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Hormonal and Histological Effects of the Inactivated SARS-CoV-2 (TURKOVAC™) Vaccine on Ovarian Reserve

İnaktif SARS-CoV-2 (TURKOVAC™) Aşısının Yumurtalık Rezervi Üzerindeki Hormonal ve Histolojik Etkileri

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ABSTRACT

Introduction: *Many countries carried out mass vaccination campaigns during the coronavirus diseases 2019 pandemic. Almost all the data on the effects of vaccines used in these campaigns on ovarian reserve come from studies to investigate the effects of their use on assisted reproductive outcomes. This study was conducted to investigate the possible effects of the inactivated SARS-CoV-2 vaccine on the number of ovarian follicle forms and serum anti-müllerian hormone (AMH) levels in rats.*

Materials and Methods: *A total of 20 female Wistar Albino rats, aged 16 to 20 weeks, were randomly assigned in a 1:1 ratio to either group 1 (control group) or group 2 (inactivated SARS-CoV-2 vaccine group). The control group was not treated. The vaccine group received two doses of the inactivated SARS-CoV-2 vaccine at 28-day intervals. On the 29th day after receiving the second dose, blood samples were taken from the hearts of the rats under anesthesia. The rats were euthanized by cervical dislocation, followed by bilateral oophorectomy performed via laparotomy. Primordial, primary, secondary, antral, preovulatory, and atretic follicle forms were counted in ovarian tissue sections using light microscopy. Serum AMH levels were measured using ELISA.*

Results: *The mean primordial and primary follicle numbers were decreased in the inactivated vaccine group compared to the control group ($p< .001$). However, the numbers of other follicle forms were similar in both groups. Serum AMH levels (ng/mL) were 9.9 (9.4-10.2) in the control group and 9.7 (9.1-10.0) in the inactivated vaccine group, and there was no difference in AMH levels between the groups ($p= 0.481$).*

Conclusion: *Our findings suggest that the inactivated SARS-CoV-2 vaccine administered to rats may reduce the primordial follicle pool. Consequently, serum AMH levels and antral follicle numbers, which remain within the normal range, may not accurately reflect ovarian reserve.*

Key Words: *Rat; Inactivated SARS-CoV-2 vaccine; Ovarian follicles; Anti-müllerian hormone; Ovarian reserve*

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ÖZ

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Giriş: *Koronavirüs hastalığı 2019 salgını sırasında birçok ülke toplu aşı kampanyaları gerçekleştirmiştir. Bu kampanyalarda kullanılan aşıların yumurtalık rezervi üzerindeki etkilerine ilişkin verilerin neredeyse tamamı, bunların kullanımının yardımcı üreme sonuçları üzerindeki etkilerini araştıran çalışmalardan oluşmaktadır. Bu çalışma, inaktif bir şiddetli akut solunum yolu sendromu koronavirüs-2 (SARS-CoV-2) aşısının sıçanlarda yumurtalık folikül formlarının sayısı ve serum anti-Müllerian hormon (AMH) düzeyleri üzerindeki olası etkilerini araştırmak için yürütülmüştür.*

Materyal ve Metod: *Çalışmaya yaşları 16-20 hafta arasında değişen toplam 20 dişi Wistar Albino sıçanı 1:1 oranında rastgele olarak grup 1 (kontrol grubu) veya grup 2'ye (inaktif SARS-CoV-2 aşısı grubu) atandı. Kontrol grubuna herhangi bir aşı uygulanmadı. Aşı grubuna 28 günlük aralıklarla iki doz inaktif SARS-CoV-2 aşısı uygulandı. İkinci dozun verilmesinden 29 gün sonra, anestezi altında sıçanların kalplerinden kan örnekleri alındı. Sıçanlar servikal dislokasyonla ötanazi edildi ve laparotomi ile bilateral ooferektomi yapıldı. Yumurtalık doku kesitlerinde primordial, primer, sekonder, antral, preovulatuar ve atretik folikül formları ışık mikroskobu ile sayıldı. Serum AMH düzeyleri ELISA ile ölçüldü.*

Bulgular: *Ortalama primordial ve primer folikül sayıları kontrol grubuyla karşılaştırıldığında inaktif aşı grubunda azaldı ($p< 0.001$). Ancak diğer folikül formlarının sayısı her iki grupta benzerdi. Serum AMH düzeyleri (ng/mL) kontrol grubunda 9.9 (9.4-10.2) ve inaktif aşı grubunda 9.7 (9.1-10.0) idi ve gruplar arasında AMH düzeylerinde fark yoktu ($p= 0.481$).*

