The ovarian follicles of matured adult mice biology essay

Science, Biology

Saheera Kamarzaman, Tg. Teh Izyan Ilyana Tg. Khajakee, Munirah Sha’ban and Suzanah Abdul RahmanDepartment of Biomedical Science, Faculty of Allied Health Sciences, International Islamic University of Malaysia (IIUM), Kuantan, Pahang, Malaysia. saheerakamarzaman@gmail. comCorrespondence concerning this article to be addressed to: Suzanah Abdul Rahman, PhDAssociate ProfessorDepartment of Biomedical ScienceFaculty of Allied Health SciencesInternational Islamic University Malaysia (IIUM)Jalan Sultan Ahmad ShahBandar Indera Mahkota25200, Kuantan, Pahang, MalaysiaTel: 09-5705203/5204, Fax: 09-5716776E-mail: suzanahrahman@yahoo. com or arsuzanah@iium. edu. myABSTRACTChemotherapy treatment has adverse effects on ovarian function at all ages. However, older women had a much higher incidence of complete ovarian failure as compared with younger women. This study aims to assess follicle preservation in mature adult female mice with the provision of Nigella Sativa oil following exposure to an alkylating agent which can cause ovarian follicular loss. Forty-eight ICR mice aged 18 weeks were divided into three main experimental groups: control, cyclophosphamide-alone (50mg/kg) and cyclophosphamide pre-treated with different doses of Nigella Sativa oil (0. 2ml, 0. 5ml and 1. 0ml/100g). The histological structure of the ovarian follicles was studied and the total number of primordial follicles remaining in the ovaries was counted. Results show that cyclophosphamide causes primordial follicle destruction in proportion to different time points of exposure ($p < 0.05$). Ten days following exposure to cyclophosphamide, the primordial follicle pool was reduced by 58. 13% (36. 33 ± 5. 86) than that in the control group (86. 67 ±32. 52). Treatment with

cyclophosphamide also induced significant toxicity as shown by a reduction in the mean number of normal primary and secondary follicles (p <0. 001), decreased in the mean ovarian diameters (p <0. 05) and showed the irregular distribution of granulosa cells, vacuolated ovarian follicles and oocyte destruction. Administration of Nigella sativa oil exhibited protection to the integrity of the ovarian follicles and induced a significant reduction in overall toxicity caused by the chemotherapeutic agent. These preliminary results showed the practical effectiveness of the proposed antioxidant that can be used in the clinical setting to minimize chemotherapy-induced ovarian failure in older women. Keywords: Nigella sativa oil, Cyclophosphamide, Ovarian follicles, Primordial follicles, Mature adult mice, InfertilityINTRODUCTIONThe advancement in the field of oncology and continued development in treatment modalities has increased the awareness on the long term effects of chemotherapy treatment on the reproductive potential of cancer survivors. It is increasingly noted that a high number of adult survivors of childhood cancer malignancies were not aware of the risk of infertility and relevant late effects (Hess et al., 2011). Chemotherapeutic agents have been shown to affect female fertility by destructing the ovarian follicles and inducing ovarian damage that can lead to infertility. Ovarian damage occurs in all age groups with older females appeared to be more affected as they have a smaller ovarian follicular reserve (Meirow et al., 1999; Meirow and Nugent, 2001). Cyclophosphamide (CPA) is one of the most damaging alkylating agents that can cause oxidative stress due to the over-production of reactive oxygen species (ROS) (Damewood and Grochow, 1986; Pryor et al., 2000; Meirow and Nugent, 2001; Mitchell et al., 2003;

