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Predictive accuracy of anti mullerian hormone as indicator of ovarian follicle loss in cyclophosphamide treated mice

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Predictive accuracy of anti mullerian hormone as indicator of ovarian follicle loss in cyclophosphamide treated mice

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Abstract

Objective: To evaluate the strength of anti-mullerian hormone in reflecting the stages of ovarian toxicity-induced by cyclophosphamide.

Methods: This study was conducted in December 2014 and comprised female mice that were divided into four groups: group A served as control, group B received three weekly injections of cyclophosphamide, group C was co administered alpha-tocopherol along with cyclophosphamide, while group D solely received alpha-tocopherol. The ovaries were evaluated for follicular dynamics, and anti-mullerian hormone was assessed using mouse enzyme-linked immunosorbent assay kit. The data was analysed using SPSS 19.

Results: There were 40 mice in the study. Histological analysis revealed severely reduced ovarian reserve in group B($p<0.01$).In group C alpha-tocopherol conserved the ovarian reserve to near normal, thus follicle count was significantly higher than group B ($p<0.05$). However, this moderate reduction was still lower than the controls ($p<0.01$). Furthermore, the number of corpus lutea and atretic follicles were significantly higher in groups B and C ($p<0.01$). Regarding hormonal analyses in comparison to controls, anti-mullerian hormone levels were low in group B ($p<0.01$), while group C reported an insignificant fall in serum anti-mullerian hormone levels ($p=0.101$).

Conclusion: There was substantial evidence that anti-mullerian hormone monitoring during chemotherapy administration may fulfil the criteria of earliest diagnostic indicator of secondary infertility.

Keywords: Anti-mullerian hormone, Chemotherapy, α-tocopherol, Ovarian reserve, Cyclophosphamide. (JPMA 67: 1470; 2017)

Introduction

The incidence of infertility is increasing day by day throughout the world. Anti-neoplastic treatment is emerging as an added cause of secondary infertility along with numerous other reasons.[1] Advancements in cancer management have undoubtedly improved the survival rates but efforts to conserve quality of life even after chemotherapy administration is still under exploration. As these agents mainly act against proliferating cancer cells, it is not surprising to observe their toxic effects on normal growing cells such as bone marrow, gastric mucosa and ovarian follicles.[2] Chemotherapeutic drugs are broadly classified into three groups based on their ability to cause ovarian toxicity. Among these, the high-risk group is associated with an overall 80% chance of premature ovarian failure (POF) which mostly comprises alkylating agents such as cyclophosphamide (CYP), busulfan and chlorambucil.[3] POF is a leading cause of infertility and increases the risk for developing cardiovascular diseases, osteoporosis, Alzheimer's disease and colorectal or ovarian cancer at an earlier age.[4]

Nowadays, massive efforts are being made to preserve fertility in young women exposed to gonadotoxic chemotherapy. As the chemotherapeutic drugs cause destruction to ovaries, it leads to defective folliculogenesis as well as steroidogenesis.[5] Various biochemical and biophysical markers are evaluated to monitor ovarian reserve (OR) that helps to ascertain patients' state of ovarian toxicity. Unfortunately, traditional markers such as follicle stimulating hormone (FSH), luteinising hormone (LH), estradiol and inhibin B, all exhibit low sensitivity in the initial stages of OR damage.[6]

Recently, anti-mullerian hormone (AMH) has arisen as the best single marker of OR quantification.[7] Numerous researches have highlighted the clinical implications of AMH analysis, including potential role in polycystic ovarian syndrome diagnosis, estimation of age at menopause and prediction of ovarian response in assisted reproduction.[8] In females, this peptide hormone is exclusively secreted by the granulosa cells of early growing ovarian follicles. It influences the folliculogenesis by regulating initial selection of follicles, their growth and threshold for FSH sensitivity.[9] Furthermore, as serum AMH deranges earlier than FSH, efforts are being made to use it as a

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diagnostic marker of acute ovarian insufficiency due to cancer treatment.[10]

The current study was planned to evaluate the strength of AMH in reflecting various stages of ovarian insufficiency. To achieve this goal, we imitated three levels of ovarian toxicity in mice model. CYP chemotherapy was administered to conceive severely reduced OR while alpha (α)-tocopherol was co-administered to ameliorate its effect, creating moderate level of ovotoxicity. These findings were compared with controls that served as a model of normal reserve. Our results confirm that as opposed to other markers, serum AMH proportionately reflects the ovarian reserve in acute ovarian insufficiency.