Sonuç: *Bulgular, sıçanlara uygulanan inaktif SARS-CoV-2 aşısının primordial folikül havuzunda azalmaya neden olabileceğini ve bu nedenle normal aralıkta olan serum AMH seviyesi ve antral folikül sayısının yumurtalık rezervini doğru bir şekilde yansıtmayacağını göstermektedir.*

Anahtar Kelimeler: *Sıçan; İnaktif SARS-CoV-2 aşısı; Yumurtalık folikülleri; Anti-müllerian hormonu; Yumurtalık rezervi*

INTRODUCTION

Coronavirus diseases-2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), emerged in December 2019, and vaccines have been produced using different functional strategies after the genome of the virus, isolated from the first cases of viral pneumonia seen in Wuhan in January 2020, was announced to the entire scientific community by Chinese researchers[1-3]. The majority of effective vaccines against SARS-CoV-2 have been produced using the following strategies: live attenuated viruses, inactivated viruses, subunit vaccines, recombinant proteins, viral vector-based vaccines and DNA/RNA vaccines[2]. SARS-CoV-2 nucleic acid vaccines, available in the form of DNA and RNA, provide our bodies with the genetic code needed for the immune system to produce antigens necessary to combat infections[4]. Viral vector-based vaccines boost the immune system and guarantee protein synthesis by introducing genome of the coronavirus into a virus that is benign and does not cause illness. In the development of vaccines that employ viral vectors, the genome of the coronavirus is integrated into a benign virus that is incapable of causing disease. This mechanism triggers an immune response and facilitates the production of proteins[4].

The best and most efficient method of preventing COVID-19 is vaccination, which is also recommended for women of reproductive age[2,5]. Unfortunately, menstrual disorders and unexpected vaginal bleeding have been reported after SARS-CoV-2 vaccines with mRNA and adenovirus vector, and a potential correlation between the SARS-CoV-2 vaccine and menstrual abnormalities has been suggested[6,7]. However, nearly all of the scientific information regarding how SARS-CoV-2 vaccines affect female fertility has come from research examining how the vaccines affect the results of assisted reproductive methods, such as the use of controlled ovarian hyperstimulation protocols in infertile women[8].

There is currently no evidence to suggest whether the administration of a vaccine against COVID-19 affects fertility in women of reproductive age[6]. Anti-Müllerian hormone (AMH) and antral follicle count are currently the simplest, most sensitive, and specific measures of ovarian reserve[9]. The aim of this study was to investigate the possible effects of an inactivated SARS-CoV-2 vaccine on the number of ovarian follicle forms and serum AMH levels in rats.

MATERIALS and METHODS

Ethics Statement

The ethical protocol was approved by the Animal Experiments Local Ethics Committee of Niğde Ömer Halisdemir University (OHU) Animal Experiments Local Ethic Committee with the number 2024/09 and decision dated 31.05.2024. The study was conducted at the OHU Experimental Animal Production and Research Laboratory.

Experimental Animals and Care

A total of 20 female Wistar Albino rats, aged between 16-20 weeks and weighing 240 ± 30 g, were used in this study. The rats were procured from the Kobay Experimental Animal Laboratory (Ankara, Türkiye). All experimental procedures were performed in accordance with the guidelines specified in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health in 1996. The sample size was determined as a total of 20 rats, 10 rats for each group, using G*Power version 3.1.9.2 software (Universität Kiel, Germany) with an effect size (d) of 1.34, power of 80% and a significance level of 0.05. At the beginning of the study, vaginal cytology was performed on the rats, and only those in the estrous phase were included. The estrous period was defined in vaginal cytology by the presence of classical keratinized, needle-like cells or round, serrated cells with irregular edges[10]. The animals were maintained at a room temperature of 22 ± 2 °C, under a 12-hour light/12-hour dark cycle, and housed in cages with a maximum of four rats per cage. Standard pellet food and tap water were provided *ad libitum*.

Experiment protocol

A total of 20 rats were randomly assigned in a 1:1 ratio to either group 1 (control group) or group 2 (inactivated SARS-CoV-2 vaccine group). Rats in the control group received no treatment during the experiment. In contrast, rats in the inactivated SARS-CoV-2 vaccine group (hereafter referred to as the inactivated vaccine group) were administered 3 µg/0.5 mL of TURKOVAC™ (ERU-CoV, Kayseri, Türkiye) subcutaneously in two doses, with a 28-day interval between doses.

The TURKOVAC™ vaccine, administered to the rats in this study, is a domestically produced inactivated SARS-CoV-2 vaccine approved for emergency use by the Turkish Medicines and Medical Devices Agency of the Ministry of Health of the Republic of Türkiye in December 2021[11]. Furthermore, in this study, the TURKOVAC™ vaccine was administered to rats in a manner similar to the recommended two-dose regimen for humans[12].

