Effect of Down-Regulation of FSH in the Presence of Müllerian Inhibitory Substance on Maturation of mice preantral follicles

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Abstract—The aim of the present study was to investigate the inhibitory and combined effect of MIS and FSH on in vitro follicle development. To determine the effect of MIS on the follicle and oocyte growth, the preantral follicles (diameter, 95 ± 5 μm) were cultured in the TCM-199 medium alone (control) and in the presence of different concentrations (100, 200, 400, 600, 800, 1000 and 1200 ng/ml) of MIS. In the presence of 800 ng/ml MIS, the follicle diameter increased from 113 to 159 μm (p<0.001) while, the survival rate showed an opposite and negative effect of MIS on the survival rate (25%) as compared to the control (28%), where p = 0.042 (p<0.01). While, oocyte maturation (23%) and GVBD rates (36%) increased significantly in the culture groups exposed to 800 ng/ml MIS as compared to the controls with 2% maturation and 9% GVBD rate (p<0.0001). To determine the effect of MIS and FSH, preantral follicles were cultured with 1) 800 ng/ml MIS, 2) 100 mIU/ml FSH and 3) 100 mIU/ml FSH + 800 ng/ml MIS. Decreased follicle diameter (171 μm) was seen in experiment 3) as compared 2)(190 μm). Oocyte maturation (28%) and GVBD rate (39%) increased significantly in the culture groups exposed to 800 ng/ml MIS + 100 mIU/ml FSH as compared to the controls (3% maturation, 9% GVBD; p<0.0001). MIS inhibits the effect of FSH on the growth and maturation of preantral follicle and enclosed oocytes in the in-vitro cultures.

Keywords—MIS, MIS-deficient, differentiation, mice, FSH, follicle, oocyte maturation.

I. INTRODUCTION

The complexity of the interrelation of the events that control oocyte growth and ultimate acquisition of developmental competence is under continuous investigation [1]. It is generally believed that follicular atresia. In fact, the default developmental pathway of preantral follicles is to undergo atresia [2]. To rescue these follicles to the preovulatory stage, the increase in circulating follicle stimulating hormone (FSH) during each cycle is crucial. According to the current understanding, FSH levels need to rise to a critical threshold concentration, which is achieved within the microenvironment of the follicle. This microenvironment is established by intra-ovarian growth factors, which can either augment or diminish FSH action [3, 4]. The precise interplay between intra-ovarian growth factors, ovarian feedback signals and gonadotropins results in the development and ovulation of a fixed, species-specific, number of follicles. These growth factors are also important for the early, hormone-independent, initial recruitment and growth of follicles. In particular, members of the TGFβ family have recently been shown to regulate follicle recruitment and oocyte maturation [5, 6].

One of the factors known to regulate initial follicle recruitment in mice is Mullerian inhibiting substance (MIS). MIS, a member of TGFβ family, is expressed in the ovary from the onset of primordial recruitment onwards in a similar pattern in women and mice [7]. MIS expression starts in the granulosa cells of primary follicles, goes to highest level in granulosa cells of preantral and small antral follicles and, gradually diminishes in the subsequent stages of follicle development [8]. MIS inhibits mouse, bovine and human follicle growth in vitro[9, 10], although conflicting results have been reported [11]. In addition to recruitment, MIS attenuates FSH sensitivity in mice[12]; albeit also for this role of MIS contrary results have been found [13]. Although it is believed that MIS reduces the FSH responsiveness of preantral and small antral follicles [4]. FSH is considered to be the fundamental driver of folliculogenesis. Elevated FSH levels in the early follicular phase stimulate recruitment and growth of preantral follicles [8].

Figure 1. Schematic diagram of the effect of MIS (AMH) on the follicle recruitment and selection.

As indicated in Figure 1, MIS attenuates the effects of FSH on growing follicles. In granulosa cell cultures, MIS inhibits the FSH-dependent induction of aromatase activity and LH receptor expression [14]. Thus; MIS is one of the
actors that indicate the size of the growing follicle pool. It controls the size of the pool by inhibiting both its growth (recruitment) and its decline (selection)[15].

The present study was designed, for the first time, to better characterize the nature and impact of MIS on the in-vitro maturation of Syrian mice preantral follicles and enclosed oocytes. In addition, a study of combined and comparative effects of MIS and FSH was also carried out.

