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Palm Tocotrienol-Rich Fraction Improves the Quality and Development of Embryos in Streptozotocin-Induced Diabetic Mice

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Palm tocotrienol-rich fraction (TRF), a well-known antioxidant, reduces the adverse effects of oxidative stress. Maternal diabetes mellitus is proposed to be responsible for impaired reproductive outcomes by increasing reactive oxygen species production, leading to oxidative stress. However, there is little evidence of whether TRF supplementation can prevent impaired reproductive outcomes in maternal diabetes. Therefore, the aim of this study was to determine the effect of palm-TRF on the quality and development of embryos and blood glucose levels in streptozotocin (STZ)-induced diabetic female mice. Forty, six-week-old female Mus musculus mice were divided into five groups: Group 1: Non-diabetic control, Group 2: STZ-induced diabetic without TRF supplementation, Group 3: Non-diabetic vehicle control, Group 4: Non-diabetic with TRF supplementation and Group 5: STZ-induced diabetic with TRF supplementation. Following induction of diabetes (by a single dose of STZ (40 mg/kg BW) given intraperitoneally for 7 days), mice were administered TRF (150 mg/kg BW) by oral gavage daily for 14 days. At the end of the treatment period, mice from all groups were superovulated, mated, and then euthanized. Embryos were retrieved and cultured. The morphology and in vitro development of embryos were monitored and recorded, and blood glucose level was analyzed. Both the percentage of normal embryos and development to the blastocyst stage was lower ($p<0.05$) in STZ-induced diabetic mice compared to that of the controls (Group 1). TRF supplementation was able to restore the percentage of normal embryos and their development in diabetic mice. However, two weeks of TRF supplementation was not able to reduce the blood glucose levels in STZ-induced diabetic mice. In conclusion, 150 mg/kg BW TRF supplementation for two weeks was able to overcome the diabetic-induced oxidative stress damage on the embryos and maintain their developmental capacity. However, the mechanism of action of TRF in STZ-induced diabetic mice remains to be elucidated.

Keywords: Tocotrienol-rich fraction; diabetes; embryo

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Tocotrienol-rich fraction (TRF) is a palm oil-derived vitamin E. It is commercially available and contains a combination of approximately 75% of tocotrienol (α, β, γ, and δ) and 25% of α-tocopherol [1]. Tocotrienol can be taken orally and has been proven in human studies to be bioavailable to all vital organs such as heart, liver and brain [2]. Our previous studies demonstrated that palm tocotrienol-rich fraction (TRF) supplementation reversed the deleterious effect of corticosteroid-induced oxidative damage on preimplantation embryonic development [3] as well as pregnancy outcome in mice [4]. The protective effect of TRF following corticosteroid-induced oxidative damage occurs by improving the catalase activity in plasma and reducing DNA damage in mouse embryos [5]. Supplementation of TRF restores the quality of oocytes [6] and embryos [7] as well as preimplantation embryonic development [8] in aging mice; an effect attributed by oxidative stress. The oxidative-induced DNA damage in mice oocytes is reduced following TRF supplementation [9]. This is proposed to occur due to the effect of TRF on the expression of DNA damage response genes in the ovary [10]. Similarly, the anti-apoptotic genes are highly expressed following TRF supplementation, a mechanism that helps to improve the quality of oocytes in aging mice [11]. These studies collectively demonstrate that the anti-oxidant properties of TRF, when given daily at a dose of 150 mg/kg BW for two weeks, could overcome the adverse effects of reactive oxygen species (ROS) that induced oxidative damage on the female reproductive system. Antioxidant supplementation in human has been reported to be able to counteract oxidative stress and its adverse consequences during foetal development

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[12]. It has been documented that antioxidant such as mulberry extract [13] and combination of vitamins C and E [14] are used to decrease the adverse effects of oxidative stress and improves foetal outcomes in diabetic rats.