Alenzi et al., 2010). It affects the DNA of replicating cells and rapidly multiplying cells especially in the gonads and pituitary. It results in miscoding, cross-linking and DNA breakage by transferring alkyl groups to the guanine compound of the DNA . In the females, the prime concern now is that cyclophosphamide can induce depletion in the primordial follicular (PMF) reserve. Since PMF pool is non-renewable, older women treated with chemotherapy have a higher incidence of ovarian failure when the chemotherapeutic agent destructs an already low follicular reserve, needed to sustain ovarian function (Kumar et al, 1972; Gosden and Faddy, 1994). A potential approach that can interfere with cyclophosphamide-induced toxicity is to lower the induced oxidative stress. Nigella Sativa, from the Ranunculacea family, has been one of the most widely used herbal medicines for the treatment of various diseases. The pharmacological properties of the oil have been reported to include anti-inflammatory, anti-cancer, anti-diabetic, anti-microbial, anti-histaminic, anti-infertility and hypotensive effects (Mukhallad, 2009; Alenzi et al., 2010). Thymoquinone is the active compound of the essential oil with anti-oxidative effect that works as a scavenger of various radical oxygen species including superoxide radical anion and hydroxyl radicals through different mechanisms (Mansour et al, 2002; Badary et al, 2003; Mahgoub 2003). The fertility-preservation effect of Nigella Sativa oil (NSO) in combination with alkylating agent of mature ovarian activity has not been explored in detail hence the present study was undertaken. The study aims to address the need of exploring the relationship between ovarian injuries as expressed by the loss of PMF population and increased follicle degeneration and the potential of NSO in

lowering the ovarian-toxicity of cyclophosphamide treatment. Data of this experiment could provide opportunities for adult cancer patients having the appropriate support to their resolution towards an improved quality of life. MATERIALS AND METHODSForty eight ICR mature adult female mice, aged 18 weeks were divided into three main experimental groups: control, cyclophosphamide-alone and cyclophosphamide pre-treated with different doses of NSO. Six control mice were injected with normal saline. Twenty-four mice were given a single intraperitoneal injection of cyclophosphamide (Sigma-Aldrich) at a dose of 50mg/kg body weight (n= 6/group). This dose was chosen based on a previous dose-response study of Meirow et al., 1999. Observation of effects was done on days-5, 10, 15 and 20 which represents a different stage of follicular growth at the time of exposure to cyclophosphamide (Meirow et al., 2001). In the third group, eighteen mice (n= 6/group) were administered intraperitoneally with 0. 2ml/100g, 0. 5ml/100g and 1. 0ml/100g body weight of NSO 6 hours before cyclophosphamide treatment, on every other day for 5 days. Observation of effects was done on day-5 post cyclophosphamide treatment. At the end of each exposure period, mice were euthanized by cervical dislocation. The ovaries were excised and trimmed free of fat before immersion in the fixative solution. Ovaries were fixed with 4% formaldehyde overnight, dehydrated in ethanol, embedded in paraffin and serially sectioned at 6-µm. The tissues were stained with haematoxylin and eosin. Ovarian follicles were counted in every section using a light microscope at a magnification of x400. Follicles were classified into four types based on the classification of Erickson (2003): (i) Primordial - characterized by an oocyte surrounded by a single

layer of flattened cells; (ii) Primary - characterized by a single layer of cuboidal pre-granulosa cells; (iii) Secondary - characterized by 2 to 5 complete layers of granulose cells and (iv) Graafian - containing cavity occupying most of the total follicular volume. Only follicles with a visible nucleus in the oocyte were considered for counting to avoid duplicate counts of a follicle. Follicles that contained an intact oocyte and intact granulosa cells were classified as normal. Follicles were classified as degenerated when they contained ruptured oocyte nuclei, shrunken ooplasm and disorganized granulosa cells. The diameters of the ovary were further measured at a magnification of x40. The quantitative information on the follicle distributions and ovarian diameters were analyzed using Olympus Analysis Image Processing. Statistical analysis was done with SPSS 18. 0 software. All the values of primordial follicles, ovarian diameters and the distribution of normal and degenerated follicles were expressed as mean ± standard deviation (S. D). Inter-group variation was measured by Student’s t-test to evaluate the significant differences between the groups. The distribution of normal and degenerated follicles in control, cyclophosphamide alone and cyclophosphamide co-treated with NSO groups were compared by one-way analysis of variance (ANOVA) and Tukey’s test. $p \leq 0.05$ was considered to be statistically significant. Work done in this study received the ethical approval from the Ethics Committee of the Faculty of Medicine of the International Islamic University Malaysia (Ref: IIUM/305/20/4/10). RESULTSHistological changes of the ovarian follicles: Control ovary showed the presence of normal ovarian architecture in the primordial (I), primary (II) and secondary (III) follicles. The oocyte (O) was surrounded by a single or two layers of