II. MATERIALS AND METHODS

A. Chemicals and Hormones
FSH (HP Metrodin; Serono, Welwyn Garden City, UK) was prepared in un-supplemented culture medium and stored in aliquots at -20 °C until used to produce final concentrations of 0-200 mIU/ml. Müllerian inhibitory substance (MIS) was purchased from Sigma chemicals (USA). All other chemicals were of analytical grade or the highest quality commercially available.

B. Animal Selection and Follicle Isolation
Female Syrian mice were housed and bred in Central Animal House. Animals were kept under controlled conditions with 14:12 hour’s photoperiod, and fed with water and food pellets ad libitum. Six weeks mice were used for the isolation of follicle-enclosed oocytes [16]. Ovarian stimulation was performed when the mice weighed 11–16 g. Ovarian stimulation was done using an i.p. injection of 10 IU/mouse (PMSG) pregnant mare’s serum gonadotrophin (Organon, Oss, The Netherlands). The animals were killed by cervical dislocation after 44-48 hours of stimulation [17, 18]. The ovaries were removed aseptically and placed in Falcon plastic petri dishes (Falcon 3037, Becton Dickinson and Co., Rutherford, NJ) filled at room temperature with TCM199 (HEPES buffered, GIBCO BRL, Tokyo, Japan) medium, supplemented with sodium pyruvate (2 mM), glutamine (2 mM), BSA (3 mg/ml), penicillin G (75 μg/ml) and streptomycin (50 μg/ml) (all chemicals from Sigma Chemicals, Poole, Dorset, UK), 1% insulin, transferrin and selenium (ITS; Gibco, UK), respectively. Preantral follicles (95 ± 5 μm), with one or two layers of granulosa cells around the oocyte and an intact basal lamina with thecal cells, were mechanically isolated from the cortical slices under a dissecting microscope. Isolated follicles, containing a centrally located healthy oocyte and a thin layer of theca cells, were randomly selected in 5 ml supplemented TCM-199 medium, overlaid with 75 μl light mineral oil (Sigma) at 25-30 °C and incubated at 37 °C, 92 % humidity and 5% CO₂ in air for 6 days. The medium was refreshed by changing half of the quantity every other day.

C. Statistical Analysis
Maximum and minimum lengths (diameter) of each follicle were measured daily with an inverted microscope (IMT-2, Olympus Corp., Tokyo, Japan). Percentages were compared using ANOVA to determine the significant differences among the group means. Homogeneity of variation, between different treatment groups in one experiment, was also evaluated where \( p<0.05 \) was considered as statistically significant.

III. RESULTS
Morphological changes in the preantral follicles of immature mice were studied during a culture period of 6 days in the presence of 10, 25, 50, 75, 100, 150 and 200 mIU/ml FSH as shown in Figure 2.

![Figure 2](image1.png)

Figure 2. Effect of different concentrations of FSH on follicle diameter (µm) and survival rate (%). Preantral follicles with a mean diameter of 95 ± 5 µm were cultured for 6 days in TCM199 medium alone (control) and in the presence of 10, 25, 50, 75, 100, 150 and 200 mIU/ml of FSH. Follicle diameter and survival rate was checked every day and degenerated follicles were removed from the medium. \( n=30 \) (total number of follicles in each experiment).

Preantral follicles harvested with 10, 25, 50, 75, 150 and 200 mIU/ml of FSH did not show significant changes in follicle diameter, survival, GVBD and oocyte maturation rates as compared to control experiment (\( p \geq 0.05 \)), as shown in Figure 3. On the contrary, in the medium treated with 100 mIU/ml of FSH, a significant increase in follicular diameter (190 µm), survival rate (91%), GVBD (81%) and oocyte maturation (59%) rates was seen as compared to the control experiment (\( p<0.0001 \)).