Female reproductive function is greatly altered in type 1 diabetes mellitus. Diabetes causes alterations in ovarian function, adversely affecting embryonic development and pregnancy outcomes which eventually leading to infertility. Alteration in female reproductive function has been implicated in the metabolic disturbances caused by hyperglycaemia and lack of insulin, an effect that was partially reversed by tungstate, an anti- diabetic agent [15]. It is suggested that the effect of maternal diabetes increases ROS that contributes to oxidative stress leading to adverse effects on embryo development [16]. Similarly, there is a significant correlation between the oxidative stress parameters i.e. malondialdehyde, superoxide dismutase and glutathione peroxidase with unfavourable pregnancy outcomes in diabetic patients [17]. The primary mechanism of diabetic embryopathy is activation of oxidative stress signalling that leads to excessive embryonic cell apoptosis [18].

Nevertheless, more data are required to determine the beneficial role of palm TRF on the reproductive outcomes in female diabetic mice. Furthermore, there has not been any study as yet to determine the effects and mechanism of action of TRF supplementation on reproductive outcomes in streptozotocin-induced oxidative damage in female mice. Streptozotocin (STZ) has been widely used in the induction of Type-1 diabetes where it can cause insulitis and β-cell death [15]. Therefore, this study aims to examine the effect of palm TRF supplementation in diabetic mice and its role in improving the adverse effects of oxidative stress-induced metabolic disturbances on reproductive outcomes.

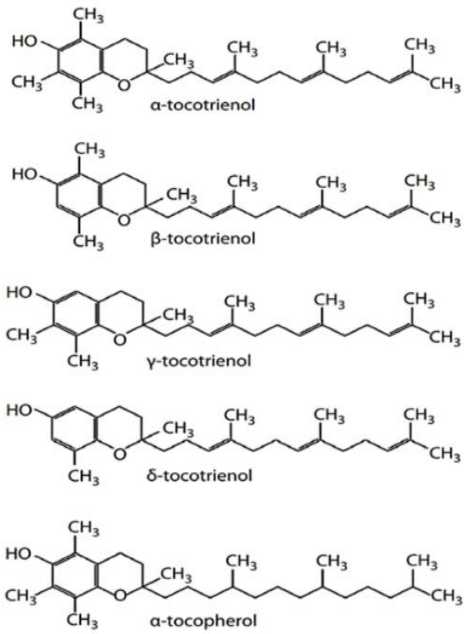

Figure 1: Chemical structures of TRF.

Table 1: The composition of palm TRF used in the experiment.

Vitamin E Components	Percentage (%)	Weight (mg/g)
α-tocotrienol	27.3	196.0
β-tocotrienol	3.4	24.0
γ-tocotrienol	35.5	255.0
δ-tocotrienol	10.4	75.0
α-tocopherol	23.4	168.0
Total	100	718.0

EXPERIMENTAL

Materials

Tocotrienol and vehicle: Palm olein and tocotrienol-rich fraction (TRF) palm oil (ExcelVite Sdn. Bhd.) was used as vehicle and treatment compound, respectively. Palm TRF used in this study comprises 76.6% (w/w) of tocotrienols (includes α-tocotrienol, β-tocotrienol, γ-tocotrienol, δ-tocotrienol) and, only 23.4% (w/w) of α-tocopherol (Figure 1; Table 1). The concentration of TRF given as supplementation in this study was 150 mg/kg body weight (BW) and was attained by dissolving 1.125 g TRF in 25 ml of palm olein [8]. Palm olein which is used to dissolve TRF is the liquid fraction of palm oil that contained oleic and linoleic acids [19]. In this study palm olein is also injected to mice in vehicle control group (Group 3).

Streptozotocin (STZ): STZ (Cas. No. 18883-66-4, Sigma-Aldrich) was used for induction of diabetes. For injection, STZ was dissolved in an ice-cold sodium citrate buffer (10 mmol/L, pH 4.5) and was given at a dose of 40 mg/kg body weight [20].

