

Serum Concentrations of Anti-Müllerian Hormone and its Expression in the Remnant Ovarian Tissue of Rats with Experimentally Induced Ovarian Remnant Syndrome

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Abstract

Anti-Müllerian hormone (AMH) is synthesised in the Sertoli cells of the testes and granulosa cells of the ovary. As the ovaries seem to be the primary source of AMH, it may be used for determination of the presence or absence of ovaries or ovarian remnants in mammals. The purpose of the present study was to compare the serum AMH concentration of rats with experimentally induced ovarian remnant syndrome and the expression of AMH in the ovarian tissue removed during ovariectomy and remnant ovarian tissue. A total of eighteen Sprague Dawley rats were used in the study. Group I consisted of 6 rats that were gone through ovarian remnant syndrome (ORS) experimentally, group II consisted of 6 rats in which both ovaries were removed and group III consisted of 6 rats that were sham-operated. Median laparotomy was performed in the all groups under general anaesthesia. AMH mRNA expression was determined using Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). AMH mRNA expression levels in group I were decreased on day 30 after surgery when compared to day 0 ($P>0.05$). Mean concentration of serum AMH on day 10 after surgery in group I, II and III were found 2.27 ± 0.52 ng/ml, <0.312 ng/ml and 3.96 ± 0.53 ng/ml, respectively ($P<0.05$). In conclusion, this finding suggests that evaluation of serum AMH concentration could be an useful method to determine the presence or absence of ovaries or ovarian remnants in the rat.

Keywords: Anti-Müllerian hormone, Ovarian remnant syndrome, Rat

DeneySEL Olarak Ovaryum Kalıntısı Sendromu Oluşturulan Ratlarda Serum Anti-Müllerian Hormon Konsantrasyonu ve Kalıntı Ovaryum Dokusunda AMH mRNA Ekspresyonu

Özet

Anti-Müllerian hormon (AMH), testislerde Sertoli hücrelerinden ve ovaryumlarda granuloza hücrelerinden sentezlenmektedir. Anti-Müllerian hormonun ovaryum kökenli olmasından dolayı, memelilerde ovaryum varlığının veya kalan ovaryum dokusunun tespit edilmesinde kullanılabilir. Bu çalışmanın amacı, deneysel olarak ovaryum kalıntısı sendromu oluşturulan ratlarda serum AMH konsantrasyonları ve ovaryohistektomi ile uzaklaştırılan ovaryum dokusunda ve kalan ovaryum dokusundaki AMH gen ekspresyonlarını karşılaştırmaktır. Çalışmada toplam 18 Sprague Dawley rat kullanıldı. Deneysel olarak ovaryum kalıntısı sendromu oluşturulan 6 rat grup I'yi, her iki ovaryumu da uzaklaştırılan 6 rat grup II'yi ve sham operasyonu yapılan 6 rat ise grup III'ü oluşturdu. Bütün gruplarda genel anestezi altında median laparotomi gerçekleştirildi. AMH mRNA ekspresyonu kantitatif gerçek zamanlı polimeraz zincir reaksiyonu (qRT-PCR) ile ölçüldü. Operasyon sonrası 30. günde, 0. güne kıyasla AMH mRNA ekspresyon düzeylerinde azalma oldu ($P>0.05$). Operasyondan 10 gün sonra ortalama AMH değeri grup I, II ve III'de sırasıyla 2.27 ± 0.52 ng/ml, <0.312 ng/ml ve 3.96 ± 0.53 ng/ml olarak bulundu ($P<0.05$). Sonuç olarak, çalışmanın bulgularına göre serum AMH konsantrasyonunun, ratlarda ovaryum varlığının veya kalıntı ovaryum dokusunun tespit edilmesinde kullanılabilir bir yöntem olduğu belirlendi.

Anahtar sözcükler: Anti-Müllerian hormon, Ovaryum kalıntısı sendromu, Rat



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INTRODUCTION

Anti-Müllerian hormone (AMH), named additionally Müllerian inhibiting substance is a member of the transforming growth factor- β (TGF- β) superfamily and is synthesised in the gonads of mammals [1-3]. AMH is produced from the Sertoli cells of the testes and granulosa cells of preantral and small antral follicles of the ovary particularly in the layer nearest to the oocyte whereas its expression is absent when follicles become atretic [4-8]. Durlinger [1] showed that AMH inhibits the initiation of primordial follicle growth in anti-Müllerian hormone-deficient female mice. Thus, it is thought that serum AMH concentration represents the size of the small antral follicles, the number of residual primordial follicles or the ovarian reserve in both women [9,10] and rodents [4,5,8,11].