Materials and Methods

This study was conducted in December 2014 at the Animal house, Ojha Campus Dow University of Health Sciences (DUHS). Ethical approval was obtained from the institutional review board of the Dow University of Health Sciences. Ten-week-old Naval Medical Research Institute (NMRI) strain female mice, weighing between 22g and 28g were included in this study. They were acclimatised for two weeks under normal circadian rhythm of 12 hours light and 12 hours dark to assess their state of health. The food and water was provided ad libitum. Diseased, pregnant or medically unfit animals were excluded on the bases of weight gain or loss.

The animals were divided into four equal groups. Group A received three weekly intraperitoneal dose of sterile water and served as controls. Group B received intraperitoneal CYP injection (Endoxane, Baxter Oncology GmbH, Frankfurt) at a dose of 75mg/kg body weight dissolved in sterile water, once a week for 3 weeks.[11] Group C received three weekly doses of CYP (75mg/kg body weight) along with daily oral dose of alpha-tocopherol (Evion, Merck, Pakistan) 150mg/kg. Group D received oral dose of alpha-tocopherol (150mg/kg) daily for three weeks by oral gavage.

Each animal was weighed at the start and on completion of the study. The mice were sacrificed under deep anaesthesia and dissected through midline abdominal incision, extending from xiphoid process to anterior pelvic wall.

Thoracic cage was retracted to collect blood via cardiac puncture. After centrifugation for 10 minutes at 3,000 revolutions per minute (rpm), the serum samples were stored at -20ºC. The serum AMH was evaluated by mouse enzyme-linked immunosorbent assay (ELISA) kit (USCN Life Science Inc., Cloud-Clone Corporation, United States).

The abdominal cavity was exposed and bi-cornuate uterus was followed to the gonads. Ovaries were dissected out from surrounding tissues and weighed on Sartorius make (MC 210 P) balance which had a minimum scale of 0.0l mg. The external features like colour, contour, consistency, vascularity and haemorrhagic necrosis were observed in the ovary of each animal. Only right ovary was evaluated to maintain uniformity and prevent any discrepancy as suggested by the literature.[11] Afterwards, the ovaries were preserved in Bouin's fluid for 12 hours followed by immersion in 70% ethanol saturated with lithium carbonate, to remove excessive picric acid. After processing, 5µm thick serial sections were prepared using rotary microtome. Every fifth section was stained with routine haematoxylin and eosin and the slides were mounted for further evaluation.

Around15-20 slides (every fifth section) were prepared for estimation of follicle count from each ovary. Blind manual counting was performed by a single observer to avoid biasness due to prior knowledge.[12] Only follicles having clearly visible nucleus within oocyte were counted to rule out the chance of recounting an oocyte. The slides were closely examined at 40x magnification and the number of various follicles was separately noted for each animal.

Data was analysed using SPSS 19. Mean ± standard deviation (SD) were reported for all measured variables. Comparison of means was made using one-way analysis of variance (ANOVA). For further investigation, Tukey's honest significant difference (HSD) test of multiple comparisons was employed to confirm the pair wise comparison at 5 % level of significance (95% confidence interval [CI]). $P \leq 0.05$ was considered statistically significant.

Results

There were 40 mice in the study. The effect of CYP was

Table: Multiple comparison of serum levels of anti-mullerian hormone amongst the groups.

Conditions	Conditions	p-value
Controls	CYP	<0.001*
	CYP+ tocopherol	0.07
	tocopherol	0.93
CYP	CYP+ tocopherol	0.01*
	tocopherol	0.002*
CYP+ tocopherol	tocopherol	0.74