Superovulating agents: Pregnant mare serum gonadotropin (PMSG) (Sigma-Aldrich) and human chorionic gonadotropin (hCG) (Sigma-Aldrich) were used as superovulating agents to induce ovulation in female mice. Both were given at a dose of 5 IU/0.1 ml.

Culture media: M2 and M16 media (Sigma-Aldrich) are modified Krebs-Ringer bicarbonate solutions, which are very similar to Whitten's medium. M2 and M16 media, which are commonly used for *in vitro* culture of preimplantation stage of embryos, were used in this study. M2 medium was used for handling embryos outside the CO_2 incubator (such as during flushing and collection of the embryos) while M16 medium was used for culturing [21].

Drugs: A drug preparation containing Ketamine (100 mg/ml) (Sigma-Aldrich) and Xylazine (20 mg/ml) (Sigma-Aldrich) (KTX) was diluted in sterile water at a ratio of 5:1; was used as a sedative agent stock solution. This mixture has been proven to produce minimal adverse effects on mice [22].

Methods

Experimental Animals and Treatment

Forty, healthy female ICR mice (*Mus musculus)* aged 6-7 weeks (20 – 25 gm) were utilized for the experiments. All animals were purchased from Laboratory Animal Facility and Management (LAFAM), Universiti Teknologi MARA (UiTM), Puncak Alam Campus. Upon arrival at Laboratory Animal Care Unit (LACU), UiTM, Sg. Buloh Campus, the basic health check or physical examination was done on each mouse before the quarantine. Then, the mice were transferred to 290 x 220 x 490 mm^2 cages and were quarantine for one week in the quarantine room at the animal house in LACU. Two mice were placed in one cage. Mice were acclimatized to the laboratory condition for one week before the actual experiment. Mice were held in quarantine as per the standard operation procedure in LACU. During the acclimatization or quarantine period, mice were housed in quarantine room in LACU under 12:12 light:dark cycles at 27 °C and 50-60% humidity conditions. The mice were placed in moulded polypropylene cages with stainless steel wire grid top with clean saw dust bedding.

After completion of the quarantine, mice were transferred into the breeding room in LACU for experimental purposes. Two mice were placed in one cage. All animals were housed at 27ºC in 12 hours of light-dark cycles and given food pellets (Gold Coin Mouse Pellets, Feedmills Sdn. Bhd.) and water *ad libitum* daily. Forty mice were divided into five groups; Group 1: Non-diabetic control, Group 2: STZ-induced diabetic without TRF supplementation, Group 3: Non-diabetic vehicle control, Group 4: Non-diabetic with TRF supplementation and Group 5: STZ-induced diabetic with TRF supplementation (Figure 2). Healthy stud male mice from the same strain aged 8-10 weeks were used for mating purposes.

Induction of diabetes was done by intraperitoneal injection (ip) of streptozotocin (STZ), 40 mg/kg 7 days before the start of TRF supplementation (Figure 3). On experimental day 1, 4 hours prior to STZ treatment, food from animal cages was removed and water was provided as normal. Streptozotocin was dissolved in ice-cold sodium citrate buffer solution (10 nmol/L, pH 4.5) at the dose of 40 mg/kg and 0.2 ml and was injected in mice using 1 ml syringe with 25 G needle. After injection, the mice were returned to the cages with free access to normal food and 10 % of sucrose water. This procedure was repeated until day 6. On experimental day 7, the 10 % of sucrose water was replaced with regular water. The non-diabetic control group received an injection of sodium citrate buffer. On experimental day 0 and 8, blood glucose levels were analysed to verify the induction of diabetes mellitus in the STZ-treated mice (Figure 3). Mice were fasted overnight, and blood were collected by the tail prick, 48 h post-STZ injection for blood glucose estimation using Sinocare Safe Accu glucometer (Sinocare Inc., China). Mice with a blood glucose level of more than 11.1 mmol/L were considered diabetic and included for further study [23].