In rats, no alterations was detected during the estrus cycle in the expression of AMH and its type II receptor (AMHRII), however some heterogeneity has been determined in AMH mRNA expression in preantral and small antral follicles at estrus and diestrus [4,5,7,12]. In addition, it has been reported that ovariectomy in regularly cycling women causes undetectable AMH concentrations [13]. Hence, the ovaries seem to be the primary source of AMH; therefore, it may be used for determination of the presence or absence of ovaries or ovarian remnants in dogs and cats [14,15].

Several researches emphasized the evaluation of AMH concentrations in various ovarian pathological conditions including polycystic ovary syndrome, granulosa cell tumors and premature ovarian failure in the women and rat and also ovarian remnant syndrome in the bitch and queen [9,16,17]. The purpose of the present study was to compare the serum concentration of AMH and the expression of AMH gene in the ovarian tissue removed during ovariectomy and in remnant ovarian tissue in rats with experimentally induced ovarian remnant syndrome.

MATERIAL and METHODS

Animals and Study Design

A total of eighteen adult female Sprague Dawley rats weighing 200-250 g were obtained from Harlan Laboratories B.V. (The Netherlands). Animals were maintained with a 12-h light/dark schedule and supplied standard rat chow (Korkuteli Food Industry, Turkey) and water *ad libitum*. The rats were divided into three groups. Group I consisted of 6 rats gone through ovarian remnant syndrome experimentally, group II consisted of 6 rats performed total ovariectomy and group III consisted of 6 rats sham operated. Median laparotomy was performed in all groups under xylazine (5 mg/kg) and ketamine (45 mg/kg) anaesthesia. In group I; uterus, left ovary and half of the right ovary were removed (total hysterectomy and unilateral hemiovariectomy) [18]. Remnant ovarian tissue was

removed 30 days later with the same technique. In group II; uterus and both ovaries were removed at the same time (total ovariectomy). All the ovarian tissue samples were stored in liquid nitrogen at -196°C until mRNA isolation. Intravenous blood samples from tail vein were obtained into plain tubes on day 0, 1, 5, 10 and also day 30 from group I and were immediately centrifuged at 1550 g for 10 min. Sera were removed and stored at -80°C until analyzed for AMH concentration. In order to compare the serum AMH concentrations of different stages, we performed vaginal cytologic examination to determine the stages of the estrus cycle in all rats. Vaginal secretion was collected with a plastic pipette filled with 10 IU of normal saline (NaCl 0.9%) by embedding the tip into the vagina. Vaginal fluid was dropped on glass slides. Unstained material was evaluated under the light microscope (Leica CME Microscope, 1349522X, NY, USA, 40x objective lenses) according to Marcondes et al. [19].

All procedures involving the use of animals were approved by the Gazi University Animal Experiments Local Ethics Committee (Approval no:15.31; Turkey) and were performed at Laboratory Animal Breeding and Experimental Researches Center of the same university.

Serum AMH Assays

The serum AMH concentration was determined using an enzyme-linked immunosorbent assay kit (MBS701712, MyBioSource, Inc. San Diego, CA 92195-3308, USA), according to the manufacturer's instructions. All serum assays were performed in duplicate. The minimum detectable concentration of the assay was 0.375 ng/ml. The lower and upper limits of detection were 0.375 ng/ml and 150 ng/ml, respectively. The intra-assay coefficient of variation (CV) was < 8% and the inter-assay CV was <10%.

Quantitative Real-Time PCR Analysis

Total RNA was extracted from each tissue using the TriReagent (peqGOLD TriFastTM, Peqlab, Erlangen, Germany) and treated with DNase I, RNase-Free (Thermo scientific, Fermentas) according to the recommendations of the supplier. The RNA concentration of each sample was measured at 260 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, USA). The reverse transcription reaction of RNA was performed with the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Samples were analyzed on a LightCycler® 480 instrument (Roche Diagnostics, Mannheim, Germany) and relative mRNA expression of the AMH was normalized to the expression levels of beta actin (ACTB) expression. The sequences of the gene-specific primers and probes for AMH and ACTB transcripts were designed using the online Universal Probe Library (UPL) Assay Design Center (<https://www.universalprobe library.com>). The sequences of the primers used for QRT-PCR experiments were as

