Gynecological Endocrinology, 2013; 29(3): 226–229

ISSN 0951-3590 print/ISSN 1473-0766 online
DOI: 10.3109/09513590.2012.738725

informa
healthcare

RAT OVARY

The effect of repeated administration of methotrexate (MTX) on rat ovary: measurement of serum antimullerian hormone (AMH) levels

Ali Benian[1], Onur Guralp[1], Duygu Derya Uzun[2], Alper Okyar[3] & Sezai Sahmay[4]

[1]Department of Obstetrics and Gynecology, Cerrahpasa School of Medicine, Istanbul University, Istanbul, Turkey, [2]Istanbul School of Medicine, Istanbul University, Istanbul, Turkey, [3]Department of Pharmacology, Faculty of Pharmacy Istanbul University, Istanbul, Turkey, and [4]Division of Reproductive Endocrinology, Department of Obstetrics and Gynecology, Cerrahpasa School of Medicine, Istanbul University, Istanbul, Turkey

***Objective*: To evaluate the possible effect of methotrexate (MTX) on rat ovaries by measuring serum antimullerian hormone (AMH), the novel marker of the ovarian reserve. *Methods*: Pretreatment serum AMH levels were measured in 15 Wistar albino rats. MTX was given in 1 mg/kg dose in days 1, 3, 5, and 7. Serum AMH levels were measured twenty-four hours after each MTX administration. Pre- and post-treatment serum AMH levels were compared. *Results*: Pretreatment median serum AMH was 102.4 ng/mL (25%: 41.9; 75%: 179.8). The median serum AMH levels were 70.6 ng/mL (25%: 54.08; 75%: 125.5); 136.1 ng/mL (25%: 57.3; 75%: 223.09); 121.2 ng/mL (25%: 52.5; 75%: 151.5); and 104.7 ng/mL (25%: 65.8; 75%: 265.5) after the first, second, third, and fourth methotrexate administrations, respectively. The ratio of the final (eighth day) median serum AMH level to the pretreatment median AMH level was 1.27 (25%: 0.84 and 75%: 2.57). Wilcoxon related samples test showed that final AMH was significantly higher as compared to the second day AMH measurement ($p = 0.041$). *Conclusion*: MTX administration did not cause a statistically significant change between pretreatment and final serum AMH levels in rats. There was no decrease in AMH levels indicating a decrease in ovarian reserve.**

Keywords: Animal study, antimullerian hormone, methotrexate, ovarian reserve

Introduction

Women who will receive any kind of medication have the same question in their minds: "Does this drug have the potential to harm my ovaries?" To answer this question, several trials were conducted with many drugs which could potentially decrease the ovarian reserve, either in humans or in animals. It is quite difficult to quantify the degree of harm, if there is any. Many methods were suggested to assess the ovarian reserve: presence of regular menses; serum levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E_2), and progesterone; and the number of primordial follicles and antimullerian hormone.

Antimullerian hormone (AMH) is produced by granulosa cells in primary to small antral follicles of the adult ovary and helps maintain primordial follicles in a dormant state. Previous studies in adult rats have shown that AMH is not expressed in primordial or small primary follicles, but is first expressed in granulosa cells of maturing large primary follicles and is at its maximal expression in the small preantral follicles [1]. In an in situ study by Baarends et al. [1], AMH mRNA expression was in granulosa cells of healthy preantral and small antral follicles of adult (30-day-old) rat ovaries, with little or no expression in oocytes, theca cells, and interstitial cells. AMH is a product of the granulosa cells of small growing follicles, whose number (and therefore circulating AMH concentrations) is reflective of the ovarian reserve [2,3].

AMH declines with age in adult women, and emerging data suggests a relationship with remaining reproductive lifespan and age at menopause. Early studies demonstrated that AMH concentrations are stable across the menstrual cycle, adding to its clinical utility [4].

