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ORIGINAL ARTICLE

The Effect of Tocotrienol-Rich Fraction Supplementation on the Ovarian Metabolome and the Quality of Oocyte in Aging Mice

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ABSTRAK

Penuaan ovari telah dikaitkan dengan tekanan oksidatif dan kehilangan fungsi ovari. Tokotrienol telah dibuktikan dapat memberi kesan yang baik terhadap sistem pembiakan wanita. Walau bagaimanapun, peranan tokotrienol ke atas metabolisma ovari dan seterusnya peningkatan kualiti oosit dalam mencit tua masih tidak diketahui. Oleh itu, hubungan antara perubahan aktiviti metabolik dalam ovari dan kualiti oosit dalam mencit tua selepas suplementasi fraksi kaya tokotrienol (TRF) telah dikaji. Mencit betina berusia enam minggu digunakan sebagai kumpulan Muda. Mencit betina berusia enam bulan dibahagikan kepada empat kumpulan iaitu kumpulan pertama yang diberikan minyak jagung-bebas tokoferol (kawalan) manakala tiga kumpulan yang lain diberi suplimen TRF pada dos 90, 120, dan 150 mg/kg. Rawatan diberikan secara oral selama dua bulan. Pada akhir rawatan, mencit dari semua kumpulan disuperovulasi dan kemudian dikorbankan. Kualiti oosit dinilai dan analisis metabolomik secara tidak disasarkan, pada tisu ovari dijalankan dengan menggunakan 'liquid chromatography tandem mass spectrometry of quadrupole time-of-flight' (LC-MS Q-TOF). Peratusan oosit normal adalah lebih tinggi ($p<0.001$) manakala peratusan oosit yang tidak normal adalah lebih rendah ($p<0.001$) dalam kumpulan terawat dengan 150 mg/kg TRF berbanding dengan kawalan. Sebanyak 17 metabolit telah dikenal pasti menunjukkan perbezaan yang nyata dalam tisu ovari kumpulan Penuaan secara berbanding dengan kumpulan Muda. Selain itu, terdapat 14 metabolit yang menunjukkan perbezaan yang nyata antara kumpulan terawat TRF dengan kumpulan kawalan. Analisa tapak jalan menunjukkan perubahan tapak jalan metabolik dalam metabolisma asid lemak

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dan asid amino yang nyata berkemungkinan dapat mempengaruhi kualiti oosit. Kesimpulannya, suplementasi TRF dapat mengaruh perubahan metabolik dalam ovari yang dapat melambatkan kesan penuaan dan seterusnya meningkatkan kualiti oosit di dalam mencit tua.

Kata kunci: metabolomik, ovari, oosit, penuaan, tokotrienol

ABSTRACT

Ovarian aging has been associated with oxidative stress and loss of ovarian function. Tocotrienol has been proven to exert beneficial effects on the female reproductive system. However, the role of tocotrienol in affecting metabolism in the ovary and subsequently improving the quality of oocytes in aging mice remains unknown. Therefore, the relationship between metabolic changes in the ovary and the quality of oocytes in aging mice following tocotrienol-rich fraction (TRF) supplementation was investigated. Six-week-old female mice were used as the Young group. Six-month-old aged female mice were divided into four groups; the first group was given tocopherol-stripped corn oil (vehicle control) while the other three groups were supplemented with TRF at the dose of 90, 120, and 150 mg/kg, respectively. The treatment was given orally for two months. At the end of the treatment, mice from all groups were superovulated and then euthanised. Oocyte quality was assessed and non-targeted metabolomic analysis of the ovarian tissues was performed using liquid chromatography-tandem mass spectrometry of quadrupole time-of-flight (LC-MS Q-TOF). Percentages of normal oocytes were higher ($p<0.001$) while abnormal oocytes were lower ($p<0.001$) in TRF 150 mg/kg group compared to that of the control. Seventeen metabolites were identified to be significantly different in the ovarian tissue of the aging group when compared to the young group. 14 metabolites were identified to be significantly different in the ovarian tissue between the control and TRF supplemented groups. Pathway analysis revealed significantly altered metabolic pathways for fatty acid and amino acid metabolism that might influence the quality of oocytes. In conclusion, TRF supplementation causes metabolic changes in the ovary that delay the consequences of aging, thus improving the quality of oocytes in aging mice.

