Serum anti-mullerian hormone: Correlation with the ovarian follicular dynamics in healthy mice

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Abstract
Objective: To evaluate the relationship between serum anti-Mullerian hormone and follicular dynamics in mice.
Methods: This experimental study was conducted in November, 2014 at the Dow University of Health Sciences, Karachi, and comprised laboratory-bred albino mice. They were sacrificed under anaesthesia and blood was collected via cardiac puncture to assess anti-Mullerian hormone while ovaries were collected for morphometric analyses. SPSS 19 was used for data analysis.
Results: There were 20 mice with a mean weight of 25±1.89 grams, while weight of the ovaries obtained from these mice was 9.6±0.92mg. The mean serum anti-Mullerian hormone was 29.89±9.7ng/ml. On average, there were 87.8±13.54 primordial follicles, 51.85±8.36 primary, 20.35±5.57 secondary, 11.30±3.38 early antral and 3.05 ± 1.27 late antral follicles (p<0.001; p=0.06).
Conclusion: Association of anti-Mullerian hormone with follicle dynamics reflected its role as a true ovarian reserve marker. Its assessment was of great significance in infertile women as well as young patients receiving chemotherapy.
Keywords: Anti-Mullerian hormone, Ovarian reserve, Follicular dynamics. (JPMA 66:1084; 2016)

Introduction
The quantity and the quality of primordial follicle within ovaries constitute the ovarian reserve (OR). Management of ovarian dysfunction requires accurate estimation of OR that assists in taking critical decisions, such as determination of best possible time and appropriate treatment protocols, in patients' counselling and for selection of cases for financial support through donations. The size and placement of primordial follicles within ovaries is such that direct assessment of their pool is not possible. Therefore, OR is typically estimated indirectly by evaluation of day 3 serum levels of follicle stimulating hormone (FSH), luteinising hormone (LH), inhibin B and estradiol. Decrement in the OR leads to a rise in the levels of FSH and a decrease in the levels of inhibin B and estradiol. There are limitations associated with the use of these hormones as indicators of OR; these include menstrual cyclic fluctuations, inter-dependence due to hypothalamic-pituitary feedback loop and relatively late alterations in their levels in response to OR decline.

Additionally, assessment of antral follicle count (AFC) by trans-vaginal ultrasonography is a consistent tool that indicates the relative number of primordial follicles, remaining within ovaries. In assisted reproductive technology (ART), AFC is considered the most reliable method to evaluate the response of ovarian stimulation. However, it requires sophisticated equipment and suffers from operator variability as well as mechanical inconsistency. Furthermore, it has higher intra- and inter-cycle variability. Thus, most researchers suggest assessment through numerous tools may increase the reliability of OR estimation tool to assess the follicle reserve.

In recent times, evaluation of anti-Mullerian hormone (AMH) has emerged as an invaluable addition to the board of OR biomarkers. It is a homodimeric glycoprotein, secreted by the granulosa cells of early growing follicles in the ovary. AMH belongs to transforming growth factor-β (TGF-β) family and its gene is located on chromosome 19p13.3. This hormone binds to the AMH type II receptor, a single transmembrane protein (TP) with serine-threonine kinase activity, expressed on target organs such as Mullerian ducts, Sertoli and Leydig cells of testis and granulosa cells of the ovary. In females, its earliest production is reported at 36th week of gestation. Since then, it gradually increases with a mild peak at puberty. The highest levels of AMH are reported between 23 to 25 years, corresponding to the most fertile era of a female. Its strength in accurately reflecting the number of growing follicles is narrated by the fact that AMH is found very high in polycystic ovaries and ovarian tumours. As a woman proceeds to menopause, serum AMH declines.
Researches have suggested that it can reflect this transition up to five years prior to finally attaining menopause. Furthermore, it does not exhibit inter- or intra-cyclic fluctuations, hence it is emerging as the only "acyclic marker" that can be accessed throughout the menstrual cycle. Currently, efforts are being made to establish AMH threshold values to classify ovarian stimulation responders, in ART clinics.

Despite multiple approaches to estimating OR with accuracy, direct assessment of primordial follicle pool that truly constitutes the reserve is considered as the best technique. The key limitation is that direct assessment of ovarian tissue on biopsy is challenging in infertile women attending ART clinics. The current study was planned to determine the relationship between serum AMH levels and dynamics of ovarian follicles using mouse model.