follows: rat ACTB forward (5'-CCCGCGAGTACAACCTTCT-3') and reverse (5'-CGTCATCCATGGCGAACT-3'), probe no 17; rat AMH forward (5'-CTGGACACCGTGCCTTTC-3') and reverse (5'-CACTGTGTGGCAGGTCCTC-3'), probe no 26. The QRT-PCR reactions with the following program: after initial denaturation at 95°C for 15 min, followed by 45 cycles consisting of 95°C for 15 sec, and 60°C for 20 sec and cooled down to 40°C. The concentration of the expression level for each sample was determined from the threshold cycle (Ct), which is the cycle where an increase in PCR product is first detected at a statistically significant level.

Statistical Analysis

The statistical significance of the differences in the AMH concentration between groups was analyzed by One-Way ANOVA analysis of variance and Duncan's multiple range test using SigmaStat (Jandel Scientific Software Inc.; San Jose, CA, USA). Data were expressed as mean \pm standard deviation (SD). A P value of <0.05 was considered statistically significant. The relative expression for each gene is obtained by comparing the CT values for each gene using the equation $2^{-\Delta\Delta CT}$ following the Pfaffl-based method with

the Relative Expression Software Tool 2008 (REST[®]) [20]. Each experiment was carried out three times.

RESULTS

Vaginal Cytologic Examination

Estrus cycle stages of the rats in all groups were determined as six proestrus, four estrus, four metestrus and four diestrus before surgery. There was no significant difference in serum AMH concentrations among the stages of the estrus cycle in all groups ($P>0.05$) (Fig. 1).

Serum AMH Concentrations

The pre-operative serum AMH concentrations in group I, II and III were 4.61 ± 1.13 ng/ml, 4.32 ± 0.71 ng/ml and 4.15 ± 0.71 ng/ml, respectively ($P>0.05$). As shown in Table 1, the pre-operative serum AMH concentrations in group I and II were significantly different compared to post-operative day 1 and 5 ($P<0.05$). In group III, there was no significant difference between pre-operative and post-operative stage in serum AMH concentrations ($P>0.05$). In

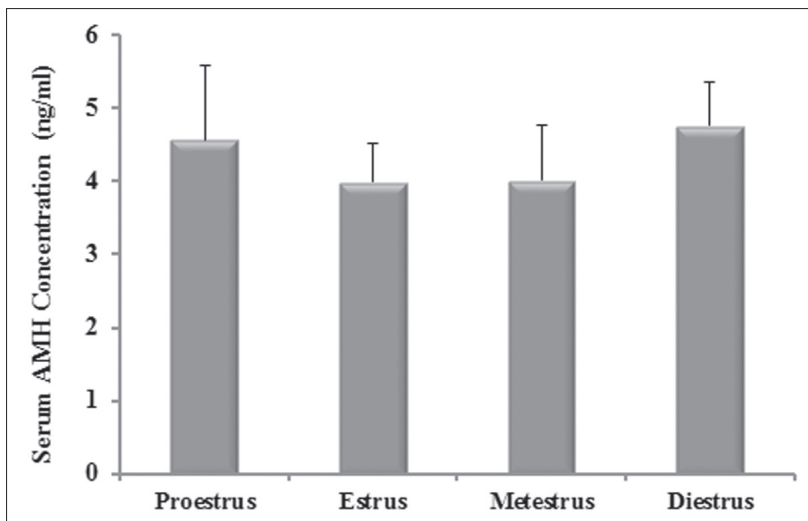


Fig 1. Mean serum AMH concentrations (ng/ml) at different stages of the estrus cycle in all groups at day 0, $P>0.05$

Şekil 1. Tüm gruplarda 0.günde farklı östrus siklus evrelerindeki serum AMH konsantrasyonları (ng/ml), $P>0.05$

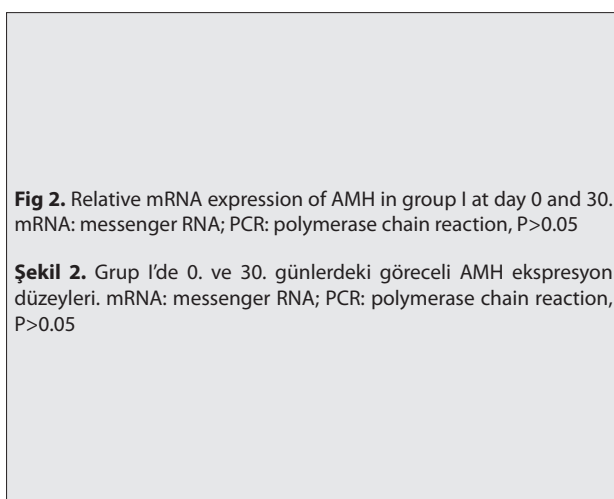


Fig 2. Relative mRNA expression of AMH in group I at day 0 and 30. mRNA: messenger RNA; PCR: polymerase chain reaction, $P>0.05$