Methotrexate (MTX) is used in the treatment of many diverse malignancies and in the therapy of diverse autoimmune diseases, such as rheumatoid arthritis and psoriasis, due to its anti-inflammatory and immunosuppressive effects [5,6], as well as ectopic pregnancy. MTX can be safely administered over a wide dose range in maintenance chemotherapy for acute lymphoblastic leukemia [7]. It exerts antineoplastic effects by competitively inhibiting folate-dependent biochemical processes, thus inhibiting DNA synthesis [8]. However, MTX is a frequently used cytotoxic agent in the clinic and is associated with acute and chronic neurotoxicity [9].

MTX selectively affects rapidly dividing cells, such as trophoblast cells, blood stem cells, and gonadal cells. On a cellular level, animal studies have shown that MTX causes cells to arrest in metaphase. Low-dose MTX is widely used in the treatment of rheumatic, gynecological, and neurological diseases. In addition, high-dose MTX (HD-MTX) is used increasingly in most childhood leukemias, breast cancer, osteosarcoma, and brain tumors.

Early studies using an AMH knock-out mouse demonstrated that perhaps its most important role is the regulation of initiation of early follicle growth [10,11], and subsequent studies have indicated that it may regulate the responsiveness of growing follicles to FSH [12]. The impact of gonadotoxic therapy, e.g. chemotherapy and radiotherapy, or of surgery on the ovarian reserve [13,14] can be assessed. AMH falls very rapidly with the onset of chemotherapy [15,16]. Lymphoma analysis allows the clear demonstration of the toxicity of alkylating agent-based therapy, as those women showed little or no recovery of AMH following chemotherapy; whereas, women receiving nonalkylating agent therapy showed a good recovery to approximate pretreatment concentrations [16].

Correspondence: Ali Benian, MD, Associate Professor, Department of Obstetrics and Gynecology, Cerrahpasa School of Medicine, Istanbul University, Cerrahpasa Tip Fakultesi, Kadin Hastaliklari ve Dogum Anabilim Dali, Fatih, Istanbul, Turkey, 34980. Tel: 00905424343032. E-mail: abenian@hotmail.com

Post-treatment follow-up studies have also shown that AMH concentrations are reduced in women following treatment for cancer, either in childhood or adulthood, with low AMH concentrations most consistently seen following total body radiation [15]. Normal concentrations have also been reported in populations of women following certain chemotherapy regimens indicating that those particular therapies do not cause loss of the ovarian reserve that is detectable using this marker [17].

In this study we aimed to evaluate the possible effect of MTX on rat ovaries by measuring serum AMH, the novel marker of the ovarian reserve.

Materials and methods

Ethical considerations

The study was performed in the Istanbul University Cerrahpasa School of Medicine, Center of Experimental Animals. The study has been carried out under approval of the Istanbul University Ethics Committee of Experimental Animals (I. U. HADYEK, Approval No. 01/2012). This study was supported by the Research Fund of Istanbul University (Project number:21638).

Animals and sample collection

Fifteen female Wistar albino rats (12 weeks old, 200–220 g) were used in our study. Animals were kept in cages, each housing up to four animals. Animals were kept in a room equipped with temperature control (22 ± 1°C) and relative humidity (60%). All rats were kept in 12/12 light/dark cycle and were fed *ad libitum*. World Health Organization recommends the use of 10 rodents in each group in toxicity studies [18]. Since we only had one group we used 15 rats in our study.

Blood samples (1 mL) were collected from each rat from the orbital vein in Li-Heparin tubes (Venoject-Italy) before MTX administration (Day 1, pretreatment collection). MTX (1 mg/kg) was administered to the rats via intramuscular (IM) route on days 1, 3, 5, and 7 as recommended by The Society of Obstetricians and Gynaecologists of Canada (SOGC) guidelines [19]. Twenty-four hours after each MTX injection, blood samples (1 mL) were taken from the orbital vein and AMH levels were determined in plasma. Blood samplings were carried out in light ether anesthesia.