*p <0.05 considered significant using Tukey post hoc analyses.
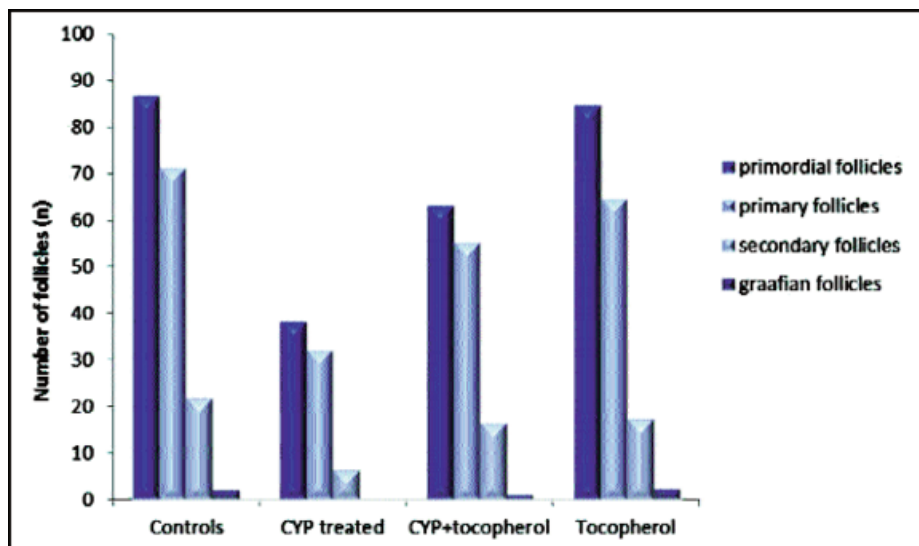CYP: Cyclophosphamide.

Figure-1: Graphical comparison between the numbers of follicles in groups.

CYP: Cyclophosphamide
AMH: Anti-mullerian hormone

Figure-2: Effective classification of study animals based on Serum AMH levels (ng/ml). Normal serum AMH values for mice were obtained from the experiment study of Kevenaar et al.[23]

difference amongst the four groups (p=0.072). The mean weight of ovaries in the control group A was 9.65±0.88mg, group B 8.62±0.83mg, group C 9.35±0.97mg, and group D 9.55±0.99mg.

In comparison to group A, group B reported the highest decline in the follicle reserve. The number of primordial, primary as well as secondary follicles were found to be significantly low (p<0.001). Regarding group C, the mean number of primordial and primary follicles showed significant reduction in comparison to controls (p<0.01), but the decline in the number of secondary follicles was insignificant (p=0.109). The follicular dynamics in group D were conserved to near normal (p=0.90). The mean number of total follicles in group A was 183.6±9.24, group B was 77.3±9.67, group C was 141.7±8.93 and group D was 170±12.97.

Furthermore, when compared with control group A, the largest number of atretic follicles was found in group B, followed by group C (12.5±2.55 and 10±4.11, respectively). This count was significantly lower in controls as well as group D (2.5±1.43 and 2.2±1.08, respectively). Similarly, corpora lutea was 2.3±0.94 in group A, 2.0±0.66 in group D, 5.7±1.88 in group B and 4.2±1.68 in group C (Figure-1).

Serum AMH in controls was 29.60±12.24 ng/ml, in group B it was 9.88±8.74 ng/ml, in group C it was 21.79±10.06 ng/ml and in group D it was 26.92±9.9 ng/ml (Figure-2).

Post hoc analysis revealed that in group B serum AMH was significantly lower in comparison to all other groups. The difference in serum AMH amongst controls and group C was insignificant (p>0.05). The serum AMH values in group D were comparable to controls (p=0.93) (Table).

observed on the morphology of ovaries of mice and was correlated with hormonal levels of AMH amongst the four groups.