Şekil 2. Grup I'de 0. ve 30. günlerdeki göreceli AMH ekspresyon düzeyleri. mRNA: messenger RNA; PCR: polymerase chain reaction, $P>0.05$

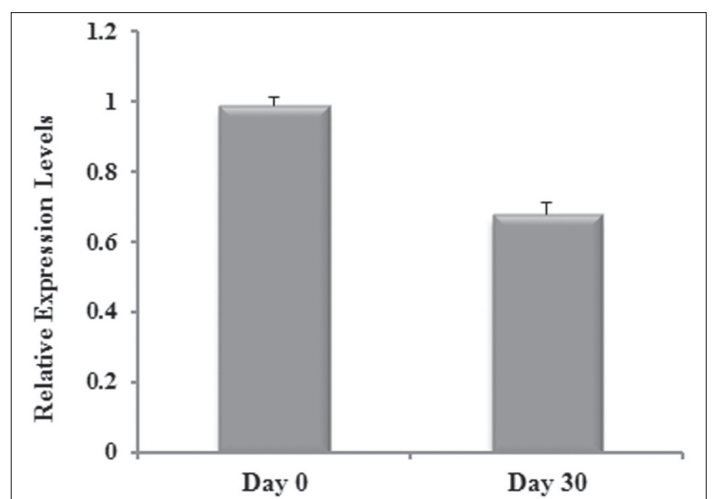
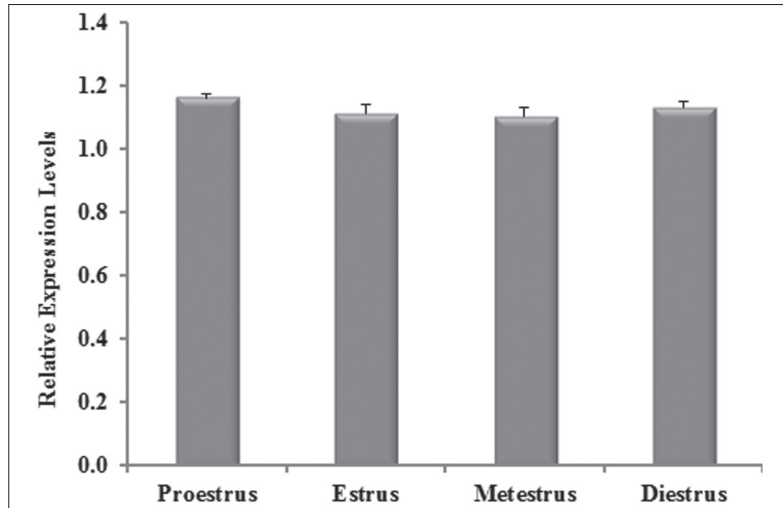


Table 1. Mean serum AMH concentrations (ng/ml) of the groups at day 0, 1, 5 and 10**Tablo 1.** Gruplardaki 0., 1., 5. ve 10. günlerdeki ortalama serum AMH konsantrasyonları (ng/ml)

Groups	Day 0	Day 1	Day 5	Day 10
Group I (ORS)	4.61±1.13 ^a	2.72±0.71 ^{A,b}	1.35±0.24 ^{A,c}	2.27±0.52 ^{B,b}
Group II (OHE)	4.32±0.71 ^a	1.78±0.24 ^{B,b}	0.52±0.13 ^{B,c}	ND
Group III (Sham)	4.15±0.71	3.98±0.42 ^c	4.09±0.50 ^c	3.96±0.53 ^c

ND: non-detectable. Different superscript capital letters indicate significant difference between the groups in the same column, Different superscript small letters indicate significant difference between the days in the same row (Duncan's test; P<0.05)

**Fig 3.** Relative mRNA expression of AMH at different stages of the estrus cycle in all groups at day 0. mRNA: messenger RNA; PCR: polymerase chain reaction, P>0.05**Şekil 3.** Tüm gruplarda 0. günde farklı östrus siklus evrelerindeki göreceli AMH ekspresyon düzeyleri. mRNA: messenger RNA; PCR: polymerase chain reaction, P>0.05

group II, serum AMH concentrations were below the non-detectable concentration (0.312 ng/ml) on day 10 after the surgery (Table 1). In addition, serum AMH concentration in group I was determined to be 2.03±0.71 ng/ml on day 30.

