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Serum anti-Mullerian hormone predicts ovarian response in (*Macaca fascicularis*) monkeys

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Abstract

AMH as a promising predictor of ovarian response has been studied extensively in women undergoing assisted reproductive technology treatment, but little is known about its prediction value in monkeys undergoing ovarian stimulation. In the current study, a total of 380 cynomolgus monkeys ranging from 5 to 12 years received 699 ovarian stimulation cycles. Serum samples were collected for AMH measure with enzyme-linked immunosorbent assay. It was found that serum AMH levels were positive correlated with the number of retrieved oocytes ($P < 0.01$) in the first, second and third stimulation cycles. In the first cycles, area under the curve (ROC_{AUC}) of AMH is 0.688 for low response and 0.612 for high response respectively, indicating the significant prediction values ($P = 0.000$ and $P = 0.005$). The optimal AMH cutoff value was 9.68 ng/mL for low ovarian response and 15.88 ng/mL for high ovarian response prediction. In the second stimulation cycles, the significance of ROC_{AUC} of AMH for high response rather than the low response was observed ($P = 0.001$ and $P = 0.468$). The optimal AMH cutoff value for high ovarian response was 15.61 ng/mL. In the third stimulation cycles, AMH lost the prediction value with no significant ROC_{AUC} . Our data demonstrated that AMH, not age, is a cycle-dependent predictor for ovarian response in form of oocyte yields, which would promote the application of AMH in assisted reproductive treatment (ART) of female cynomolgus monkeys. AMH evaluation would optimize candidate selection for ART and individualize the ovarian stimulation strategies, and consequentially improve the efficiency in monkeys.

Key Words

- ▶ anti-Mullerian hormone
- ▶ prediction
- ▶ number of retrieved oocytes
- ▶ ovarian response
- ▶ cynomolgus monkeys

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Introduction

Due to the similarities to human beings in reproductive physiology and endocrinology, nonhuman primates have been served as an ideal model for human reproductive biology, gynecology and other diseases (1, 2, 3), especially the cynomolgus monkeys with no seasonal breeding. To date, assisted reproductive technology (ART) was routinely applied in nonhuman primates to produce *in vitro*-derived embryos and offspring, which

largely advanced the research of somatic cell nuclear transfer and the embryo stem cell establishment (4, 5). These achievements were dependent on an amount of oocytes retrieval in ovarian stimulation. However, ovarian stimulation regimen in nonhuman primates has not been set up as well as human beings, and the high cost of medicine used also limited the availability of large number of oocytes. Regarding ovarian stimulation,

the efficiency of ART in nonhuman primates has the great potential to improve.

In controlled ovarian stimulations, the characteristics, such as age, menstrual cycle length and results from previous *in vitro* fertilization (IVF) cycles are generally considered for ovarian stimulation strategies. Apart from these factors, several ovarian markers, such as antral follicle count (AFC) (6), estradiol concentration (7), basal FSH (8), inhibin and AMH (9), have been used to estimate the ovarian response to tailor ovarian stimulation protocols in human beings (10). As for the advancement in clinical practices to obtain satisfactory results, it is valuable to evaluate ovarian response to gonadotropin before ART in monkeys.

AMH is a homodimeric glycoprotein and secreted from preantral and small antral follicles as recruited from the follicle pool in females (11, 12). Thus, it is considered as the reliable marker of ovarian reserve, reflecting both the quantity and the quality of the resting primordial ovarian follicle pool (13). Both woman and female monkeys have the similar AMH expression pattern, with a peak during the fertility period and becoming undetectable around the menopause (14, 15, 16). Further, AMH exhibited lower inter- and intra-cycle variability (17, 18), which make it a better predictor of ovarian response in IVF cycles than both AFC and FSH level (10). To date, AMH is widely used in prediction of ovarian response and clinical outcomes in humans (19) and other species, such as cow (20), sheep (21) and goats (22). However, the role of AMH in prediction of ovarian response in monkeys has not been uncovered.