Assay of plasma AMH concentrations

Plasma AMH levels were measured in duplicate aliquots, using a human enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions [Rat Mullerian Inhibiting Substance/Anti-Mullerian hormone, MIS/AMH ELISA Kit 96 TEST, EIAab®, China]. The coefficients of intra- and inter-assay variations were 4.2% ($n = 10$) and 7.3% ($n = 10$), respectively.

Statistical analysis

AMH levels were defined as ng/mL. Since the number of samples was below 30, medians (25 and 75%) and nonparametric tests were used. The homogeneity was assessed by Kolmogorov–Smirnov test. The medians were compared by Wilcoxon related samples test. $p < 0.05$ was considered significant.

Results

Pretreatment median serum AMH was 102.4 ng/mL (25%: 41.9; 75%: 179.8; Table I). The median serum AMH levels were 70.6 ng/mL (25%: 54.08; 75%: 125.5); 136.1 ng/mL (25%: 57.3; 75%: 223.09); 121.2 ng/mL (25%: 52.5; 75%: 151.5); and 104.7 ng/mL (25%: 65.8; 75%: 265.5) after the first, second, third, and fourth MTX administrations, respectively (Figure 1). Wilcoxon related samples test showed that final serum AMH was significantly higher compared to the second day AMH measurement ($p = 0.041$). There was no other significant difference between any other days. The results of the Wilcoxon related samples test was shown in Table II. The ratio of the final (eighth day) median serum AMH level to the pretreatment median serum AMH level was 1.27 (25%: 0.84 and 75%: 2.57).

Table I. The AMH levels in each measurement.

	Pretreatment sample (ng/mL)	2nd sample (ng/mL)	3rd sample (ng/mL)	4th sample (ng/mL)	Final (5th sample) (ng/mL)
1	41.99	39.41	57.31	52.54	32.74
2	229.23	75.94	105.90	151.51	206.63
3	162.92	93.86	191.44	129.95	60.19
4	56.76	65.17	155.20	80.84	150.00
5	212.02	125.51	136.16	261.86	270.00
6	11.39	64.52	29.30	128.45	104.73
7	67.85	43.15	60.79	45.61	69.59
8	201.41	361.09	244.89	169.02	370.54
9	28.57	128.45	223,09	151.51	264.01
10	131.47	62.00	55.67	121.26	264.20
11	102.44	54.08	155.20	52.54	86.13
12	124.07	226.25	115.86	103.58	65.83
13	179.80	70.66	53.05	49.63	265.56
14	11.08	42.76	308.80	13.19	16.79
15	102.44	79.16	265.43	350.28	102.46
Median	102.40	70.60	136.10	121.20	104.70
25%	41.90	54.08	57.30	52.50	65.80
75%	179.80	125.50	223.09	151.50	265.50

Discussion

In our study, the median serum AMH was 102.4 ng/mL, 70.6 ng/mL; 136.1 ng/mL; 121.2 ng/mL; and 104.7 ng/mL in the pretreatment, second, third, fourth, and fifth AMH measurements, respectively. There was no decrease in AMH; instead it slightly increased. The median of the ratio of the final (eighth day, fifth measurement) serum AMH level to the pretreatment AMH level was 1.27. Wilcoxon related samples test showed that final AMH was significantly higher compared to the second day AMH measurement ($p = 0.041$). There was no other significant difference between any other days.

Many methods were suggested to assess the ovarian reserve after radiotherapy or chemotherapy. These include the presence of regular menses; serum levels of FSH, LH, E_2, and progesterone; number of primordial follicles on histologic examination, antral follicle count (AFC) on ultrasound, and serum AMH. Regular menses is known to continue after unilateral oophorectomy, so it cannot quantify the degree of harm; rather it may show complete dysfunction of the ovaries. Although histologic sections give accurate results, it cannot be used on humans. There are many factors affecting serum levels of FSH, LH, E_2 and progesterone; none of those correlate with ovarian reserve as well as AFC and AMH. The intercycle change of AFC makes AMH the most favorable marker.