Keywords: aging, metabolomics, ovary, oocytes, tocotrienol

INTRODUCTION

Over the past decade, female fertility is declining at an alarming rate in Malaysia as well as globally (Mascarenhas et al. 2012; Vital Statistics Malaysia 2018). Along with educational improvement and social changes, the age of marriage has been steadily rising to cause most women to delay childbearing (Mascarenhas et al. 2012). Therefore, aging is one of the

factors for the reduction in fecundity as fertility diminishes in parallel with increasing age. Women lose their fertility in their middle age (around 35 years old) and their fecundity expires soon thereafter (at more than 40 years old) (Baird et al. 2005; Alviggi et al. 2009) fertility decreases with increasing maternal age. A slow but steady decrease in fertility is observed in women aged between 30 and 35 years, which is followed by an accelerated decline among women aged over 35 years. A combination of delayed childbearing and reduced fecundity with increasing age has resulted in an increased number and proportion of women of greater than or equal to 35 years of age seeking assisted reproductive technology (ART). Eventually, ovarian aging may result in ovarian failure, leading to a decrease in follicle number and diminishes oocyte quality. It has been suggested that the loss of oocyte quality is due to an age-related increase in meiotic non-disjunction, causing an increased aneuploidy rate in the early embryo of females aged between 35-38 years. Mechanisms that may underlie the age-related decline in oocyte quality include possible differences between germ cells at the time of formation during fetal life, accumulated damage of oocytes and the changes in the quality of granulosa cells surrounding the oocyte (Broekmans et al. 2009) followed by subsequent stages of overt cycle irregularity. The gradual decline in the size of the antral follicle cohort is best represented by decreasing levels of anti-Mullerian hormone. The variability of ovarian ageing among women is evident from the large variation in age at menopause. The identification of women who have severely decreased ovarian reserve for their age is clinically relevant. Ovarian reserve tests have appeared to be fairly accurate in predicting response to ovarian stimulation in the ART. As a result, women may possibly experience a loss in ovarian function by producing low quality oocytes and embryo leading to poor embryonic development. This could eventually lead to infertility, recurrent abortion, and aneuploidy.

During aging, an imbalance between reactive oxygen species (ROS) and antioxidant systems occurs leading to oxidative stress, which negatively impacts reproductive processes such as ovulation, fertilization and embryonic development (Agarwal et al. 2006; Mohd Mutalip et al. 2018). Oocyte protection against ROS by antioxidants may play an important role in pre-implantation embryonic development where it scavenges excessive ROS, thereby helping to maintain the oocyte's oxidant/antioxidant balance. Khazaei et al. (2017) reported that the addition of antioxidant substances such as melatonin to in vitro maturation (IVM) media produced a cytoprotective effect on the cumulus-oocyte complexes, thus supporting the subsequent embryonic development. Therefore, attenuation of oxidative stress by antioxidant supplementation could potentially reduce oxidative damage due to the aging process, thus maintaining the number and quality of oocytes and follicles in women (Liu et al. 2012; Wang et al. 2017) and also has

been implicated in reproductive aging. Telomere shortening is also involved in aging, and telomeres are particularly susceptible to ROS-induced damage. Previously, we had shown that antioxidant N-acetyl-L-cysteine (NAC). Tocotrienol, a component of natural vitamin E, was a potent antioxidant. Its molecular structure allows for more efficient penetration into tissues with saturated fatty layers, leading to the powerful disordering of membrane lipids and scavenging peroxyl radicals (Serbinova et al. 1991). According to Kanchi et al. (2017), tocotrienol as compared to tocopherol, exerts a more potent antioxidant activity in vitro and is more efficient in suppressing ROS levels. The antioxidant activity of palm oil derived tocotrienol-rich fraction (TRF) occurs through the inhibition of lipid peroxidation (Rajikin et al. 2012) and protein oxidation (Rajikin et al. (2012). There is increase in evidence of the beneficial effects of tocotrienol supplementation, especially in the female reproductive system. To date, several studies have demonstrated that following exposure to oxidative stress, TRF supplementation improves the quality of embryos and embryonic development (Hamdan et al. 2017; Nasibah et al. 2012) telomerase activity and oocytes qualities (morphology and deoxyribonucleic acid damage and preserves ovarian function (Saleh et al. 2015).

Metabolomics is an emerging technology and has been widely used to identify biomarkers and to build metabolic profiles associated with biological activities and physiological status (Ke et al. 2015). In metabolomics analysis, it was shown that metabolic dysfunction is a common hallmark of the aging process. Studies describing the complete overview of metabolic changes related to the reproductive system in healthy aging mammals remains lacking. Analysis of ovarian metabolites would be useful to understand the biochemical changes that take place during aging and how it affects the quality of oocytes.

Therefore, this study performed a metabolomics analysis in aging mice supplemented with TRF to identify the metabolites involved in female reproductive aging and infertility and also to elucidate the metabolic pathways which promote the protective effects of TRF as antioxidants.

MATERIALS AND METHODS

Materials

The following chemicals were obtained from the indicated source: tocopherol-stripped corn oil (MP Biomedicals, USA), and TRF palm oil (Sime Darby Bioganic Sdn Bhd, Malaysia). Tocotrienol-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%). TRF was diluted with tocopherol-stripped corn oil as a vehicle (Mokhtar et al. 2008) to obtain the desired concentrations of 90 mg/kg, 120 mg/kg and 150 mg/kg body weight. Supplementation of TRF was administered by the force-feeding technique (oral gavage) using a 20-gauge, 3.8 cm straight feeding

needle, with a smooth ball on the tip (Kent Scientific Corporation, USA). The dispensing volume was 0.1 ml.