Following induction of diabetes (by a single dose of STZ (40 mg/kg BW) given intraperitoneally for 7 days), mice were administered TRF (150 mg/kg BW) by oral gavage daily for 14 days (Figure 3). Mice were given TRF after 7 days induction of diabetes by oral gavage daily at the dose of 150 mg/kg BW (0.1 ml) for duration of 14 days, between 8 am to 10 am. The TRF dose of 150 mg/kg used in the present study was carefully selected as numerous prior studies have shown it to be effective especially

looking on its effect in female mice reproductive system [8,11]. The TRF dose used in this study is the optimum dosage obtained from previous study on the effect of TRF supplementation in mice in improving the quality of embryo [8] and oocytes [11].

Blood glucose levels were analysed again two weeks after induction of diabetes and following 14 days of TRF supplementation (Figure 3).

Experimental procedures were in accordance with regulations recommended by the Research Committee on the Ethical Use of Animals (UiTM CARE: 388/2022).

Collection and Classification of Embryos

Female mice were superovulated by intraperitoneal injection (0.1 ml) of pregnant mare serum gonadotropin (PMSG) followed by human chorionic gonadotropin (hCG) (ip, 0.1 ml), 46 – 48 hours after PMSG injection [24]. For collection of embryos, following hCG injection, female mice were cohabited overnight with stud male mice at a ratio 1:1. Successfully mated female mice were confirmed by observing the presence of vaginal plug or copulation plug, when checked the following morning. The presence of vaginal plug indicated day one of pregnancy. Upon confirmation of successful mating, embryos were flushed out and cultured in a culture dish. The embryos were collected 48 hours after mating, mice will be euthanized via cervical dislocation, the fallopian tubes were excised and flushed with the M2 medium to collect the 2-cell embryos. The presence of normal and abnormal embryos was counted and recorded. All normal embryos were cultured *in vitro* in the M2 medium, and the cultures were kept in an incubator with humidified atmosphere (at 37°C, 90% air and 5% CO2) for 6 days. *In vitro* development of embryos were monitored every 20-24 hours (using an inverted microscope, Leica DM IRB) and the number of blastocysts were recorded [25].

In Vitro Development of the Embryo

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 reserved in a humidified atmosphere incubator (at 37ºC, 90% air and 5% CO2) for 6 days (Figure 3) [20]. The *in vitro* development of the embryos was observed and counted under an inverted microscope (100× magnification) (Leica DM IRB), and photos were taken using Pixe-LINK Megapixel FireWire Camera (Canada).

