embryonic cleavage [41, 42], the number of retrieved embryos [43], the number of hatched blastocysts, and the rate of implantation [21 40].

Intervention with palm-TRF enhances embryonic development both in the presence and absence of nicotine. This promising effects of palm-TRF is in agreement with previous studies. For instance, concurrent treatment with 90 mg/kg and 120 mg/kg of palm-TCT and 10 mg/kg corticosterone (CORT) reduced the numbers of abnormal embryos induced by CORT [44]. A study by [21] also reported that embryonic

development in nicotine-induced mice were improved following treatment with palm γ-TCT. Further, one-month supplementation with 150 mg/kg/day of palm-TCT was reported to significantly increase the percentage of normal embryos [45].

Concurrent treatment with annatto δ-TCT ceased the embryonic development at morula stage. This result is similar to our previous study [29] where the embryonic development was terminated at the morula stage. However, when δ-TCT supplemented alone (G5), the development was completed to the hatched blastocyst stage. This is an important finding that illustrates the tendency of annatto δ-TCT to attenuate; as opposed to reverse hence improve, the embryos of nicotine-treated mice from developing.

To understand the differences between the effects of palm-TRF, nicotine + palm-TRF/δ-TCT and δ-TCT alone, further studies have to be conducted to gain more comprehensive understanding on the basis of their effects in the presence and absence of nicotine.

In general, studies on vitamin E are still superficial, and require thorough studies before it can be considered for use in infertility management. Studies also need to be conducted on clinical aspects and take into account some of the important impelling factors including the optimum dose intakes, synergistic effects of vitamin E with other compounds/drugs, conditions of the patients (infertile couples/people with reproductive disorders) and other related factors.

CONCLUSION

Palm-TRF and annatto δ-TCT are exerting different effects on the body weight and pre-implantation embryonic development in nicotine-treated mice. In both parameters, palm-TRF supported while annatto δ-TCT tend to supress the body weight gain and development of pre-implantation embryos following nicotine treatment. Further study on gene signalling pathways is warranted to reveal the mechanism of actions by both palm-TRF and annatto δ-TCT in nicotine-treated mouse model.

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