Considering the great importance of nonhuman primate models and promising applications of AMH in ART, its predictability in monkeys deserved to be assessed. The present study aimed to explore the correlation between serum AMH levels and the number of retrieved oocytes after ovarian stimulation and to determine the role of AMH as a predictor for the ovarian response in monkeys undergoing IVF cycles, which will help to individualize and optimize the ovarian stimulation regimen in nonhuman primates.

Materials and methods

Animals

A total of 380 cynomolgus monkeys and 699 cycles were included. Animals were maintained in social groups and housed in outdoor compounds with attached indoor

quarters. The use and care of animals complied with the guideline of the Biomedical Research Ethics Committee at the Shanghai Institutes for Biological Science (CAS) under the approval application entitled 'Reproductive physiology of cynomolgus monkeys and establishment of transgenic monkeys' (#ER-SIBS-221106P). They had menstrual cycles between 22 and 36 days and had no hormone exposure during the last 2 months before testing AMH levels. Our data showed that ovarian stimulation had no effect on AMH levels. No significant differences were observed in serum AMH levels among the groups receiving different times of ovarian stimulation cycles at given age (Supplementary Fig. 1, see section on [supplementary data](#) given at the end of this article, $P>0.5$).

Ovarian stimulation treatment and oocyte retrieval

Ovarian stimulation was conducted as the routine protocol. The initial dose of 15–30 IU FSH (Recombinant Human Follicle Stimulating Hormone; Merck Serono SA Aubonne Branch, Co.) was administered twice a day from menstrual cycle day 3. Ovarian sizes of monkeys were monitored by B-scan ultrasonography from menstrual cycle day 10 onward. 1000–1500 IU HCG (chorionic gonadotropin human; SIGMA-ALDRICH, Co.) were used to trigger ovulation when more than ten follicles were larger than 3 mm in diameter. 33–38 h after hCG administration, monkeys were anesthetized with tiletamine hydrochloride (4 to 6 mg/kg, Zoletil 50; Virbac S.A.) intramuscularly, and cumulus-oocyte complexes were collected by laparoscopic follicular aspiration.

AMH assay

Blood was withdrawn via an intravenous catheter and transferred into tubes for centrifugation. All serum was isolated from blood samples and was measured using an ELISA kit according to the manufacturer's instructions (AMH ELISA, AL-105, Ansh Labs, USA) (15, 16). The assay was validated by demonstrating parallelism between the standard curve and the serially diluted monkey serum samples. High and low AMH calibrators were run in all assays and the values were within the assay's acceptable ranges. The range of the AMH standards used in the assay was from 0.09 to 16.4 ng/mL. The intra-assay coefficients of variation were less than 8%, and inter-assay coefficient was less than 12%.

Statistical analysis

One-way ANOVA and *t* tests were used to compare the differences in three and two groups respectively. Pearson correlation coefficients were performed to examine the associations among serum AMH, age and oocyte outcome in different stimulation cycles. Receiver operator curves (ROC) were used to analyze the sensitivity and specificity of AMH at different levels in predicting low ovarian response (number of retrieved oocytes ≤ 14) and high ovarian response (number of retrieved oocytes ≥ 51) respectively (about 10% of the cohort was defined as the low or high responders in our study). High and normal response data were used for cutoff values analysis for high response, and low and normal response data were used for cutoff values analysis for low response. Significance was assigned at $P < 0.05$. All analyses were performed using SPSS software package (24.0) or SigmaPlot (10.0).

Results

Characteristics and oocyte retrieval

A total of 380 female cynomolgus monkeys were included in this study. The statistical characteristics of age, AMH and oocytes retrieved from 699 ovarian stimulation cycles were categorized according to the number of cycle times and summarized in Table 1. Both age and serum AMH levels were similar among the three different stimulation cycles ($P = 0.089$, $P = 0.706$, respectively, Table 1). There was no significance in the number of retrieved and metaphase II (MII) oocytes between the first and second stimulation cycles and also between the second and third cycles (Table 1). Compared with the first cycles, the significant decline in the number of retrieved oocytes rather than metaphase II was observed in the third cycle (33.6 ± 20.5 vs 27.6 ± 18.9 , $P < 0.01$, Table 1).