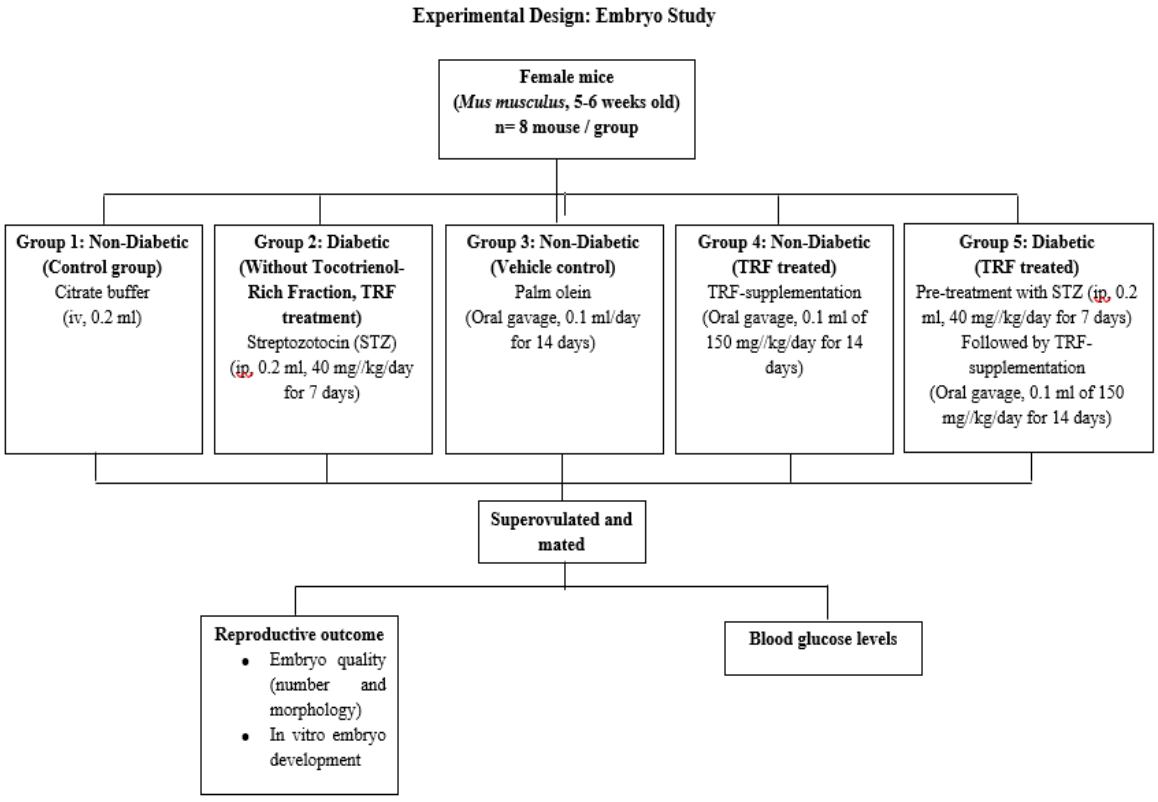

Figure 2: Experimental design.

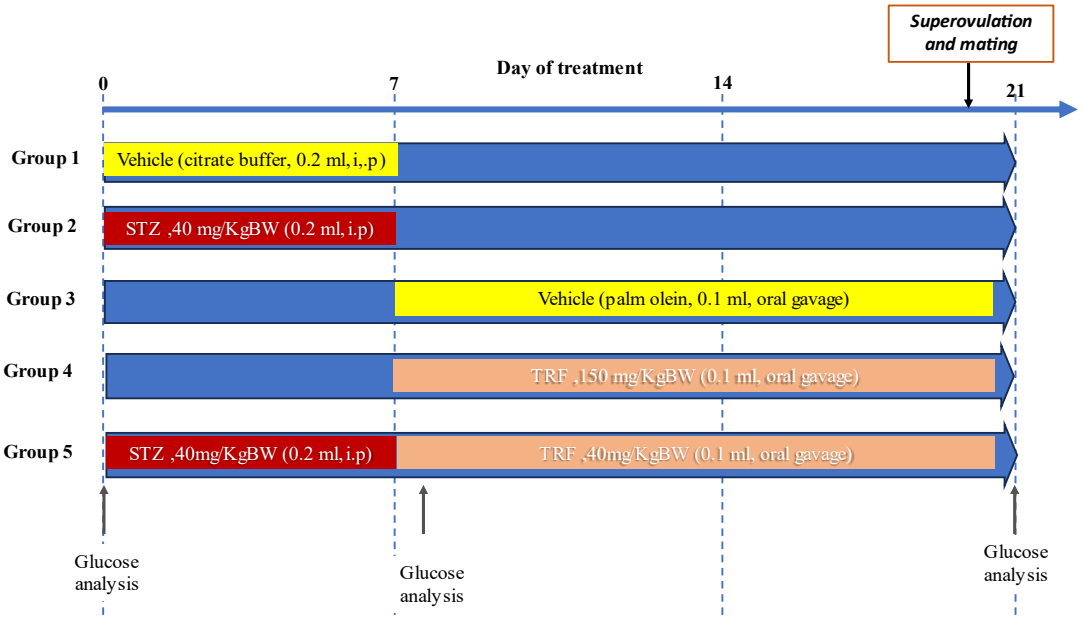

Figure 3: Treatment timeline.