Method

Mice Feeding, Mating and Tocotrienol Supplementation

The experimental protocol was in strict accordance with the regulations and prescribed animal ethical procedures outlined by the Research Committee on the Ethical Use of Animals (UiTM Care: 159/2016). In this study, female *Mus musculus* mice aged (i) six weeks old (Young) with an average body weight of 14.8-9.7 g and (ii) six months old (Aging) with an average body weight of 26-30 g were used for experiments. According to Dutta & Sengupta (2016), six weeks old mice are equivalent to 18-24-years of human age whereas six-months mice are equivalent to 35-40-years of human age. Thus, the aging group in this study is defined as mature adult mice that are undergoing the aging process but have not reached the aged category yet. The mice were divided into two main groups, a control group and an experimental group, which consisted of eight mice per group (n=8). Mice at the age of 6 weeks (Young group) (Group A) were used as a negative control group, while mice at the age of 6 months were used as a positive control group (Group B1). In the vehicle control group (Group B2), mice were given 0.1 ml tocopherol-stripped corn oil as a vehicle control daily for two months using the force-feeding technique (oral gavage). The tocopherol-stripped corn oil that was used in this study is crude oil without the addition of any antioxidants (MP Biomedicals, USA). In the experimental group, 6 months old mice were supplemented daily using the force-feeding technique (oral gavage) for 2 months with 0.1 ml TRF at the dose of 90 mg/kg BW (Group C1), 120 mg/kg BW (Group C2), and 150 mg/kg BW (Group C3), respectively. The TRF dose used in this study is the optimum dosage obtained from previous studies on the effect of TRF supplementation in aging mice on the quality of embryo (Norerlyda et al. 2015). The animals were housed at 27˚C under 12-hour light-dark cycles. The mice were given food pellets and water ad-libitum daily.

Collection of Oocytes

After 2 months, all mice from aging (Group B1), vehicle control (Group B2), and TRF supplemented groups (Group C1, C2 and C3) were superovulated to synchronize the time for collection of oocytes. Female mice were superovulated intraperitoneally with 5 IU of pregnant mare serum gonadotropin (PMSG) (Sigma, Aldrich), followed by 5 IU of human chorionic gonadotropin (hCG) (Sigma, Aldrich) 48 hours later. Subsequently, 14 hours after hCG injection, the mice were euthanised and oocytes were collected by tearing the ampullae from the oviduct. The cumulus cells from oocytes were removed using hyaluronidase (Sigma, Aldrich) and oocytes were kept in the M2 medium until further analysis. M2 medium is a modified Krebs-Ringer solution with

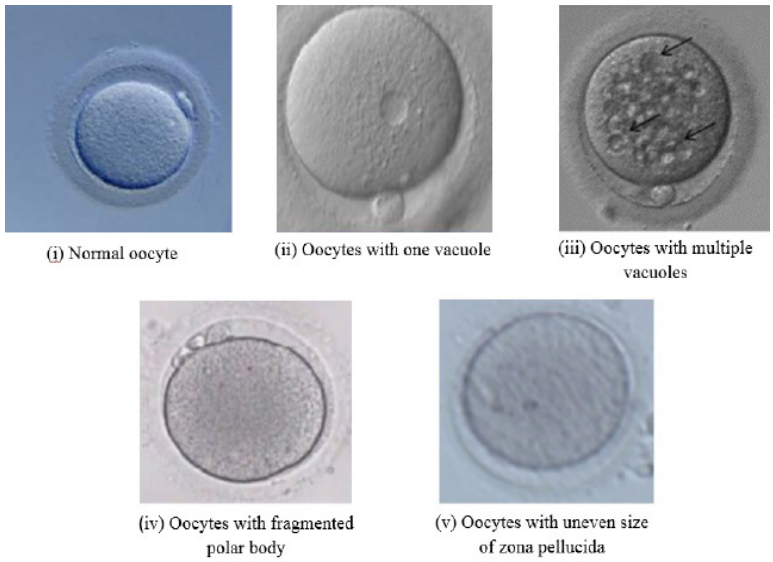

Figure 1: The photomicrograph of normal and abnormal morphology in oocytes retrieved from young and aging mice (100x magnification). (i) oocyte with normal morphology: with the presence of a polar body and evenly-sized zona pellucida (ii - v) oocytes with abnormal morphology: with the uneven size of zona pellucida, the appearance of one or multiple vacuoles, and a fragmented polar body.

some of the bicarbonate substituted with HEPES buffer to maintain the correct pH and this medium was used for collecting and keeping the embryo (Nagy et al. 2003). The numbers of normal and abnormal oocytes were counted and recorded. The morphology of normal and abnormal oocytes retrieved from young and aging mice is shown in Figure 1.