granulosa cells (G). The oocyte was separated from the surrounding follicular cells by a well developed glycoprotein layer called zona pellucida (ZP). The outermost layer of follicular cells rests on a well defined basement membrane (BM) that separates it from the ovarian stroma. At the periphery, the connective tissue stroma surrounding the follicle begins to condense and form a theca folliculi layer (TL). Theca layer is usually distributed out of the healthy granulosa basement membrane in one, or more rarely, two continuous layers (Figure 1). Some injury was observed in the CPA group including the disruption of intercellular contacts among granulosa cells and the oocyte of primary and secondary follicles at day 5 (Figure 2). These follicles also exhibited numerous cytoplasmic vacuoles, abnormal shapes of the granulosa cells, some with absent or not well developed zona pellucida and oocyte, as well as vacuolated oocyte with nuclear shrinkage. NSO-treated groups showed an improved histological appearance in the CPA-exposed mice. The morphology of the follicles and the structure of the oocytes were well preserved, similar to the control group (Figure 3). The CPA-exposed groups of 15 and 20 days showed less signs of degeneration and injury in pre-antral (primary and secondary) follicles when compared to that in the groups of 5 and 10 days. The total numbers of primordial follicle (PMF): At 5 days of exposure, although there were differences in the mean number of PMF in the cyclophosphamide groups pre-treated with NSO as compared to the cyclophosphamide alone group, the differences were not statistically significant (Figure 4). A significant relationship was observed between the different time points of cyclophosphamide exposure and the total number of PMF counted in the ovaries (Figure 5). The mean ± SD

number of PMF in the ovaries of mice exposed to 50mg/kg cyclophosphamide at day-10 (36. 33 ± 5. 86) was reduced than that in the control group (86. 67 ±32. 52), a reduction of 58. 13%, p <0. 05. On days 15 and 20, follicle densities were further decreased in the cyclophosphamide exposed groups (23. 67 ± 9. 07, 12. 00 ± 1. 00) in comparison to the controls, p <0. 05. The total number of normal and degenerated follicles: The total number of normal and degenerated follicles in the control, cyclophosphamide alone and cyclophosphamide co-treated with 0. 2, 0. 5 and 1. 0ml/100g NSO at day 5 are presented in Table 1. The data were normally distributed to each group. The mean ± number of normal and degenerated primary and secondary follicles were statistically significantly different in the control and treatment groups (p <0. 001) as determined by one-way ANOVA. The mean number of degenerated primary, secondary and graafian follicles was significantly higher after 5 days of cyclophosphamide exposure compared to the control and NSO pre-treated groups (p <0. 05, p <0. 001). A Tukey post-hoc test further revealed that the mean number of normal primary and secondary follicles in the three groups of cyclophosphamide co-treated with NSO were significantly higher than the control and cyclophosphamide alone group (p <0. 001). There was no statistical significant difference in the total number of normal Graafian follicles between control and the test groups. Morphometrical analysis: The study revealed a significant reduction in the mean ovarian diameters of the cyclophosphamide-treated groups observed at 15 and 20 days; 674. 37±39. 12 and 417. 43±14. 51 respectively (p <0. 05), compared to an average of 1055. 56±35. 32µm in the control group (Table 2). Cyclophosphamide