Statistical Analysis

Data were analysed using the SPSS package programme (SPSS 29.0, New York, USA). Data on the quality and *in vitro* development of embryos were presented as percentage and data were analysed using the Chi-square test. Blood glucose levels were presented as mean ± SEM and data were analysed using one-way ANOVA. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Embryo Qualities and *In Vitro* Development of Embryos

In this study, a total of 185 embryos were retrieved which 72 % were found to be normal according to the criteria set by Ertzeid & Storeng [25]. Embryos were characterised based on the presence of a first polar body, the number of cells, cytoplasmic cavity, the percentage of the volume of the embryo fragmented, compaction, blastomeres with symmetrical shape and size and the absence of vacuoles and multinucleation [26]. Abnormal embryos were determined by the non-symmetrical blastomere number and size, cytoplasmic deformities (vacuoles, fragmentation), extracytoplasmic abnormalities (zona, shape, and perivitelline space irregularities), absence of first polar body and compaction [27] (Figure 4). Only normal embryos at two cell stage were cultured to monitor its development until blastocyst stage (Figure 5).

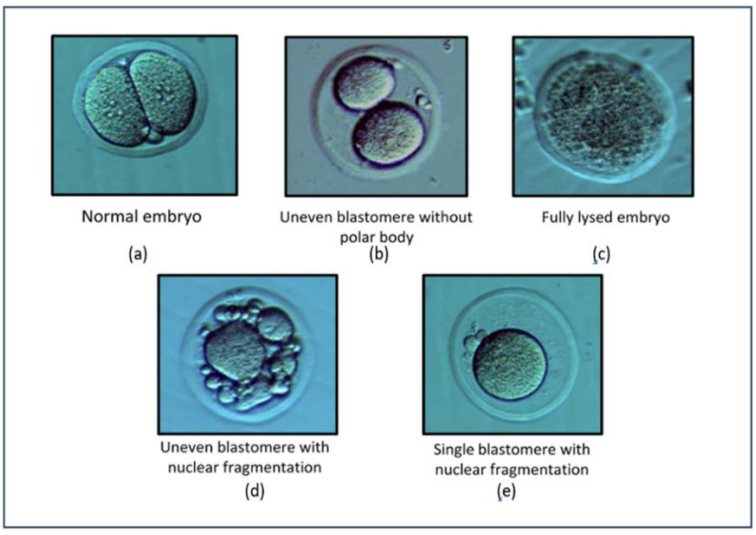

Figure 4: Representative images of (a) a normal embryo and (b-e) abnormal embryos at the 2-cell (zygote) stage (100x magnification), n = 185; normal embryos=133, abnormal embryos=52.

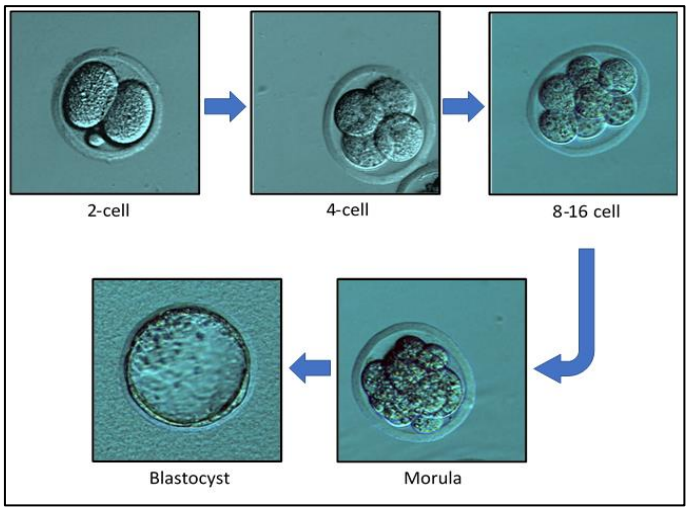

Figure 5: Embryonic development *in vitro* of mice embryo observed using an inverted microscope (100x magnification), n = 133.