The role of AMH as an ovarian reserve marker has been investigated in some studies. AMH was shown to reflect the decrease in ovarian reserve after cisplatin treatment in female rats [20]. Gracia et al. [21] performed a prospective study on 71 cancer

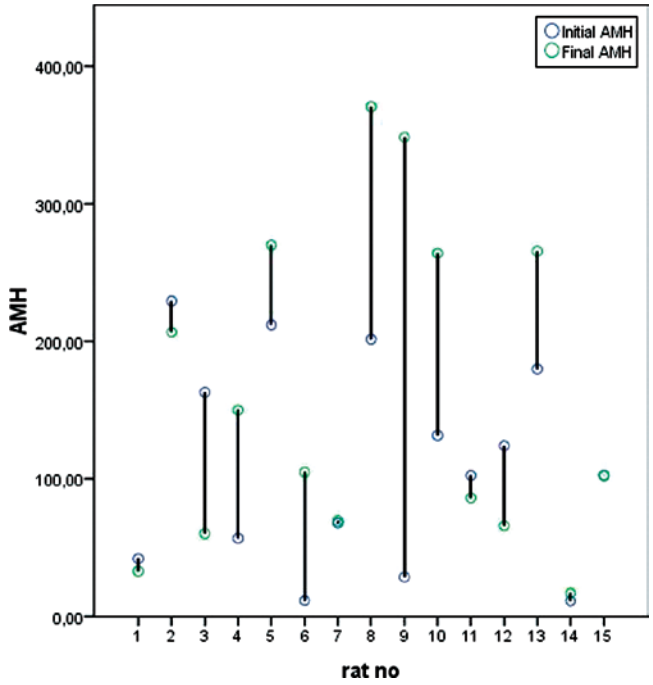

Figure 1. After the MTX administration, AMH was increased in 10, decreased in three, and did not change in two rats.

survivors aged 15–39 years who used alkylating agents including carmustine, busulfan, lomustine, chlorambucil, cyclophosphamide, ifosfamide, melphalan, nitrogen mustard, procarbazine, and thiotepa; 67 healthy, similarly aged unexposed subjects; and 69 regularly menstruating women of late reproductive age (40–52 years). The study measured various markers of ovarian reserve such as FSH, E_2, inhibin B, AMH, ovarian volume and AFC. They observed that after the exposure to alkylating agents, the change in FSH, AMH, and AFC were significant; whereas E_2, inhibin B, and ovarian volume were not. Sahambi et al. [22] conducted a study on mice and showed that repeated exposure of mice to 4-vinylcyclohexene diepoxide (VCD) induced loss of primordial and earliest growing ovarian follicles; AMH levels in VCD-exposed mice were significantly lower or undetectable after day 16.

In 1981, Shamberger et al. [23] conducted a prospective study on seven women who received high dose MTX (five of those also received vincristine) and observed that all seven women had regular cyclic menses; moreover, serum FSH, LH, estradiol, and progesteron levels were also found to be normal after the treatment. However, the number of patients was too small to draw definitive conclusions.

Gol et al. [24] showed that high-dose (5 g/m^2) MTX causes damage to the primordial follicles of the ovaries of mice. They used nine inbred Balb/c mice aged 7–8 weeks in the study group and administered 5 g/m^2 MTX as a single agent intraperitoneally; and nine mice in the control group received saline. Seven days later, the total number of the primordial follicles remaining in both ovaries was counted. They observed a significant decrease in the number of primordial follicles. In our study, we operated the molar pregnancy protocol with recurrent low doses (1 mg/kg) of MTX on days 1, 3, 5, and 7, instead of a single high dose MTX administration (5 g/m^2).