Correlations of age, AMH and oocyte outcomes

To determine the effect of ovarian stimulation on AMH level, samples from the monkeys who received repeated ovarian stimulation were analyzed. There was no difference observed in AMH levels among different age groups from 5 to 12 years. In the same age section, no significant differences were observed in AMH levels among the groups undergoing different times of ovarian stimulation (Supplementary Fig. 1), indicating that AMH was relatively stable across several stimulation cycles. Then, Pearson correlation analysis was conducted to examine the relationships of AMH and age in the total participants. Our data revealed that AMH levels were positively correlated with age ($r = 0.151$, $P = 0.002$, Table 2).

Univariate analysis showed that both the number of retrieved oocytes and MII oocytes had no relationships with age ($P > 0.05$, Table 2) in all stimulation cycles, but showed a positive correlation with AMH level in the first and second stimulation cycles ($P < 0.05$, Table 2). In the third cycle, although there also was a positive correlation between AMH levels and the number of retrieved oocytes ($r = 0.316$, $P = 0.009$), the number of MII oocytes showed no significant correlation with AMH ($r = 0.211$, $P = 0.083$, Table 2).

AMH for prediction of low and high ovarian response

ROCs were plotted to depict the predictive potential of AMH in low and high ovarian response in stimulation cycles. The area under the curve of ROC (ROC_{AUC}) exhibited the ability of AMH levels for predicting the high and low response in the first cycles, and the high response in the second cycles ($P < 0.05$), while no significant ROCAUC was observed in the third cycles (Table 3). The sensitivity and specificity of AMH at different levels were analyzed in predicting value. The cutoff values were determined as well.

Table 1 Characteristics of age, AMH and oocyte retrieval in different cycles.

	First cycle ($n = 380$)	Second cycle ($n = 251$)	Third cycle ($n = 68$)	P value
Age (years)	8.2 ± 2.5	8.4 ± 2.4	8.9 ± 2.6	0.089
AMH (ng/mL)	15.3 ± 5.0	15.5 ± 4.8	15.8 ± 4.4	0.706
Retrieved oocytes	33.6 ± 20.5	31.3 ± 12.3	$27.6 \pm 18.9^{##}$	0.048*
MII oocytes	13.2 ± 10.9	12.3 ± 10.6	10.4 ± 10.0	0.112

Values are presented as mean \pm s.d. P value by one-way ANOVA. * $P < 0.05$. Compared with the second cycle, $^{##}P < 0.001$. AMH, anti-Mullerian hormone; MII, metaphase II.

Table 2 Correlation analyses among age, AMH, the number of retrieved oocytes and metaphase II oocytes in different ovarian stimulation cycles.

	Age (years)		AMH (ng/mL)	
	Pearson correlation coefficient (<i>r</i>)	<i>P</i> value	Pearson correlation coefficient (<i>r</i>)	<i>P</i> value
Age (5–12 years)		–	0.151	0.002**
First cycle (<i>n</i> =380)				
No. of retrieved oocytes	–0.028	0.583	0.344	0.000**
No. of MII oocytes	–0.062	0.231	0.262	0.000**
Second cycle (<i>n</i> =251)				
No. of retrieved oocytes	–0.034	0.594	0.259	0.000**
No. of MII oocytes	0.020	0.753	0.179	0.004**
Third cycle (<i>n</i> =68)				
No. of retrieved oocytes	–0.219	0.072	0.316	0.009**
No. of MII oocytes	–0.160	0.192	0.211	0.083

Pearson correlation analysis was performed. ***P*<0.01. AMH, anti-Mullerian hormone; MII, metaphase II.

AMH cutoff values in the first stimulation cycles

The ROC curves of AMH concentrations predicting female monkeys with low and high ovarian response in the first cycles compared with normal ovarian response are showed in Fig. 1 and Table 3. ROC_{AUC} of AMH is 0.688 for low response (95% CI: 0.604–0.771) and 0.612 for high response (95% CI: 0.541–0.683) respectively, indicating a useful potential for prediction (*P*=0.000 and *P*=0.005, Table 3). The optimal AMH cutoff value predicting low and normal ovarian was 9.68 ng/mL (sensitivity: 45.5%, specificity: 87.8%), and high and normal ovarian was 15.88 ng/mL (sensitivity: 66.7%, specificity: 52.9%).