Collection of Ovaries for Metabolomic Analysis

For metabolomic analysis, ovaries were collected from the Young (Group A), Aging (Group B1), vehicle control (Group B2), and TRF 150 mg/kg supplemented group (Group C3). Ovarian samples for metabolomic analysis was selected only from the group supplemented with TRF 150 mg/kg (Group C3) as the TRF 150 mg/kg supplemented group showed the best results for oocytes quality. The mice were humanely sacrificed and the ovaries were collected. Ovarian tissues were perfused with phosphate buffer saline (PBS). Samples were snap-frozen in Eppendorf tubes in liquid nitrogen and stored at -80°C until processing. For processing, frozen samples were weighed around 50 mg then pulverized to a fine powder (freeze fracturing) with a mortar and pestle in liquid nitrogen. Metabolites were extracted using the mixture of chloroform: methanol: deionized water with the ratio of 2:2:1 (v/v). Approximately 200 µl of chloroform was added to each sample together with 200 µl of methanol and 100 µl of deionized

water. Samples were vortexed briefly before centrifugation at 10,000 x g for 10 minutes at 4°C. Portions of the aqueous layer and a portion of the organic layer were removed and pooled together in a new microcentrifuge tube. Supernatants were dried using a vacuum concentrator. All extracted samples were stored at -80°C until required.

LC-MS Q-TOF Analysis

The chromatography, separation and detection of small molecules were performed on an Agilent Series 1200 SL Rapid Resolution Liquid Chromatography system coupled to an Agilent 6520 accurate mass spectrometer equipped with dual electrospray ionization (ESI) source. For the detection of molecules, Zobrax Eclipse Plus C18-ID of 1.8 µm particle size and 2.1 x 100 mm column dimensions were used. The temperature was maintained at 40°C during the run. The mobile phase composed of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The flow rate was set at 0.25 ml/minutes and the injection volume was 2 µl. A linear gradient was developed over 36 minutes from 5% to 95% of the mobile phase (B). The total run time was set at 48 minutes for each analysis. To produce ion for detection, ESI source settings were used as follows: V Cap 4000 V, skimmer 65 V and fragmentor 125 V. Mass spectral acquisition range was set from 50 to 1400 m/z. The nebulizer was set at 45 psig and the nitrogen drying gas was set at a flow rate of 12 L/minutes. Drying gas temperature was maintained at 350°C. Data were acquired at a rate of 2.5 spectra/second with a stored mass range of m/z 50-1000. Internal reference ions were used to correct mass accuracy. Auto calibration parameters were chosen to average 5 scans and reference mass correction was enabled throughout the run. The mass spectrometer was tuned to allow the detection of compounds to an accuracy of $\pm$ 2 ppm before the analysis. To assure the desired mass accuracy, internal reference ions (m/z 121.0509 and 922.0098) were used throughout the run.

Data Processing

MassHunter Workstation software, including Qualitative Analysis (version 3.01) was used for processing both raw MS and MS/MS data, including molecular feature extraction, background subtraction and data filtering. To perform subtraction of molecular features (MFs) originating from the background, analysis of a blank sample (deionized water) was carried out under identical instrument settings and background MFs were removed. Using background-subtracted data, files in compound exchange format (CEF files) were created for each sample and exported into the Mass Profiler Professional (MPP) software package for further processing.

To minimize the number of missing values, only metabolites that were consistently detected in at least 50% of samples were included in the statistical analyses (Xia & Warshart 2016). All known artifact peaks, such as internal

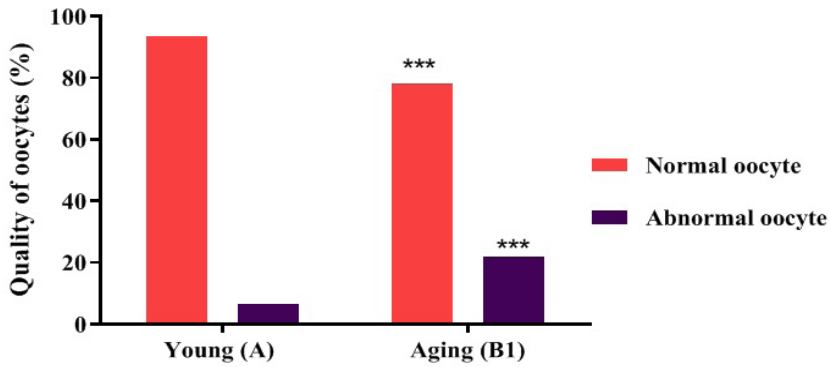

Figure 2: The quality of oocytes retrieved from young and aging mice. The percentage of normal oocytes was significantly lower ($p<0.001$) and the abnormal oocytes were significantly higher in percentage ($p<0.001$) in the aging group when compared to the young group. Values were expressed as a percentage. Data were analysed using Chi-square test. ***$p<0.001$ significant difference from the Young group, n = 8

standards, column bleed, plasticizers, or reagent peaks were excluded from the result sheets. All metabolite data were normalized relative to the sum of all known metabolites in each sample and were log transformed. The metabolites were detected using the Mass Profiler Professional (MPP) software package and were identified based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The identified metabolites were used to check on their associated pathways in the KEGG database and the metabolite enrichment analysis was analyzed using MetaboAnalyst.