In our study, we detected that final AMH was significantly higher compared to the second day AMH measurement (p = 0.041). However, although significant, this increase has very little clinical value.

Table II. Comparison of the AMH measurements.

AMH sample number	Pretreatment	2nd	3rd	4th	Final
Pretreatment AMH	–	$p = 0.650$	$p = 0.334$	$p = 0.955$	$p = 0.158$
2nd	$p = 0.650$	–	$p = 0.173$	$p = 0.233$	$p = 0.041$*
3rd	$p = 0.334$	$p = 0.173$	–	$p = 0.532$	$p = 0.570$
4th	$p = 0.955$	$p = 0.233$	$p = 0.532$	–	$p = 0.233$
Final AMH	$p = 0.158$	$p = 0.041$*	$p = 0.570$	$p = 0.233$	–

Wilcoxon related samples test.
$p < 0.05$ is significant.

We do not exactly know if the AMH levels did not change significantly because the ovaries were not harmed or because serum AMH failed to show the damage by MTX. Moreover we do not have the long-term results; however, considering the study of Gol et al. [24], we may conclude that seven days is adequate to assess the damage in the ovaries. Our results must be challenged by larger trials with more animals conducted over a longer period of time as well as by trials on humans.

Our search in PubMed with the keywords "methotrexate, antimullerian hormone" in English language between 1960 and 2012 showed that there is only one study by Oriol et al. [25], which compared AMH levels before and after single dose MTX (1 mg/kg IM) in 25 women with ectopic pregnancy. In this study by Oriol et al. [25], AMH levels did not change significantly after MTX administration; moreover, the final AMH levels were higher than the pretreatment levels (3.7 ± 0.3 ng/mL vs. 3.9 ± 0.3 ng/mL, $p >$ 0.05) as is the case in our study. There is no study which compared AMH levels before and after "recurrent" MTX treatment in either animals or in humans.

In conclusion, MTX administration on days 1, 3, 5, and 7 did not cause a statistically significant change between pretreatment and final serum AMH levels in rats. Final measurement was significantly higher than the second measurement. There was no decrease in AMH levels indicating a decrease in ovarian reserve.

Acknowledgments

This study was supported by the Research Fund of Istanbul University (Project number:21638)

Declaration of Interest: The authors report no conflicts of interest

References

1. Baarends WM, Uilenbroek JT, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen AP, Grootegoed JA. Anti-müllerian hormone and anti-müllerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. Endocrinology 1995;136:4951–4962.
2. Bonilla-Musoles F, Castillo JC, Caballero O, Pérez-Panades J, Bonilla F Jr, Dolz M, Osborne N. Predicting ovarian reserve and reproductive outcome using antimüllerian hormone (AMH) and antral follicle count (AFC) in patients with previous assisted reproduction technique (ART) failure. Clin Exp Obstet Gynecol 2012;39:13–18.
3. Visser JA, Schipper I, Laven JS, Themmen AP. Anti-Müllerian hormone: an ovarian reserve marker in primary ovarian insufficiency. Nat Rev Endocrinol 2012;8:331–341.
4. Gilchrist RB, Ritter LJ, Armstrong DT. Oocyte-somatic cell interactions during follicle development in mammals. Anim Reprod Sci 2004;82-83:431–446.
5. Carbone PP. The role of chemotherapy in the treatment of cancer of the breast. Am J Clin Pathol 1975;64:774–779.