![Figure 3](image2.png)

Figure 3. Effect of different concentrations of FSH on oocyte maturation and GVBD. Preantral follicles with a mean diameter of 95 ± 5 µm were cultured for 6 days in TCM199 medium alone (control) and in the presence of 10, 25, 50, 75, 100, 125, 150 and 200 mIU/ml of FSH. Rate of oocyte maturation and GVBD (germinal vesicle breakdown) was checked every day and degenerated cells were removed from the medium. \( n \approx 30 \) (total number of follicles in each experiment).
To determine the effect of MIS, the preantral follicles (90±5 µm) were cultured in the medium alone (control) and in the presence of different concentrations (100, 200, 400, 600, 800, 1000 and 1200 ng/ml) of MIS. In all the experimental groups, the addition of exogenous MIS caused a slightly increasing follicular growth patterns in a concentration-dependent manner, indicated by the slightly increasing curve of follicular growth in the presence of MIS (Figure 4). Growth pattern tended to increase up to 800 ng/ml but after this concentration, the diameter curve started showing the decline. Diameter increased from 113 µm to 159 µm (P < 0.001) while, the follicle survival rate showed an opposite and negative effect of MIS on the survival rate (25%) as compared to the control (28%), where p = 0.042 when control experiment was compared to MIS-experiment. These results show a negative effect of MIS on the survival rate of the growing follicles, as shown by the survival curve of Figure 4.

Figure 4. Effect of different concentrations of MIS on follicle survival rate and diameter during 6 days culture. Preantral follicles with a mean diameter of 95 ± 5 µm were cultured for 6 days in TCM199 medium alone (control) and in the presence of 100, 200, 400, 600, 800, 1000 and 1200 ng/ml of MIS. Follicle diameter and survival rate was checked every day and degenerated follicles were removed from the medium. n = 30 (total number of follicles in each experiment).

Histological observations were made using the follicles on day 6 of culture using control follicles and follicles treated with MIS (100, 200, 400 600, 800, 1000 and 1200 ng/ml). At the end of the 6-day culture period, the follicles were carefully opened with two fine needles and the cumulus-oocyte complexes (COCs) were morphologically evaluated. An oocyte complex with continuous and compact layers of cumulus cells was considered to be an intact cumulus enclosed oocyte complex. The GVBD and oocyte maturation was determined. Oocyte maturation (23%) and GVBD (36%) rates increased significantly in the culture groups exposed to 800 ng/ml MIS + 100 mIU/ml FSH as compared to the controls with 2% maturation and 9% GVBD rate (P<0.0001), as shown in Figure 5.

In both experimental groups, the addition of exogenous MIS caused a marked inhibition of FSH-stimulated preantral follicle growth in a time-dependent manner, indicated by the significantly smaller diameter (171 µm) of follicles cultured in the presence of MIS + FSH as compared to those cultured in the presence of FSH (190 µm) alone (Fig. 6). The follicle diameter with MIS (145 µm) was significantly less than that seen in the presence of FSH + MIS (159 µm, p<0.05), which but the combined effect of MIS + FSH was not significant as compared to the effect of FSH alone on the growth of follicles (P≥0.05), which indicates that the increase of diameter during MIS + FSH treated follicles was because of the growth enhancing effect of FSH. On the other hand, oocyte maturation (28%) and GVBD rate (39%) increased significantly in the culture groups exposed to 800 ng/ml MIS + 100 mIU/ml FSH as compared to the controls with 3% maturation and 9% GVBD rate (p<0.0001), as shown in Figure 6.
in the presence of 2) 100 mIU/ml of FSH, 3) 800 ng/ml MIS and 4) 100 mIU/ml of FSH + 800 ng/ml MIS. Follicle diameter, survival, oocyte maturation and GVBD rates were checked every day and degenerated follicles were removed from the medium. n = 30 (total number of follicles in each experiment).

IV. DISCUSSION

Several studies show that ovarian growth factors play an important role in initiation of primordial follicle growth [19]. In our study, it became evident that MIS affects the growth and survival of preantral follicles. In the present study, the effect of MIS on the preantral follicle recruitment was tested using the follicles of 6-week-old mice in an in-vitro culture system. The in-vitro system provides to be useful for the study of different factors that influence the follicular growth [20]. To exclude the possibility that the lower number of growing follicles found in MIS-treated cultures is caused by MIS-induced retardation of preantral follicle growth, we also looked at the maximal mean diameter that was reached in control and MIS-treated cultures [21, 8]. Because under MIS-treated experiment, the surviving follicles [25%] showed decreased number, we conclude that MIS has a negative effect on the follicle survival. This inhibition is probably due to a direct effect of MIS on the preantral follicles [8, 22].