Statistical Analysis

For oocyte quality, the significant differences between groups were analyzed using the Chi-Square Test and considered significant at $p<0.05$. The data were analyzed using Statistical Package for Social Science (SPSS) version 25 and data were expressed as a percentage.

For metabolomic analysis, Mass Profiler Professional (version B.02.00) was used for statistical analysis by Analysis of One-way Analysis of Variance (ANOVA) and Partial Least Squares-Discriminant Analysis (PLS-DA), followed by the construction of the predictive classification model, molecular formula estimation, and database searching.

RESULTS AND DISCUSSION

In this study, the quality of oocytes was evaluated based on the morphology of normal and abnormal oocytes, which were observed using an inverted microscope (Figure 1). Oocytes with the presence of a polar body and evenly sized zona pellucida are defined as normal oocytes. Meanwhile, the oocytes with no polar body or uneven size of zona pellucida, the appearance of one or multiple vacuoles and fragmented polar body are defined as abnormal oocytes. Figure 2 shows a lower percentage of normal oocytes in

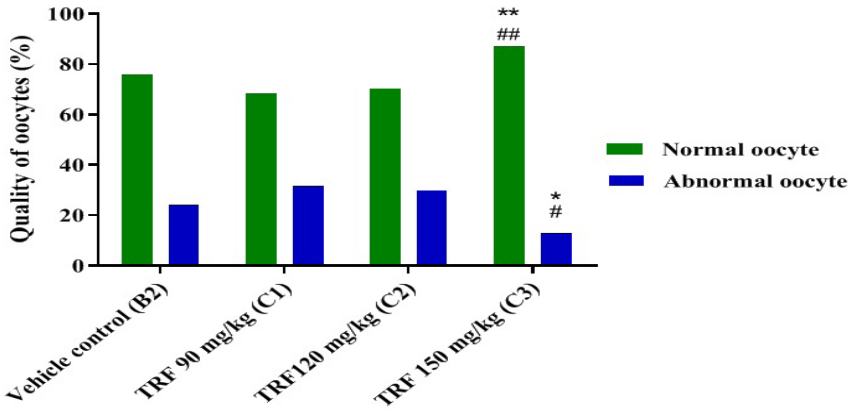

Figure 3: The quality of oocytes retrieved from aging mice supplemented with TRF. The percentage of normal oocytes in the group supplemented with TRF 150 mg/kg BW was significantly higher ($p<0.05$) and the abnormal oocytes were significantly lower ($p<0.05$) when compared to vehicle control and the other TRF Groups. Values were expressed as a percentage. Data were analysed using Chi-square test. *$p<0.05$, **$p<0.01$ significant difference from the vehicle control group, ##$p<0.01$ significant difference from the other TRF groups, #$p<0.05$ significant difference from the other TRF groups, n = 8.

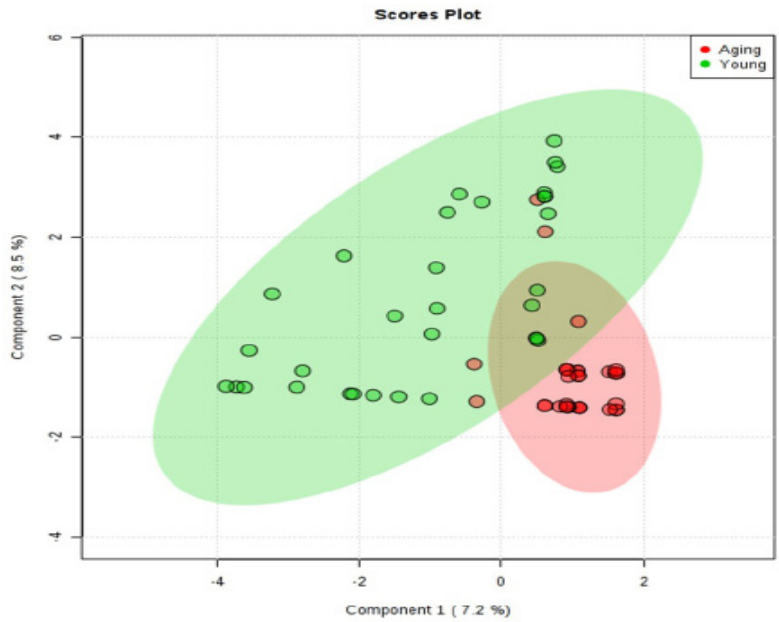

Figure 4: PLS-DA Score plot between Young and Aging groups. The explained variances are shown in brackets. Ovarian metabolomics profiles examined by supervised PLS-DA demonstrated a clear differentiation between the Young and Aging groups. Plots show Component 1 (x-axis) and Component 2 (y-axis) with the percentage of the variance explained by each component labelled on the axis. Ovals indicate the 95% confidence interval ($p<0.05$). Discrimination of metabolite regulation is presented as fold change between the Young and Aging groups.