6. Furst DE. The rational use of methotrexate in rheumatoid arthritis and other rheumatic diseases. Br J Rheumatol 1997;36:1196–1204.
7. Widemann BC, Adamson PC. Understanding and managing methotrexate nephrotoxicity. Oncologist 2006;11:694–703.
8. Kamen BA, Moulder JE, Kun LE, Ring BJ, Adams SM, Fish BL, Holcenberg JS. Effects of single-dose and fractionated cranial irradiation on rat brain accumulation of methotrexate. Cancer Res 1984;44:5092–5094.
9. Vezmar S, Becker A, Bode U, Jaehde U. Biochemical and clinical aspects of methotrexate neurotoxicity. Chemotherapy 2003;49:92–104.
10. Durlinger AL, Kramer P, Karels B, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen AP. Control of primordial follicle recruitment by anti-Müllerian hormone in the mouse ovary. Endocrinology 1999;140:5789–5796.
11. Gigli I, Cushman RA, Wahl CM, Fortune JE. Evidence for a role for anti-Mullerian hormone in the suppression of follicle activation in mouse ovaries and bovine ovarian cortex grafted beneath the chick chorioallantoic membrane. Mol Reprod Dev 2005;71: 480–488.
12. Durlinger AL, Gruijters MJ, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, et al. Anti-Müllerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology 2001;142:4891–4899.
13. Meirow D, Biederman H, Anderson RA, Wallace WH. Toxicity of chemotherapy and radiation on female reproduction. Clin Obstet Gynecol 2010;53:727–739.
14. Chang HJ, Han SH, Lee JR, Jee BC, Lee BI, Suh CS, Kim SH. Impact of laparoscopic cystectomy on ovarian reserve: serial changes of serum anti-Müllerian hormone levels. Fertil Steril 2010;94:343–349.
15. Anderson RA, Themmen AP, Al-Qahtani A, Groome NP, Cameron DA. The effects of chemotherapy and long-term gonadotrophin suppression on the ovarian reserve in premenopausal women with breast cancer. Hum Reprod 2006;21:2583–2592.
16. Decanter C, Morschhauser F, Pigny P, Lefebvre C, Gallo C, Dewailly D. Anti-Müllerian hormone follow-up in young women treated by chemotherapy for lymphoma: preliminary results. Reprod Biomed Online 2010;20:280–285.
17. Rosendahl M, Andersen CY, Ernst E, Westergaard LG, Rasmussen PE, Loft A, Andersen AN. Ovarian function after removal of an entire ovary for cryopreservation of pieces of cortex prior to gonadotoxic treatment: a follow-up study. Hum Reprod 2008;23:2475–2483.
18. The 54th meeting of the WHO Expert Committee on Biological Standardization. WHO Guidelines on Nonclinical Evaluation of Vaccines; 2003. pp 10.
19. Gestational Trophoblastic Disease, SOGC Clinical Practice Guidelines, No. 114; 2002.
20. Yeh J, Kim BS, Liang YJ, Peresie J. Gonadotropin stimulation as a challenge to calibrate cisplatin induced ovarian damage in the female rat. Reprod Toxicol 2009;28:556–562.
21. Gracia CR, Sammel MD, Freeman E, Prewitt M, Carlson C, Ray A, Vance A, Ginsberg JP. Impact of cancer therapies on ovarian reserve. Fertil Steril 2012;97:134–40.e1.
22. Sahambi SK, Visser JA, Themmen AP, Mayer LP, Devine PJ. Correlation of serum anti-Müllerian hormone with accelerated follicle loss following 4-vinylcyclohexene diepoxide-induced follicle loss in mice. Reprod Toxicol 2008;26:116–122.
23. Shamberger RC, Rosenberg SA, Seipp CA, Sherins RJ. Effects of high-dose methotrexate and vincristine on ovarian and testicular functions in patients undergoing postoperative adjuvant treatment of osteosarcoma. Cancer Treat Rep 1981;65:739–746.
24. Gol M, Saygili U, Koyuncuoglu M, Uslu T. Influence of high-dose methotrexate therapy on the primordial follicles of the mouse ovary. J Obstet Gynaecol Res 2009;35:429–433.
25. Oriol B, Barrio A, Pacheco A, Serna J, Zuzuarregui JL, Garcia-Velasco JA. Systemic methotrexate to treat ectopic pregnancy does not affect ovarian reserve. Fertil Steril 2008;90:1579–1582.