The Effect of Palm TRF on Embryo Qualities and *In Vitro* Development in STZ-induced Diabetic Mice

It was found that the percentage of normal embryos was lower (5.4%) ($p<0.05$) in STZ-induced diabetic mice (Gp 2) compared to that of the controls (13.5%) (Gp 1) (Figure 5). It was previously reported that embryo malformation rates were high in STZ-induced diabetic mice [18]. Another study also reported that hyperglycaemia in STZ-injected female rats impaired reproductive function by lowering the serum LH and FSH and reduced the percentage of parturition [15]. It has been reported that the effect of maternal diabetes is attributed to oxidative stress that contributed to low oocytes quality [28] and high embryo malformation [16].

Embryos were not able to develop until blastocyst stage (0.0%) in STZ-induced diabetic mice (Gp 2) where it was significantly different ($p<0.05$) as compared to that of the control (Gp 1) (3.6%). TRF supplementation was able to restore the embryonic development in diabetic mice (Gp 5) (3.0 %) as compared to control group (Gp 1) (3.6%) (Table 2). Previously, it has been reported that *in vitro* hyperglycaemia condition caused mouse embryonic growth retardation [29]. It has also been reported that maternal diabetes caused a delay in embryonic development in mice [30]. Maternal hyperglycaemia in mice induced oxidative stress in embryos leading to defective embryo development [16]. Other study reported that maternal diabetes in rats impair the antioxidant defence system

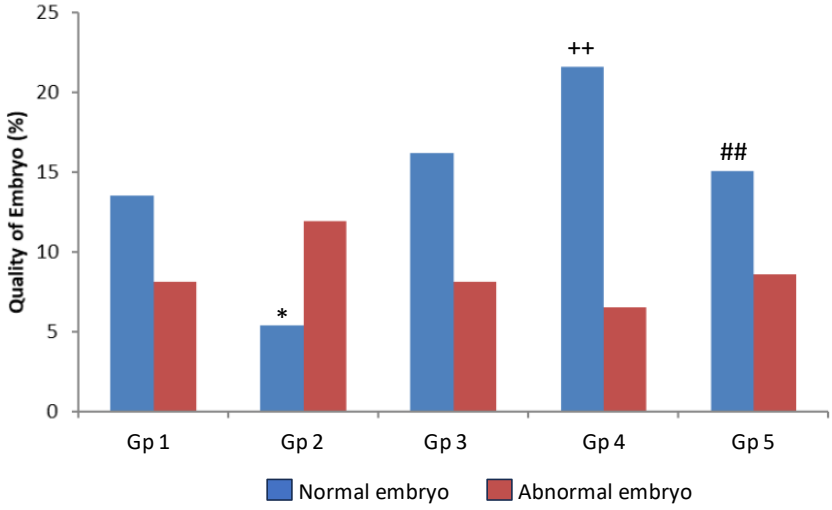

Figure 6: Quality of embryos in STZ-induced diabetic mice following palm TRF supplementation. Gp 1: Non-diabetic control, Gp 2: STZ-induced diabetic without TRF supplementation, Gp 3: Non-diabetic vehicle control, Gp 4: Non-diabetic with TRF supplementation and Gp 5: STZ-induced diabetic with TRF supplementation. The number of normal embyros was expressed as a percentage. Data were analysed using Chi-square. *$p<0.05$ compared to Gp 1; $^{++}p<0.01$ compared to Gp 3, $^{\#\#}p<0.01$ compared to Gp 2. n = 8 for each group.

Table 2: Preimplantation embryo development in STZ-induced diabetic mice following palm TRF supplementation. Gp1: Non-diabetic control, Gp 2: STZ-induced diabetic without TRF supplementation, Gp 3: Non-diabetic vehicle control, Gp 4: Non-diabetic with TRF supplementation and Gp 5: STZ-induced diabetic with TRF supplementation. The number of normal embyros were expressed as a percentage. Data were analysed using Chi-square. *p<0.05; ***p<0.001 compared to Gp 1, n = 8 for each group.