Group A mice gained significant weight (p<0.05), whereas there was no weight change in group B. Group C reported an insignificant weight gain (p=0.34) and group D gained the weight similar to group A. Regarding the absolute weight of the ovaries, there was an insignificant
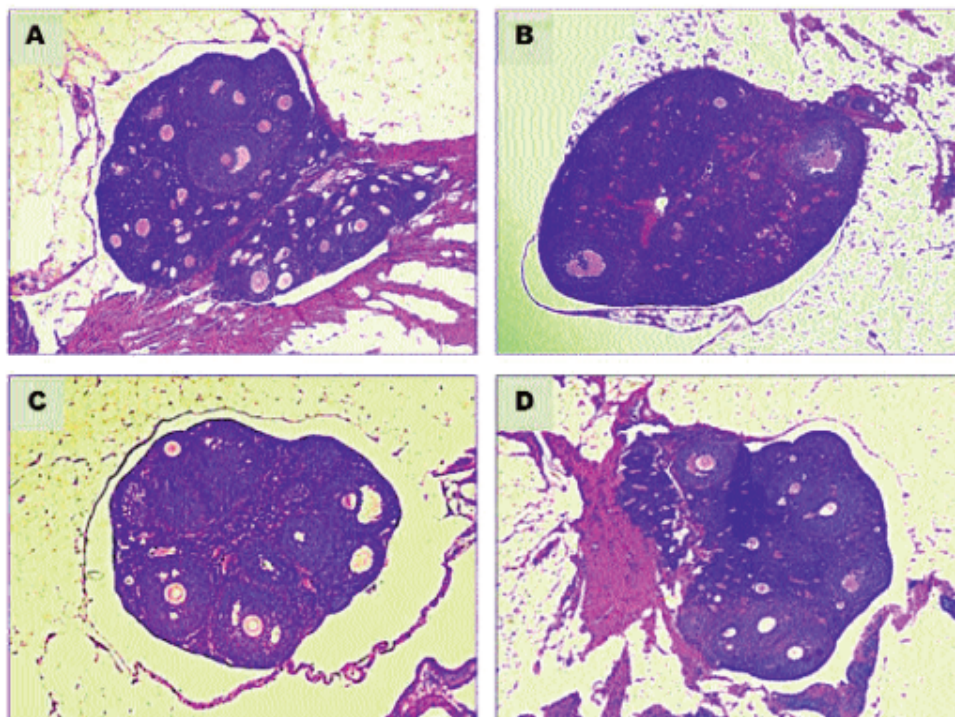
CYP: Cyclophosphamide

Figure-3: H & E stained photomicrograph at 10x magnification showing ovaries collected from various groups. Control group (A), CYP treated group (B), CYP + α-tocopherol treated group (C) and α-tocopherol treated group(D). The loss of contour nodularity and follicular pool in visible in B.

Discussion

This study evaluated the role of AMH in accurate reflection of OR in various stages of acute ovarian insufficiency. Hence, to create this model of ovotoxicity, we administrated CYP chemotherapy to mice.

In rodents, the effects of CYP have been observed at varied strengths, ranging from 40 to 300 mg/kg, in single or multiple dosages.[13,14] We administered three weekly doses (75mg/kg) to replicate model of human ovarian damage caused by multiple doses of chemotherapy. This non-sterilising dose of CYP created an ideal model to study the follicular and hormonal dynamics spanning over five to six estrous cycles. As the literature supported the protective role of α-tocopherol, we employed it against CYP-induced ovarian toxicity in group C to imitate moderate ovarian damage.[11] Aiming to exploit the potential impact of acute ovarian damage on follicular dynamics and serum AMH production, we were able to study three levels of OR; normal (group A and D), moderately decreased (group C) and severely decreased (group B).

In our study, gross morphological features of ovaries such

as colour, consistency and vascularity were found to be conserved. After treatment, there was an insignificant difference in weights of ovaries amongst the four groups. A slightly lower weight and loss of nodularity of ovaries was noticed in the CYP-treated group (Figure-3).

In agreement to previous data, we suggest that acute damage may not alter size or external features of gonads.[11] Therefore, the absence of gross changes suggests that the monitoring of ovarian volume is not an ideal marker to scan ovarian injury.

The crucial step to ascertain reproductive capability of ovaries is to ascertain the number of various follicles with cortex, especially, primordial follicles.[15] We manually counted the follicles to observe their dynamics. CYP administration affected entire cohort leading to drastic fall in group B. The greatest destruction was noticed on primordial and primary follicles, strongly predicting premature ovarian failure in the near future. Consumption of primordial follicles logically predicts a decline of all other growing follicles. As reported by Saleh HS, the primary and secondary follicles expresses highest mitotic index, thus are prone to deoxyribonucleic acid(DNA) cross-linking induced by anti-tumour drugs.[16] Our findings are consistent with the study of Yan Jiang et al., who reported upto 56% reduction in the number of primordial and primary follicles in CYP-treated mice.[17] Furthermore, we observed a rise in the number of atretic follicles and corpora lutea in group B. This suggests higher turnover of follicles that finally ends into atresia. With the administration of CYP that led to exhaustion of ovarian pool, we were able to create a model of severely reduced OR in vivo.