Table 1: The differential metabolites in the ovary between the Young and Aging groups

Metabolites	Regulation	Log Fold Change	p-value
Prostaglandin A2	Up	3.2969	3.77E-05
17alpha-Hydroxypregnenolone	Up	1.0654542	9.05E-05
Retinoate	Up	6.5015	0.0001745
Spermine	Up	7.252243	0.0005523
alpha-Linolenic acid	Up	7.2743425	0.0009626
Thymidine	Up	5.5446	0.0010138
Taurocholic acid	Up	3.8329222	0.0015217
S-Acetyldihydrolipoamide-E	Down	-4.6399693	0.0015635
17alpha-Hydroxyprogesterone	Up	5.4235144	0.0016782
Cholic acid	Up	6.4932	0.002076
N-Acetylornithine	Up	6.4932	0.002076
5-Hydroxykynurenamine	Up	6.2964	0.0020764
Hypoxanthine	Down	-6.7602	0.0020765
Linoleic acid	Down	-6.3692	0.0020772
UDP-N-acetyl-alpha-D-glucosamine	Down	-6.113	0.0021012
3-Dehydrosphinganine	Up	4.6620464	0.002138
Prostaglandin D2	Up	5.1589217	0.0028375

aging ($p<0.001$) as compared to Young group, while in Figure 3, the percentage of normal oocytes in aging mice supplemented with TRF at 150 mg/kg BW was found to be higher ($p<0.01$) as compared to the vehicle control group. The percentage of normal oocytes in aging mice supplemented with TRF at 150 mg/kg BW was found to be significantly higher as compared to other TRF-supplemented groups.

Using non-targeted metabolomics analysis, thousands of metabolite signals were captured whereby 17 metabolites were identified to be significantly different in ovarian tissue of the aging group when compared to the Young group (Table 1). Meanwhile, 14 metabolites were identified to be significantly different in the ovarian tissue of vehicle control and TRF supplemented groups (Table 2). Ovarian metabolomics profiles examined by supervised PLS-DA demonstrated a clear differentiation between the Young and Aging groups. This differentiation was mainly described along with the first component of the PLS-DA. Discrimination of metabolites regulation was presented in fold-change in Young and Aging groups as shown in Figure 4, where metabolites in the young group were found to be down regulated.

The aging process affects ovarian function causing ovarian failure that may lead to a decrease in the number of follicles, loss of follicular functions, a decline in oocyte reserve as well as oocyte quality (Sukur et al. 2014). This finding indicates that oxidative stress-induced ovarian aging results

Table 2: The differential metabolites in the ovary between vehicle control and TRF-supplemented group

Metabolites	Regulation	Log Fold Change	p-value
Androsterone glucuronide	Down	-7.2097	0.000103
Alpha-Linolenic acid	Down	-4.3562	0.000345
Resiniferatoxin	Down	-7.9118	0.000388
5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid	Down	-1.2582	0.000471
R-S-Lactoylglutathione	Up	4.6552	0.000821
S-Acetyldihydrolipoamide-E	Up	7.3555	0.002078
Nicotinamide adenine dinucleotide	Up	6.5757	0.002303
Urocanate	Up	2.7953	0.003983
gamma-Glutamyl-gamma-aminobutyraldehyde	Up	6.5958	0.004213
17-Hydroxylinolenic acid	Down	-7.4267	0.004528
Arachidonic acid	Up	6.8773	0.004552
Docosahexaenoic acid	Down	-3.5048	0.005002
beta-D-Glucuronoside	Down	-2.9536	0.005562
11-Deoxycorticosterone	Up	2.7811	0.005917

in the dysfunction of the reproductive process (Fujii et al. 2005). Based on Figure 2, the loss of oocyte quality in aging is believed to be due to an increase in meiotic non-disjunction, thus resulting in dysfunction of the reproductive process (Broekmans et al. 2009). Using metabolomics analysis, it was shown that metabolic dysfunction is a common hallmark of the aging process (Houtkooper et al. 2011).