Group/ Developmental stages (%)	2-4 cells	4-8 cells	8-16 cells	Morula	Blastocyst
Gp 1	18.8	15.0	11.3	7.5	3.6
Gp 2	7.5*	4.5*	2.3*	0.0*	0.0*
Gp 3	22.6	18.8	17.3	13.5*	11.3*
Gp 4	30.1*	27.1*	24.8*	22.6*	21.1***
Gp 5	21.1	15.0	22.6	6.0	3.0

that caused an increase in embryonic death and foetal malformation [31]. The direct harmful effect of hyperglycaemia in avian model has also been reported where hyperglycaemia slowed the cell cycle progression during embryonic development [32].

In this study, palm TRF supplementation in non-diabetic mice (Gp 4) significantly increased the percentage of normal embryos (21.6%) ($p<0.01$) as compared to its control (Gp 3) (16.2%). Palm TRF supplementation in diabetic mice (Gp 5) on the other hand was able to restore the percentage of normal embryos (15.1%) towards control value (Gp 3) (16.2%) (Figure 6). Previously, it has been reported that antioxidants such as mulberry extract [13] and combination of vitamins C and E [14] were used to decrease the adverse effects of oxidative stress and improves foetal outcomes in diabetic rats. Maternal supplementation of diabetic mice with thymoquinone, a plant-based antioxidant was reported to increase the number of successful pregnancies and delivery of neonates [33]. Treatment with tungstate which is proposed to have normoglycemic effect improve the fertility rates in STZ-induced diabetic rats [15]. Similarly in this study, supplementation of palm TRF in maternal diabetic mice is able to prevent the adverse effects of hyperglycemia induced-oxidative damage on the embryo and improve the quality of embryos.

The Effect of Palm TRF on Blood Glucose Levels in STZ-induced Diabetic Mice

Two weeks of TRF supplementation was not able to reduce the blood glucose levels in STZ-induced diabetic mice (Table 3). Previously, it was reported that injection of STZ for 5 days induced maternal diabetes in mice and the high blood glucose levels remains throughout the pregnancy until lactation [33]. Similar with finding of this study, treatment with tungstate was found to improve the fertility rates in diabetic rats but failed to restore the blood glucose levels towards normal levels [15]. In contrast, thymoquinone supplementation in maternal diabetic mice was proposed to improve the reproductive outcome by exerting antioxidant effect and reducing the blood glucose levels [33].

Table 3: The effect of palm TRF on the blood glucose levels in STZ-induced diabetic mice. Gp 2: STZ-induced diabetic without TRF supplementation Gp 5: STZ-induced diabetic with TRF supplementation. Values were expressed as mean ± SEM. Data were analysed using one-way ANOVA. ***p<0.001 compared to Day 0 (before induction of diabetes). n = 8 for each group.

Groups	Blood glucose levels (mmol/L)		
	Day 0	Day 8	Day 21
Gp 2	5.89 ± 0.33	17.75 ± 0.71***	-
Gp 5	6.01 ± 0.28	16.29 ± 0.50***	12.58 ± 0.23***

CONCLUSION

In conclusion, TRF supplementation for two weeks was able to overcome the diabetic-induced oxidative stress damage on the embryos and maintain their developmental capacity. However, TRF is not able to lower the blood glucose levels in diabetic mice. Although the levels of oxidative stress biomarkers are not measured and reported here, previous studies have shown that maternal hyperglycaemia increases ROS that contributes to oxidative stress leading to adverse effects on female reproductive system. This study also showed that palm TRF is potentially to be used to improve the quality and development of embryos in STZ-induced diabetic mice, and, surprisingly, suggests that palm TRF alone may enhance development of normal embryos. However, the mechanism of action of TRF in STZ-induced diabetic mice remains to be elucidated.

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