The protective role of α-tocopherol is well established in different biological systems such as maintenance of cell structure, ischaemia and reperfusion injuries.[18] It is effective against ovarian toxicity induced by chromium, carbon monoxide pneumo-peritoneum and

chemotherapies.[19] In order to utilise its ameliorating effect, we co-administered it with CYP in group C. In line with a previous study conducted by Seren Gulsen, we endorse that α-tocopherol conserves ovarian parameters, when co administered with CYP.[11] In common agreement to our results, he reported that most obvious protective effect was seen on early follicles. The secondary follicles were conserved to near normal as well. On the other hand, group C reported higher count of atretic follicles and corpora lutea in comparison to controls. This suggests that although α-tocopherol prevented follicles from CYP-induced destruction, there was high turnover of follicles from dormant to growing stage. Group C served as a useful model of moderate ovarian damage with a greater OR in comparison with group B. The protective role of α-tocopherol might be explained by the fact that it balanced excessively produced reactive oxygen species (ROS) under influence of CYP, conserving the cell architecture against free radical injury. While maintaining the balance, it has shown promising results in preserving the number of primordial, primary and secondary follicles; however, it has not been able to reduce the number of atretic follicles within CYP exposed ovaries.

As ovaries secrete a number of biomarkers, ideally its destruction is reflected by deranged hormonal levels. Regrettably, literature suggests normal levels of FSH or LH in acute ovarian injury.[20] As they become abnormal, the reduction in OR has already reached to such a critical level that any intervention to preserve fertility proves unsuccessful.[21] This gap prompted us to investigate AMH, a relatively new marker that may predict ovarian damage in time. We found that over a period of a month, CYP treatment led to a significant fall in the serum AMH, proportionate with the drastic decline in various follicles. Our results support the work of Kevenaar et al., who reported strong correlation between serum and primordial follicles.[22] It is worth mentioning that in group C serum AMH was significantly higher than group B ($p < 0.001$), however, it was lower than AMH levels obtained from controls ($p = 0.101$). To the best of our knowledge, our study reports the strength of AMH in reflecting protective effects of α-tocopherol against CYP, for the first time. We support the role of α-tocopherol in preserving normal reproductive physiology and likewise it advocates the role of AMH as an accurate maker of OR that has the potential to reflect acute injury to the gonads (Figure-2).

As there was no previous study on association between α-tocopherol administration and AMH, α-tocopherol was solely administered to group D and possible effects were noticed. The histo-morphologic analysis and ELISA confirmed that there was insignificant difference in the follicular profile or hormonal levels between controls and group D. Therefore, it could be inferred that α-tocopherol does not have a potential role in down-regulating AMH secretion. Moreover, there was mild variation in the levels of AMH recorded in mice amongst group A and D, but it can be explained by the fact that literature reports as much as ten-fold difference in reserve amongst animals of the same age group.[23] This explains the possibility of wider variation in serum AMH, truly depicting the ovarian reserve.

Our results are in line with a human study conducted by Yu B. et al. that reported a steady fall in the levels of AMH in cancer patients, treated with CYP. As the literature highlights that AMH is solely produced by the granulosa cells of early growing follicles.[24] and is devoid of any feedback loop from the hypothalamus-pituitary axis, it is emerging as an accurate indicator, devoid of inter-cyclic variations.[25] Furthermore, AMH is establishing as an early indicator of decreased OR, making it ideal for screening patients for timely referral to infertility clinics. Moreover, various occupational hazards such as metals (lead and mercury), solvents (toluene and glycol ethers), chemical exposures to anaesthetic gases and formaldehyde are assumed to be associated with a decline in fertility. Therefore, we propose that women exposed to such occupational hazards might be routinely evaluated for serum AMH, in order to screen ovarian insufficiency.

One limitation of our study included the use of animal model, as it was not feasible to collect the entire ovary from human samples to evaluate a comprehensive follicle profile.

Conclusion

There was substantial evidence that the monitoring of serum AMH may pave the way to screen high-risk patients who may progress to premature ovarian failure as a result of acute ovarian injury. Its monitoring may be employed for screening female workers against environmental and occupational hazards in various industries. Therefore, further studies on humans may ascertain the role of serum AMH evaluation as a suitable marker of ovarian insufficiency in numerous conditions.

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Conflict of Interest: None.

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