Collection of blood and tissue samples

After the second dose, a 28-day period was allowed for antibody formation. At the end of this period, all rats in both groups were anesthetized with 50 mg/kg of 10% ketamine hydrochloride (Ketasol; Richter Pharma) and 10 mg/kg of 2% xylazine (Rompun; Bayer Healthcare). All rats were euthanized by cervical dislocation following the extraction of two milliliters of blood from the apex of the heart, and bilateral oophorectomy was performed via emergency laparotomy. The blood was then centrifuged at 3000 rpm for 15 minutes and stored in a refrigerator at -80 °C.

The ovarian tissues were promptly fixed in 10% formaldehyde.

AMH analysis

The serum AMH level was determined using ELISA kits (Rat ELISA Kit, 201-11-1246, Baoshan District, Shanghai, China). AMH measurement was conducted in the Biochemistry Laboratory of OHU Training and Research Hospital. All procedures followed the manufacturer's instructions, with a precision of 0.101 ng/mL and a coefficient of variation of less than 5%.

Histological procedure

All histological procedures and examinations of ovarian slides were performed by an experienced histologist who was blinded to the study. The ovarian tissues, fixed in formaldehyde solution, were dehydrated in a graded alcohol series, cleared with xylene and embedded in paraffin blocks. Five µm thick sections were obtained using a microtome. Standard Hematoxylin & Eosin (H&E) and Masson's Trichrome (MT) staining procedures were applied to all sections, which were subsequently examined under an Olympus BX51 light microscope and photographed using an Olympus DP72 digital camera.

Follicle count

Follicle counting was performed by randomly selecting 10 out of the 30 prepared slides for each rat. The follicles were classified based on previously established histological criteria as follows: primordial follicles (PrmF), primary follicles (PriF), secondary follicles (SF), antral follicles (AF), preovulatory follicles (PF), and atretic follicle (ATF)[13,14].

Statistical Analysis

SPSS 24.0 for Windows were used for all statistical analyses. Group conformity to normal distribution was assessed using the Shapiro-Wilk test, histograms, and skewness and kurtosis analyses. The Mann-Whitney U test was used for intergroup comparisons of variables that did not follow a normal distribution. The Spearman correlation test was used to assess the relationship between parameters within the groups. The alpha level of significance was set at $p< 0.05$.

RESULTS

When the MT staining results were evaluated under a light microscope, the ovarian tissue of the control group exhibited a normal appearance, with a cortex containing follicles at various stages of development on the outer region and a medulla layer consisting of loose connective tissue and a diffuse vascular network on the inner region (Figure 1A, C). In the inactivated vaccine group, a slight enlargement in follicular diameters was observed (Figure 1B, D).

During the light microscopic examination of H&E and MT-stained tissue sections, it was noteworthy that PrmFs and PriFs were observed less frequently in the sections from the inactivated vaccine group (Figure 2B, D) compared to those from the control group (Figure 2A, C).

When the mean numbers of follicle forms were compared between the groups, the numbers of PrmFs and PriFs were lower in the inactivated vaccine group compared to the control group ($p< 0.001$). A comparison of the groups in terms of the mean number of follicle forms is presented in Table 1.

The serum AMH level (ng/mŞ) was 9.9 (9.4-10.2) in the control group and 9.7 (9.1-10.0) in the inactivated vaccine group, with no significant difference between the groups ($p= 0.481$). There was a negative correlation between the numbers of primordial follicles and the numbers of atretic follicles in the control group ($r= -0.745$, $p= 0$), but there was a positive correlation between the number of follicular forms and serum AMH levels in both groups when the within-group relationships were analyzed. Aside from this correlation, neither the number of follicular forms nor the number of follicular forms and serum AMH levels in either group were related.

DISCUSSION

In this study, follicle forms in ovarian tissue were counted, and serum AMH levels were measured to investigate the potential impact of the inactivated SARS-CoV-2 vaccine on ovarian reserve in rats. A significant decrease in the numbers of PrmFs and PriFs was observed in rats that received two doses of the vaccine three months after the initial administration.