We used two types of quantitative analysis of MIS-effect on the preantral stages of development, which was MIS effect on the follicle itself in terms of diameter and survival rate. While other factor was the MIS effect on the percentege oocyte maturation and GVBD rate. Interestingly, when the presence or absence of MIS was noted, the effect of the inhibition of FSH-activity was prominently seen [8]. Because the MIS expression may affect FSH sensitivity of follicles, MIS may play a role in determining whether follicles undergo selection or atresia. In the present study, we have observed a negative effect on the follicle survival in the MIS + FSH treated cultures [23]. A possible gradient of MIS expression within the follicle may reflect functional differences between the follicle and oocyte requirements. Moreover, because communication between the oocyte, follicular cells and the extra-cellular matrix all contribute to follicular development, alterations in gonadotropin stimulation may disrupt these processes. Additional studies are required to further elucidate such mechanisms. The first study to evaluate the effects of MIS on intact ovarian follicles showed that MIS treatment enhanced preantral Follicle grows in-vitro by increasing both follicle size and cell number [24]. This finding is consistent with the highest expression of MIS in populations of cells that are most rapidly dividing. In addition, MIS promoted the follicle growth (diameter) without enhancing the survival. This may have a long-lasting effect; when a follicle stimulated by MIS eventually differentiates, it will be larger and contain more granulosa cells and thus have a greater capacity for producing estrogen and angiogenic factors, which in turn should provide a competitive advantage compared with other follicles in its cohort. Therefore, it may be expected that the inhibitory effect of MIS on the FSH-treatment, in the present study, would be to decrease MIS expression in growing follicles, because these treatments ultimately result in arrested follicular development [24-26]. It should be noted, however, that another study has shown that MIS inhibits FSH-simulated development of murine preantral follicles in-vitro [23]. In contrast, in the male, administration of recombinant FSH to pre-pubertal mice results in an increase in testicular MIS output [26].

The negative effect of MIS on the FSH stimulation may be a down-regulation of MIS expression in preantral, either directly as a result of a shutdown of gonadotropin stimulation or via a disruption in paracrine interactions between neighboring follicles. As previously stated by our research group, activin A and FSH regulate the effect of each other and also have a positive influence on the follicle and oocyte growth and maturation. It can be thought that MIS affects such paracrine factors, which in turn leads to a decreased follicle survival rate in the presence of the MIS + FSH combination [19]. However, it is also possible that in the absence of MIS, the follicles display an increased sensitivity towards FSH [9]. These finding are in accordance with the observations of in-vitro study of Visseret al., (2006) that FSH dependent follicle growth of cultured mouse preantral follicles was inhibited by MIS [21]. Nevertheless, MIS showed an increased maturation capacity of oocytes and GVBD rate, which was not significant as compared to the cultures grown in the presence of FSH alone. These findings show that the decreased follicle survival in the MIS-treated follicles is not dependent on the oocyte maturation.

In an in-vitro follicle culture, we found that MIS inhibits FSH-stimulated growth of mouse preantral follicles. However, in another in vitro study [13], it was reported that MIS enhanced FSH-stimulated growth of rat preantral follicles. Two possible explanations for these contradictory results can be put forward. Animal age (prepubertal vs. adult) could be the cause. More insight into the signaling pathway of MIS will be gained after identification of the MIS type I receptor [26]. The inhibitory mechanism could involve an effect of MIS on FSH receptor expression. A change in the expression of the FSH receptor may change the sensitivity of a follicle to FSH, as was demonstrated by studies in the bovine. However, several studies have shown that, besides FSH effects on the follicles, MIS can also inhibit similar effects induced by cAMP, which is the second messenger of FSH. This would suggest that the molecular target site of MIS action is downstream of the FSH receptor [25].

V. CONCLUSIONS

Our results suggest that MIS plays a role in fine-tuning the balance of follicle survival and death, either directly or indirectly through the regulation of other factors. A better understanding of the process of follicle recruitment might provide tools to control early folliculogenesis, which in the long run might help to preserve ovarian function in women at risk for premature ovarian failure, such as young women treated for childhood cancer. Furthermore, our results suggest that MIS contributes in determining the FSH threshold levels required for cyclic follicle recruitment. Increased knowledge of the mechanisms underlying cyclic recruitment of the Syrianmice preantral follicles may prove to be useful in the future in-vitro maturation studies.
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REFERENCES