TRF supplementation has been previously shown to improve the quality of oocytes in aging mice by lowering the percentage of oxidative deoxyribonucleic acid (DNA) damage, thereby restoring the telomere length and telomerase activity. Consequently, mitochondrial function is protected during oxidative stress conditions (Hamdan et al. 2017). Therefore, the results of this study confirm that aging is highly correlated with oxidative stress in the ovary thus leading to the production of poor quality oocytes. Metabolomic analysis from ovaries shows that most affected pathways are fatty acid and amino acid metabolism. The most remarkable changes in metabolism in Aging groups were defined by a significant change in fatty acid-related metabolism, specifically in alpha-linolenic acid metabolism (Figure 5). Previously it was reported that in aging, as an alternative for oxidative phosphorylation, fatty acid was utilized for ATP production (Fong et al. 2011). Accumulation of fatty acids intermediates in the cardiomyocyte cytosol could result in lipotoxicity and could result in a decrease in fertility (Wu et al. 2000).

Meanwhile, ovarian metabolomic profiles when compared between the vehicle control group and TRF supplemented group (examined by supervised PLS-DA) demonstrated a clear differentiation between both

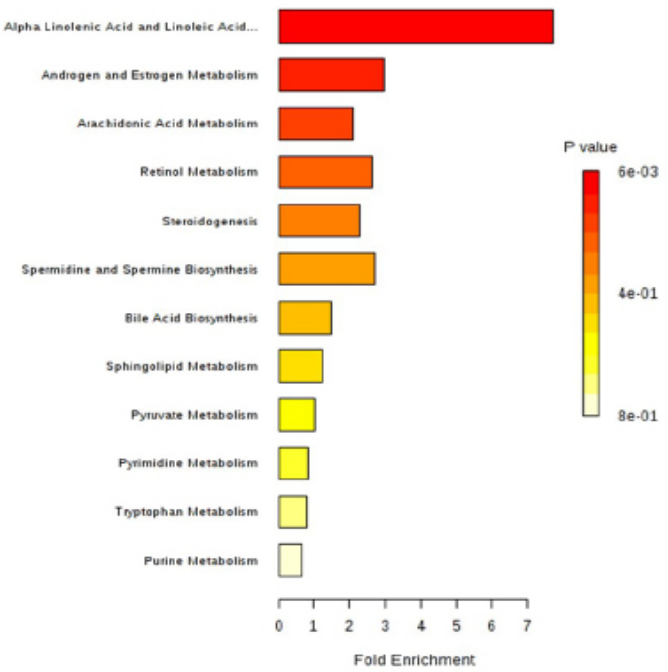

Figure 5: Results of metabolite set enrichment analysis (MSEA) in Young vs Aging group. The horizontal bar graph summarizes the metabolic pathways that were strongly affected in the Aging group compared to the Young group. Metabolite sets are ranked according to Holm p-value with hatched lines showing the cut off Holm p-value. The bars are colored based on their p-values and the length is based on the fold enrichment.

groups (Figure 6). This differentiation was mainly described along with the first component of the PLS-DA. Discrimination of metabolites regulation was presented as fold-change in vehicle control and TRF supplemented groups as shown in Figure 5, where metabolites in the young group were found to be down regulated. TRF-supplemented group showed a significant down-regulation in alpha-linolenic acid metabolism. Alpha-linolenic acid metabolism is important and needs to be provided in the diet. Alpha-linolenic acid is necessary for numerous processes, including growth, reproduction, improved reproductive lifespan, oocyte quality and aging (Dunning et al. 2014). However, the imbalance of fatty acids (FAs) in cell membranes where the metabolism is highly regulated can cause damage from free radicals leading to oxidative stress (Tsaluchidu et al. 2008). Furthermore, fatty acids are used as an energy source during oocyte maturation and the extended period of embryo development before implantation (Wathes et al. 2007).

In the Aging group, amino acid metabolism was mostly up-regulated (Figure 6) (Table 1). These findings suggest that due to oxidative stress, amino acid metabolism was upregulated, causing damage to cell structures leading to low viability of