Relative Expression Levels of AMH

AMH mRNA expression levels in group I showed no significant difference in comparison with the group II on day 0 (P>0.05). In addition, AMH mRNA expression levels in group I were decreased on day 30 after surgery when compared to day 0, but this reduction was not statistically significant (P>0.05) (Fig. 2). There was no significant difference between the stages of the estrus cycle in AMH mRNA expression levels in all groups (P>0.05) (Fig. 3).

DISCUSSION

Anti-Müllerian hormone (AMH) is only produced by granulosa cells of the ovary [6,7,8,21]. AMH may be used for determination of the presence or absence of ovaries or ovarian remnants in mammals since the ovaries appear to be the primary source of AMH. It has been determined that serum AMH concentration was undetectable on day 3-5 after bilateral ovariectomy in women [13]. Similarly, the present study showed that serum AMH concentrations gradually decreased from day 1 to 5 and were undetectable on day 10 after ovariectomy and this finding made us thought that serum AMH concentration might be affected by the absence of the ovaries.

Recent studies revealed that serum AMH concentrations were not significantly different during the menstrual cycle in normocycling women [22,23]. However, a comparison of serum AMH concentrations between the stages of the estrus cycle in the rat has not been investigated before. In our study, we demonstrated that serum AMH concentrations before surgery were not statistically different amongst all groups considering the stages of the estrus cycle (P>0.05).

AMH and AMHR11 mRNA expression in preantral follicles did not vary during the estrus cycle, despite the heterogeneous decrease at estrus in rats [12]. Hirobe et al. [7] also presented that AMH mRNA expression are wide and uniform in recruited growing antral follicles on the morning of estrus but become heterogeneous on diestrus, when selection for atresia occurs. However, expression of AMH gene in the ovarian tissues at all stages of the estrus cycle in rats has been identically strong and homogenous in our study.

AMH mRNA expression initiates at the peri-natal period, declines along reproductive life and becomes undetectable at the postmenopausal period in women [1,24]. In rodents, AMH expression in the ovary has been observed by postnatal day 3 in granulosa cells of growing primordial follicles [5,25]. Kevenaar et al. [8] found that serum AMH concentrations decrease in mice with increasing age and this reduction in serum AMH is not represented by a similar change in AMH mRNA expression level. Immunostaining intensity of AMH expression in granulosa cells of growing follicles does not show an alteration with age in mice [8]. The

rats used in the study were particularly chosen from the young adults already reached puberty and aged 2.5 to 3.0 months to exclude the diversity of results due to different ages. Therefore, both the measurement of serum AMH concentrations and gene expressions were performed when the rats were active reproductively.

AMH mRNA expression levels in group I declined on day 30 after surgery when compared to day 0, but this reduction was not statistically significant. This is because the amount of ovarian tissues taken at day 0 and 30 in order for measurements of AMH mRNA expression level were same. Therefore, it was thought that the statistically insignificant difference of AMH mRNA expression was not dependent upon the amount of tissue. The reason for the measurement of AMH mRNA expression of remnant ovarian tissue at day 30 was to wait the tissue to complete its repairment. One major drawback of our study is lack of comparison of AMH mRNA expression in remnant ovarian tissue on day 1, 5 and 10 after surgery.

In a study by Minke et al.^[26], seventy-five percent of the devascularized ovarian tissue revascularized and forty-three percent of them demonstrated follicular growth. One step ahead, our present study indicated that devascularized ovarian tissue remained functional and this was confirmed by increased serum AMH concentration between 5th and 10th postoperative days in group I, which was attributed to the revascularization of remnant ovarian tissue.

In conclusion, the present study revealed that serum AMH concentrations were below the non-detectable concentration on day 10 after ovariohysterectomy. This finding suggests that evaluation of serum AMH concentration could be an useful method to determine the presence or absence of ovaries or ovarian remnants in the rat. This result may be considered for future studies to clarify serum AMH concentrations and AMH mRNA expression levels in the stages of the estrus cycle in rats.

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