AMH cutoff values in the second stimulation cycles

The ROC curves of AMH concentrations predicting female monkeys with low and high ovarian response in the second cycles compared with normal ovarian response are showed in Fig. 1 and Table 3. The ROC_{AUC} of AMH is 0.535 for low response (95% CI: 0.435–0.636) and 0.673 for high response (95% CI: 0.584–0.784) respectively. The significance of ROC_{AUC} of AMH for high response rather than the low response was observed in the second cycles (*P*=0.001, and *P*=0.468, respectively, Table 3), indicating the potential predictive utility. The optimal AMH

cutoff value for high ovarian response was 15.61 ng/mL (sensitivity: 82.4%, specificity: 54.3%) compared with the normal response.

Discussion

This study represents the first published report of AMH prediction of ovarian response in monkeys undergoing ovarian hyperstimulation. Our data demonstrated a positive association between serum AMH and the number of retrieved oocytes in 699 stimulation cycles of cynomolgus monkeys ranged from 5 to 12 years (*P*<0.01). Allowing for chronologic age, AMH can be regarded as a significant predictor of poor and high ovarian response in the first stimulation cycles and high ovarian response in the second cycles (*P*<0.001), but lost the prediction role of low ovarian response in the second and both responses in the third stimulation cycles from ROC analysis. It is possible due to the decrease in the number of retrieved oocytes after repeated cycles, but AMH is still maintained stably between the cycles shown in our data.

Regarding the ovarian response in repeated cycles, there are disagreements in the results reported. Some studies showed that the ovarian responses were similar in consecutive cycles (23), while others reported that ovarian

Table 3 Potential of AMH to predict low and high response in different ovarian stimulation cycles.

Cycle	Low response			High response		
	ROC _{AUC}	95% CI	<i>P</i> value	ROC _{AUC}	95% CI	<i>P</i> value
First	0.688	(0.604, 0.771)	0.000***	0.612	(0.541, 0.683)	0.005**
Second	0.535	(0.435, 0.636)	0.468	0.673	(0.584, 0.761)	0.001**
Third	0.642	(0.499, 0.784)	0.077	0.618	(0.418, 0.818)	0.102

P value means significance of ROC_{AUC} compared with 0.5. ***P*<0.01. ****P*<0.001. AMH, anti-Mullerian hormone

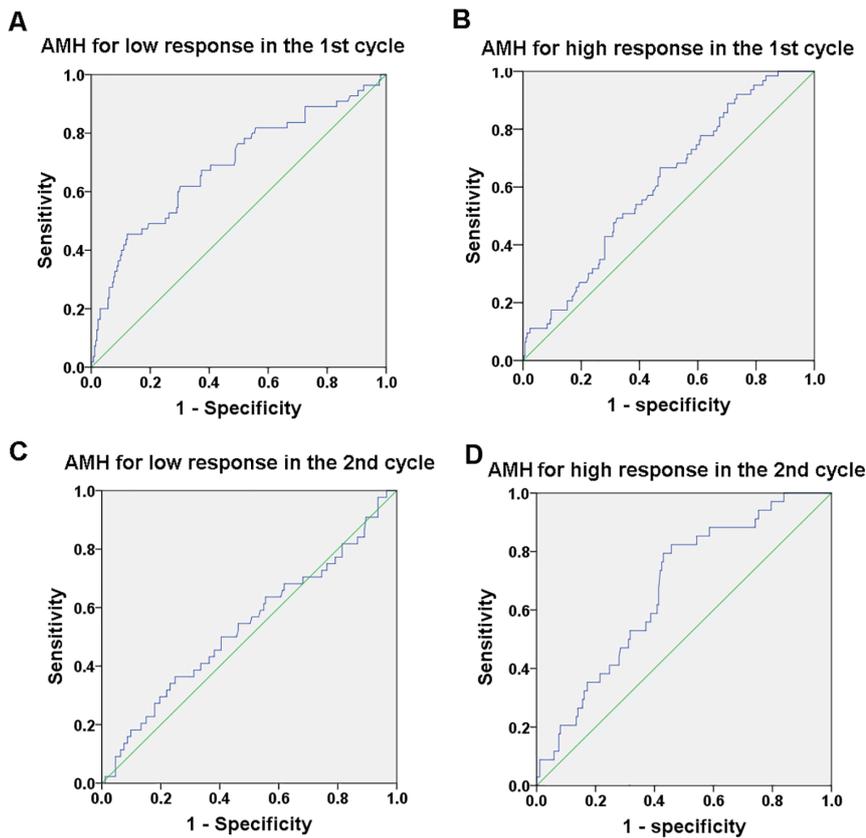


Figure 1
AMH receiver operator characteristic (ROC) curves for low and high ovarian response predictions in the first and second cycle respectively.