groups that were pre-treated with 0. 5ml and 1. 0ml/100g of NSO for 5 days showed a significant higher ovarian diameters (1113. 10 ± 14. 68 and 1215. 70 ± 14. 50, respectively, p <0. 05) compared to the group that received cyclophosphamide alone (919. 83 ± 96. 43). DISCUSSION AND CONCLUSIONThe antioxidant property of NSO and its active compound, thymoquinone, has been elucidated previously in different experimental settings (Ismail, 2009). In this study, we reported the potential therapeutic role of NSO against ovarian toxicity induced by cyclophosphamide in matured mice. The results revealed that co-administration of NSO during cyclophosphamide treatment can lower or prevent cyclophosphamide-induced ovarian toxicity. Data obtained from the histological study showed that the most important ovarian content which is the primordial follicle population decreased significantly after cyclophosphamide injection. Reduction of primordial follicle reserve showed that cyclophosphamide treatment induces damage to the reproductive organs through the destruction of the germ cells, resulting in infertility (Aguilar-Mahecha et al, 2002). Several studies have reported the impact of chemotherapy agents on the PMF reserve. A study by Farokhi et al. (2006) on the effects of cyclophosphamide administration on mouse ovaries revealed that a dose of 75mg/kg destroyed more than 50% of PMF pool, caused a reduction in diameter and size of ovary and decreased thickness of the endometrium. A study by Meirow et al., 1999 reported that significant damage to the PMF population resulted even following administration of low doses of cyclophosphamide (20mg/kg). A xenograft model used to illustrate the impact of chemotherapy drugs on human primordial follicular reserve

reported that animals received a single dose of 200mg/kg cyclophosphamide showed a 12% reduction in the PMF density by 12 hours following treatment ($p < 0.05$) and significantly increased in follicle loss at 24 hours (53%, $p < 0.01$). The percentage of follicular loss was peaked at 48 hours (93%, $p < 0.0001$) (Oktem and Oktay, 2007). Ovarian function depends on the follicular reserve as they sustain the ovarian function. Thus, the depletion of PMF reserve as presented in this study indicates ovarian failure in mice treated with cyclophosphamide. Histological counting of PMF number reflects the damage caused by chemotherapy agent to the ovary more directly rather than reproductive performance i. e ovulation, mating and pregnancy rates (Meirow et al., 1999). In human, the incidence of complete ovarian failure and permanent infertility is much higher in older women than in younger women (Sanders et al., 1996). The PMF populations fall below a key threshold number required for ovarian function at the age of 45-50 years, at which point the menstrual cycle ceases and menopause occurs. In contrast, young females have a larger reserve of PMF; therefore the chemotherapy-induced loss of PMF may not be significant to cause immediate ovarian failure. Nevertheless, the reduction in PMF reserve in addition to natural atretic follicular loss may lead to an increase risk of premature menopause in these patients (Meirow et al., 1999). The use of the mature adult animal model is ideal for this study, as it would not be practical to correlate ovarian injury in adult women using young female mice. The mature adult group (3 to 6 months of age) consists of mice that are are fully developed but still not affected by reproductive senescence. Mice of age 3 to 6 months can be extrapolated to women of age 20 to 30 years (Flurkey et al, 2007). The

results obtained using animal model would contribute to medical progress and thus benefited humans. Toxicity related to anticancer drugs is usually associated with the over-production of reactive oxygen species (ROS) that cause oxidative stress (Mitchell et al., 2003). Oxidative stress has been implicated in a number of different reproductive scenarios such as endometriosis, folliculogenesis, oocyte maturation, hydrosalpingeal fluid, necrozoospermia, asthenozoospermia and sperm DNA damage (Guerin et al, 2001). In the context of female infertility, oxidative stress has been poorly characterized (Agarwal and Allamaneni, 2004). Nevertheless, oxidative stress in the female reproductive system has been demonstrated to correlate with fertility. Markers of oxidative stress in follicular fluid such as lipid peroxidation, total antioxidant capacity and superoxide dismutase activity are strongly correlated with oocyte fertilization and pregnancy rates following IVF (Pasqualotto et al., 2004). A decrease in their total antioxidant capacity may lead to oxidative stress. The combination of NSO with cyclophosphamide seemed to suggest that NSO may have contributed to the protection against follicular destruction induced by the alkylating agent. The anti-oxidative properties of NSO may have also protected the follicular cells from cyclophosphamide which is known to cause the over-production of ROS (Alenzi et al., 2010). Assessment of the total follicular count at different stages of growth revealed that the normality of the primary and secondary follicles in the three groups of cyclophosphamide co-treated with NSO were significantly higher than the control and cyclophosphamide-alone group. In agreement with this, the histological structures of the primary, secondary and graafian follicles derived from the groups of cyclophosphamide pre-