Materials and Methods
This experimental study was conducted in November, 2014, at the Dow University of Health Sciences (DUHS), Karachi, and comprised laboratory-bred albino mice. Ethical clearance was obtained from the institution review board (IRB) of the university. Adult BALB/c female mice, aged eight to nine weeks, weighing 24 to 28 grams, were included. The animals were kept in tagged cages, under standard laboratory conditions of 12 hours light and 12 hours dark. Pelleted form of laboratory diet and water was provided to the mice ad libitum. Animals were acclimatised for two weeks to assess the state of health based on weight gain or loss. Diseased, pregnant or medically unfit animals were replaced.

The animals were sacrificed under deep anaesthesia. Abdomen was dissected using a midline incision. Thoracic cage was retracted to expose the heart. Blood was collected via cardiac puncture and allowed to clot for 30 minutes, followed by centrifugation for 10 minutes at 3,000 rpm. Afterwards, the serum samples were stored in aliquots at -20°C. The serum AMH was evaluated by mouse enzyme-linked immunosorbent assay (ELISA) kit [USCN Life Science Inc. Cloud-Clone Corporation USA]. The minimum detection dose for AMH was 0.095ng/ml.

The abdominal cavity was exposed to locate the bicornuate uterus, followed laterally to reach the experimental organ. The ovaries were dissected out from surrounding tissues and weighed on Sartorius make (MC 210 P) balance. To maintain uniformity, only right ovary of each animal was collected. External features like colour, contour, consistency, vascularity and haemorrhagic necrosis were recorded. Later, the ovaries were fixed in formalin solution and processed for subsequent histological evaluation. The embedded tissue was cut into 5 µm thick serial sections and every fifth section was stained with haematoxylin and eosin (H&E) for morphometric analysis. The follicles were classified as primordial if the oocyte was surrounded by a single layer of squamous cells while primary follicle had a single layer of cuboidal cells. Secondary follicles possessed multiple layers of granulosa cells, without any antrum. Early antral follicles exhibited one or more small fluid filled antrum while large antral follicles possessed a single confluent antral cavity (Figure). Follicle counting was conducted by a single observer at 40x magnification. Data was analysed using SPSS 19. The number of follicles and serum AMH were represented as mean ± standard deviation (SD). Analysis was performed on the whole group of mice to assess correlation between hormonal levels and follicle count. P<0.05 was considered significant.

Results
There were 20 mice with a mean weight of 25±1.89 grams. The mean weight of the ovaries obtained from these mice was 9.6±0.92mg. On average, there were 87.8±13.54 primordial follicles, 51.85±8.36 primary, 20.35±5.57 secondary, 11.30±3.38 early antral and 3.05 ± 1.27 late antral follicles (Table).

AMH level was correlated with mean number of follicles in various stages of development: primordial follicles (r=0.82; p<0.001), primary (r=0.79; p<0.001), secondary (r=0.62; p=0.004), early antral (r=0.66; p=0.002) and late antral (r=0.41; p=0.065).

<table>
<thead>
<tr>
<th>Types of Follicles</th>
<th>Mean ±SD</th>
<th>Minimum count (n)</th>
<th>Maximum count</th>
<th>Correlation with AMH (r)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primordial</td>
<td>87.80 ± 13.54</td>
<td>65</td>
<td>115</td>
<td>0.82</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Primary</td>
<td>51.85 ± 8.36</td>
<td>37</td>
<td>66</td>
<td>0.79</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Secondary</td>
<td>20.35 ± 5.57</td>
<td>7</td>
<td>29</td>
<td>0.62</td>
<td>0.004*</td>
</tr>
<tr>
<td>Early Antral</td>
<td>11.30 ± 3.38</td>
<td>6</td>
<td>18</td>
<td>0.66</td>
<td>0.002*</td>
</tr>
<tr>
<td>Late Antral</td>
<td>3.05 ± 1.27</td>
<td>1</td>
<td>6</td>
<td>0.41</td>
<td>0.065</td>
</tr>
</tbody>
</table>

Data expressed as Mean ± S.D. Pearson correlation was used to assess coefficient. *p <0.05 considered significant.

AMH: Anti-Mullerian Hormone.
The correlation between the number of primordial follicles and developing follicles (the sum of primary, secondary, early antral and late antral follicles) was significant \( r=0.53; p=0.004 \).

**Discussion**

The OR reflects the capability of a female to achieve conception. A decline in OR is suggestive of initiation of menopause, representing an end of the reproductive period and is associated with an increased risk of conditions such as osteoporosis, cardiovascular disorders and Alzheimer's disease. Estimation of OR can be a critical indicator of capability of a female to achieve conception, and thus assist in the management of infertility.\(^{12}\)

AMH is an endocrine and a paracrine hormone that is secreted by and acts upon the ovarian follicles.\(^{13}\) In this study, the mean AMH level was recorded at 29.89±9.7 ng/ml. It was observed that amongst the cohort, hormonal level ranged from as low as 17.35ng/ml to as high as 49.20ng/ml. This highlights the fact that even in age-matched population there can be diversity in AMH level, reflecting a varied range of ovarian reserve. This supports the role of AMH as superior to the age of animal that is considered significant in reflecting true OR.