stimulations led to the decrease of the retrieved oocytes in rhesus monkey (24). Our data showed that, there was a decline in the number of retrieved oocytes with the growing stimulation accounts, which consequently led to the different predictability of AMH in the first, second and third stimulation cycles. The decrease of oocyte yields was also observed in rhesus monkeys undergoing repeated ovarian stimulation (24). It is proved that repeat ovarian stimulations only altered the expression of proteins in ovaries, but not ovarian structure and function (25), which might be the reason why AMH level was maintained stably as indicated in our data.

Age is one of the traditional factors used for measuring the ovarian reservation in humans (10, 26). With age, ovarian reservation falls to a critical level, finally leading to menopause. As well, nonhuman primates have the similar characteristics (15). In the cohort of cynomolgus monkeys during the primary fertility period, age showed the positive correlation with AMH levels, but had no significant correlation with the number of retrieved oocytes observed in our data. Even though age can partially reflect ovarian aging related to the decline of the quantity and quality of the follicle pool, it is not an appropriate predictor in ovarian response in monkeys that was also

proved in humans (10). In addition, the comparison of predictive value between AMH and other ovarian markers, such as AFC and FSH, has not been performed in the current study due to lack of strict hormone monitor as in humans during ovarian stimulation, which limited the acknowledgement of other ovarian response predictors in monkeys.

Using cutoffs of AMH to select appropriate candidates for ovarian stimulation will improve the efficiency of ART in monkeys. Definitely, the cutoff values with high sensitivity and specificity can only be considered as screening markers. In the first stimulation cycles, the AMH cutoff value was 9.68 ng/mL for predicting low ovarian response with a specificity of 87.8% and a sensitivity of 45.5%. It is clear that AMH had a modest sensitivity for poor response, and thus, it would include the candidates with poor response for ovarian stimulation. The higher cutoff values than 9.68 ng/mL should be considered for exclusion of false negative ones in practice. On the other hand, the individual adjustment of the dose of gonadotropins in poor responders is also effective in increasing the success rate of ovarian stimulation. In regard to high response, the cutoff values of AMH were similar in the first and second stimulation cycles,

while had different sensitivity and specificity. Considering the potential risk of ovarian hyperstimulation syndrome (OHSS) in primates, the high sensitivity was preferable.

It is well known that AMH levels, as a marker of severity of ovarian dysfunction, are elevated up to two- to three-fold in PCOS patients (27). PCOS is the most common cause of infertility, and the occurrence reached 6–10% in women of reproductive age (28). The PCOS like status is believed to be existing in an unselected cohort of cynomolgus monkeys during fertility period. Thus, high AMH level, especially much higher than the cutoff value for high ovarian response should be ill advised for ovarian stimulation, due to the potential bad quality of retrieved oocytes and risk of OHSS.

Taken together, serum AMH can be used as a cycle-dependent predictor of ovarian response in cynomolgus monkeys treated with ovarian stimulations. It will provide the reference criteria for candidate selection for ART in monkeys. The optimal AMH cutoff values gained in this study will promote the individualization and optimization of ovarian stimulation regimen and therefore improve the efficiency of ART in cynomolgus monkeys.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/EC-18-0189>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Hui Long, Yanhong Nie, Li Wang and Miaomiao Jia participated in experiment execution and manuscript drafting; Yong Lu and Yan Wang participated in experiment execution and data analysis; Yijun Cai and Zhen Liu participated in data analysis and critical discussion; Qifeng Lyu, Yanping Kuang and Qiang Sun participated in study design and critical discussion.

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