treated with NSO were more preserved than that achieved from cyclophosphamide-alone group. In contrast to our observations, Yadav and Agarwal (2011) reported that the ovarian follicles showed degenerative changes following administration of aqueous extract of Nigella Sativa. This different result is due to differences in the treatment regimen of which the rats were administered with high concentration of Nigella Sativa extract (200mg/kg body weight) for the duration of 40 days, without exposure to anticancer drug. There are controversial reports regarding the safety of Nigella Sativa. Its seed powder did not produce any toxic effects at high doses (28 gm/kg orally) in rabbits (Tissera et al, 1997); its oil was also safe when given orally to rats (LD50 of 28. 8ml/kg) (Zaoui et al., 2002) and oral thymoquinone was also found to be quite safe (LD50 of 2. 4 g/kg) (Badary et al, 1998). However, the LD50 of thymoquinone given intraperitoneally to rats or mice varies from 10mg/kg to 90. 3 mg/kg (Mansour et al., 2001). A study on oral and intraperitoneal LD50 of thymoquinone on rats noted the LD50 after oral ingestion and intraperitoneal injection were 57. 5mg/kg and 794mg/kg, respectively. Therefore, thymoquinone can be concluded as a safe compound when given orally to experimental animals (Al-Ali et al, 2008). Zaghlol et al. (2012) reported rats that received large doses of NSO (15 and 25ml/kg) for 1 month has toxic effects on the histological structure of the kidney and liver and therefore concluded that NSO should be used in proper doses. Nevertheless, other studies have demonstrated the antioxidant effects of the NSO and thymoquinone on the reproductive organs following toxicity exposure. A study by Kanter (2011) on the protective effects of thymoquinone on spermatogenesis demonstrated that pre-

treatment of 50mg/kg of thymoquinone was effective in improving the histological appearance in chronic toluene exposure in rats as well as preventing mitochondrial degeneration and dilatation of smooth endoplasmic reticulum. It was also found to prevent enlarged intercellular spaces in Sertoli and spermatogenic cells. Administration of NSO or thymoquinone in male albino rats was also found to limit the changes in haemoglobin, blood sugar, liver function, serum lipid profile and hepatic lipid peroxidation induced by cyclophosphamide treatment (Alenzi et al., 2010). Therefore, antioxidant can be used as a supplement to reduce the risk of oxidative stress and subsequent DNA damage. In conclusion, our morphological observation in correlation with morphometrical and follicular viability analysis showed cyclophosphamide exposure has induced some alteration on the fine structure of mature ovarian follicles with reduced number of PMF and higher total count of degenerated follicles. The results of the present work indicated that the administration of NSO prior to anticancer agent can reduce the toxicity through induction of antioxidant mechanism in the mature ovary. Thus, the integrity of mouse ovarian tissue affected by chemotherapy treatment can be well preserved till the senescent years making it a valuable natural product for the development in the field of assisted reproductive technique. ACKNOWLEDGEMENTThe authors are grateful to Mdm. Sri Viorwarti Binti Noerdin from Kulliyyah of Pharmacy, IIUM for the histology technical assistance. This work was supported by a grant from the International Islamic University Malaysia (IIUM) Research Endowment Fund.