Kevenaar et al. reported normal range of AMH at 28.34±7.12ng/ml in healthy mice aged between 2 and 8 months.\(^{14}\) We also found similar mean levels of AMH.

When AMH levels were correlated with the ovarian follicle dynamics, we found that the number of primordial and primary follicles exhibited strongest positive correlation with the hormones (Table). Similar findings have been reported by other studies which, using immunohistochemistry (IHC),\(^{6}\) reported highest expression of AMH on small growing follicles (measuring between 4 and 8mm).

In clinical practice, assessment of AFC on transvaginal scans is often used to evaluate the OR. But due to the smaller size of primordial follicles, mere count of primary, secondary, early and late antral follicles is assessed to reflect the pool of follicles.\(^{15}\) As studies suggest strong positive correlation between the number of resting primordial follicles and growing follicles, AFC count is considered as a reliable tool to roughly estimate the OR.\(^{4}\) Our results were inline with these findings as we reported moderate positive correlation between primordial follicles and the total number of growing follicles \( r=0.53, p=0.004 \). Furthermore, we support evaluation of AMH along with AFC in increasing the predictability of OR, as one reflects primordial and primary follicles while the other represents early growing follicles (including primary, secondary as well as antral follicles).

Regarding ovarian physiology, AMH is considered as a factor that controls follicle recruitment and thus testified to preserves the overall OR.\(^{16}\) Studies on AMH null mice have reported that in the absence of AMH, an early consumption of follicular pool leads to exhaustion of the OR at a younger age.\(^{17}\) But little is known about the mechanism through which AMH decelerates the rate of follicle recruitment as during early reproductive life, as an increased AMH level has been reported even in the presence of a reasonable quantity of follicle recruitment.\(^{14}\)
Therefore, further studies are required to establish this inhibitory pathway and to then link AMH with a growth factor that may have inhibitory effect on primordial follicle recruitment.

Our study reported a strong relationship of AMH with the primordial follicles, thus supporting the existing evidences that suggest AMH as the earliest marker to predict menopause. A decline in count of primordial follicle pool is proportionate to the time of menopause in mammals as well as in humans.

This study also testifies that as the size of follicles increased, a steady decline in the correlation with AMH level was observed. The secondary and early antral follicles were found to have a lower correlation \((r=0.62\) and 0.66, respectively) in this experiment. This finding is in agreement with existing data that as the follicle comes under influence of FSH, a decline in the AMH secretion is witnessed. Conversely, in our study AMH levels revealed a slightly weaker correlation with secondary follicles in comparison to early antral follicles. This discrepancy may be further investigated using IHC. Interestingly, we found a statistically insignificant correlation \((r=0.41, \ p=0.065)\) between late antral follicles and AMH. This further strengthens our findings, thus inferring a decline in AMH secretion with each step of follicular development.

Currently, there is an ongoing debate in the literature regarding variation in AMH levels over the period of menstrual cycle. Numerous studies have concluded that AMH levels remain steady throughout the cycle while others suggest a mid-cycle AMH drop. It is possible that as the larger follicles are selected for ovulation, they lose the ability to secrete AMH. This is reflected by a drop in the hormonal levels corresponding to mid-luteal phase; but at that moment in time, other primordial and early growing follicles still keep on secreting AMH, thus conserving its level.

To study the follicle dynamics, we effectively used the mice model as genetically it closely resembles the human. Furthermore, similarity in endocrinology and reproductive physiology of mice to that of humans enables the use of mouse model as an effective and efficient model to explore the role of various reproductive markers. In mice, the oestrous cycle corresponds to the menstrual cycle while at the end of the reproductive life they enter a permanent vaginal phase termed anestrus that corresponds to the menopause. In addition, collection of ovarian samples from females is not feasible to estimate follicular count. Thus establishment of AMH correlation with follicular dynamics in mice and then its translation in clinical set-ups is a useful method to explore the role of AMH as a marker of OR in human samples.

**Conclusion**

AMH seemed to have a strong potential to be used as an indicator of ovarian follicles that constitute the OR. Monitoring of AMH levels can thus enable us to predict the follicular dynamics in conditions that may result in ovarian toxicity, like chemotherapy, radiotherapy or ovarian surgeries. Further studies to estimate AMH levels in such patients can be of great significance, particularly in women undergoing infertility treatment where accurate estimation of OR is critical to plan future interventions.

**Disclosure:** No.

**Conflict of Interest:** No.

**Funding Sources:** No.

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