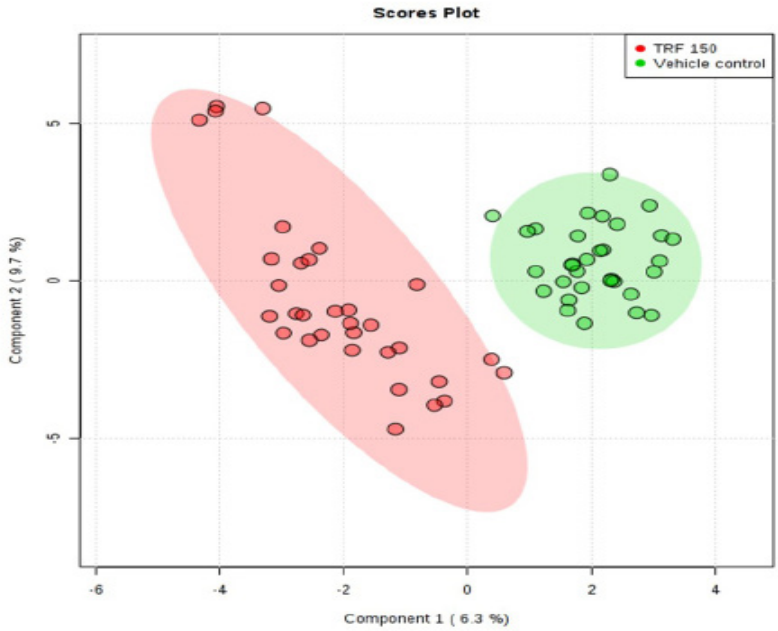

Figure 6: PLS-DA Score plot between vehicle control and TRF 150 groups. The explained variances are shown in brackets. Ovarian metabolomics profiles examined by supervised PLS-DA demonstrate a clear differentiation between vehicle control and TRF-supplemented group. Plots show Component 1 (x-axis) and Component 2 (y-axis) with the percentage of the variance explained by each component labelled on the axis. Ovals indicate the 95% confidence interval ($p<0.05$). Discrimination of metabolites regulation was presented as fold change between vehicle control and TRF-supplemented groups.

cells (Fujii et al. 2005). An increase regulation of most amino acid metabolism in the ovary of the aging group, as was found in the present study. This could result from an increase in the rate of a whole-body protein breakdown, insulin resistance and subsequent oxidation of their carbon skeletons in the citric acid cycle (Lawton et al. 2008).

However, a significant down-regulation in amino acid metabolism was observed in the TRF-supplemented group (Figure 7) (Table 2). This finding is comparable to that reported by Botros et al. (2008), which found that embryos with TRF viability had a lower amino acid metabolism needed for successful implantation than did the embryos that were arrested. Although the metabolism of amino acids on ovarian function and aging is still not well understood, a recent study on the metabolic profiles of ovarian follicular fluid collected from patients undergoing in vitro fertilization (IVF) revealed that women with repeated IVF failure had increased concentrations of amino acids (Xia et al. 2014). Interestingly, the process was reversed in the TRF-supplemented group by down regulation of amino acid metabolism, thus suggesting the effectiveness of TRF in combating oxidative stress and enhancing antioxidant defence mechanisms. This was in accordance

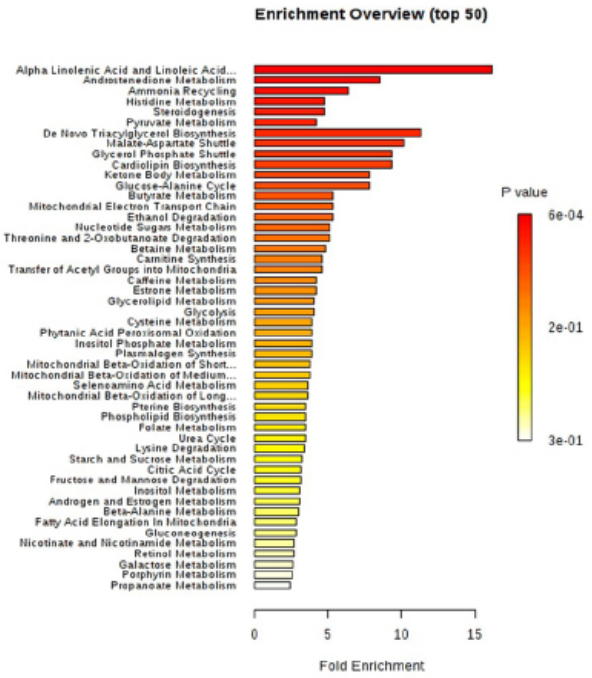

Figure 7: MSEA in TRF-supplemented group vs vehicle control. The horizontal bar graph summarizes the metabolic pathways that were strongly affected in TRF 150 mg/kg supplemented group compared to vehicle control. Metabolite sets are ranked according to Holm p-value with hatched lines show the cut-off Holm p-value. The bars are colored based on their p-values and the length is based on the fold enrichment.

with the study of Khor et al. (2017) where oxidative stress affects the normal function of satellite cells, with subsequent regeneration defects that lead to sarcopenia. This study aimed to evaluate TRF, where they found similar outcomes in senescent human myoblast tissue supplemented with TRF.

CONCLUSION

In conclusion, TRF supplementation at a dose of 150 mg/kg body weight (BW) for two months was effective in delaying the effect of aging on oocyte quality. Additionally, metabolic changes in the aging ovary had a negative impact on cellular energy storage, energy metabolism and oxidative stress that subsequently affects female fertility. Furthermore, supplementation with TRF prevented the negative impact of age-related metabolic changes in the ovary. Thus, TRF is proposed to exert a protective effect on female reproductive aging. The pathways identified also suggest a positive relationship between metabolic changes in the ovary and the quality of oocytes in mice supplemented with TRF.

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