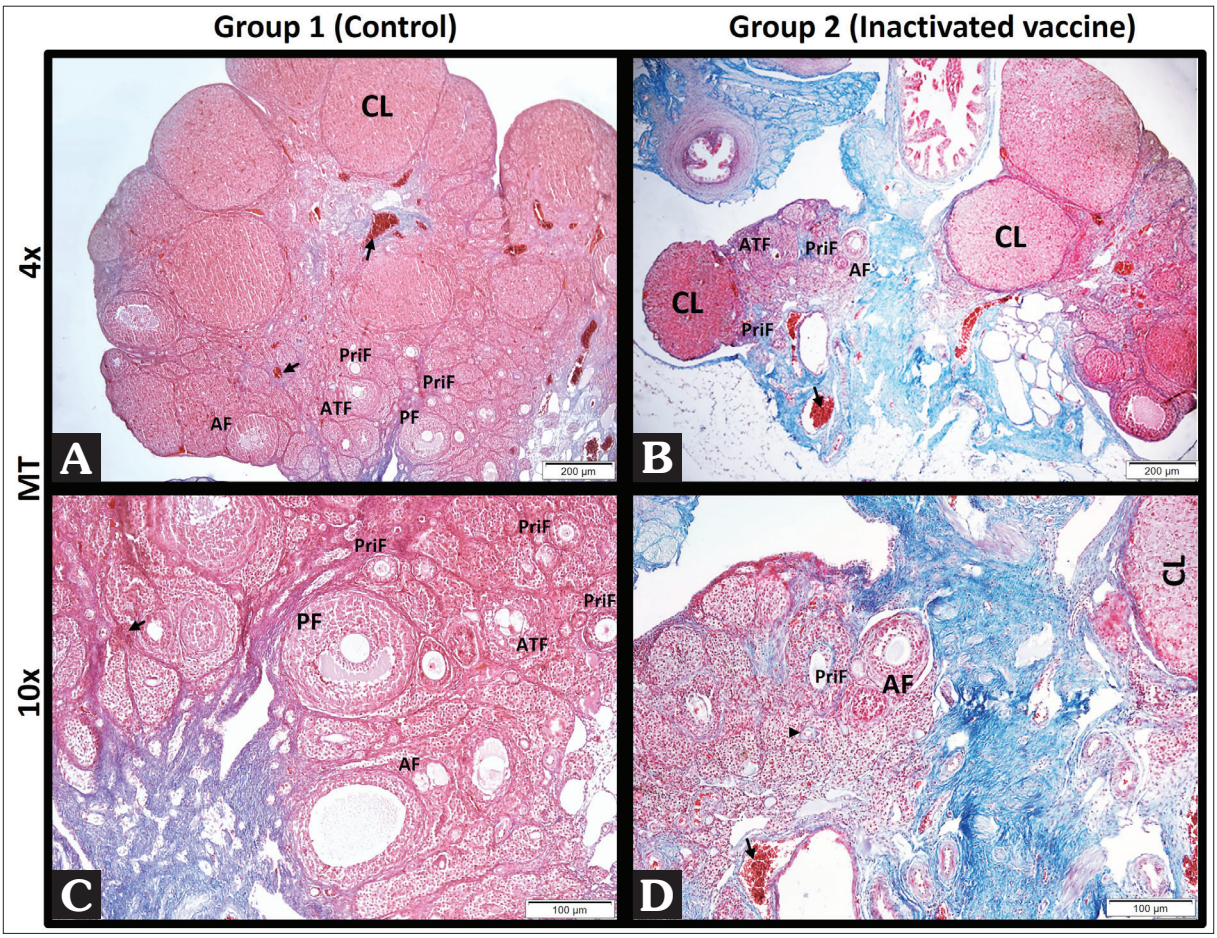

Figure 1. Light microscopic images of the control and inactivated vaccine groups (MT, X4; MT, X10).

MT: Masson's trichrome, CL: Corpus luteum, PriF: Primary follicle, SF: Secondary follicle, AF: Antral follicle, PF: Preovulatory follicle, ATF: Atretic follicle, Arrow: Blood vessel, Arrowhead: Primordial follicle.

However, neither the number of other follicle forms nor the levels of serum AMH changed. This study is the first investigation counting the follicle forms in rats following vaccination. The data showing that the counts of antral follicles and serum anti-Müllerian hormone AMH levels, both serving as indicators of ovarian reserve, remained unchanged three months after the first administration suggest that ovarian reserve was preserved. Nonetheless, the reduction in primordial follicle numbers, which is a significant measure of ovarian reserve, raises concerns about the potential vulnerability of ovarian reserve throughout the reproductive phase.

The impact of SARS-CoV-2 vaccines on the morphology of ovarian follicles in rats has not yet been investigated. Nevertheless, this subject has been explored in human cycles utilizing assisted reproductive technology (ART). A recent meta-analysis of 15 studies, seven of which employed inactivated vaccines, was conducted to understand the impact of SARS-CoV-2 vaccination on assisted reproductive outcomes. These studies included 1506 vaccinated and 3626 unvaccinated ART cycles. The analysis showed that the administration of SARS-CoV-2 vaccines did not have a significant impact on the number of oocytes retrieved. According to the subgroup analysis, the pooled estimate for the number of oocytes retrieved showed consistency across the various types of SARS-CoV-2 vaccines, including mRNA, inactivated, and protein-based formulations[8].

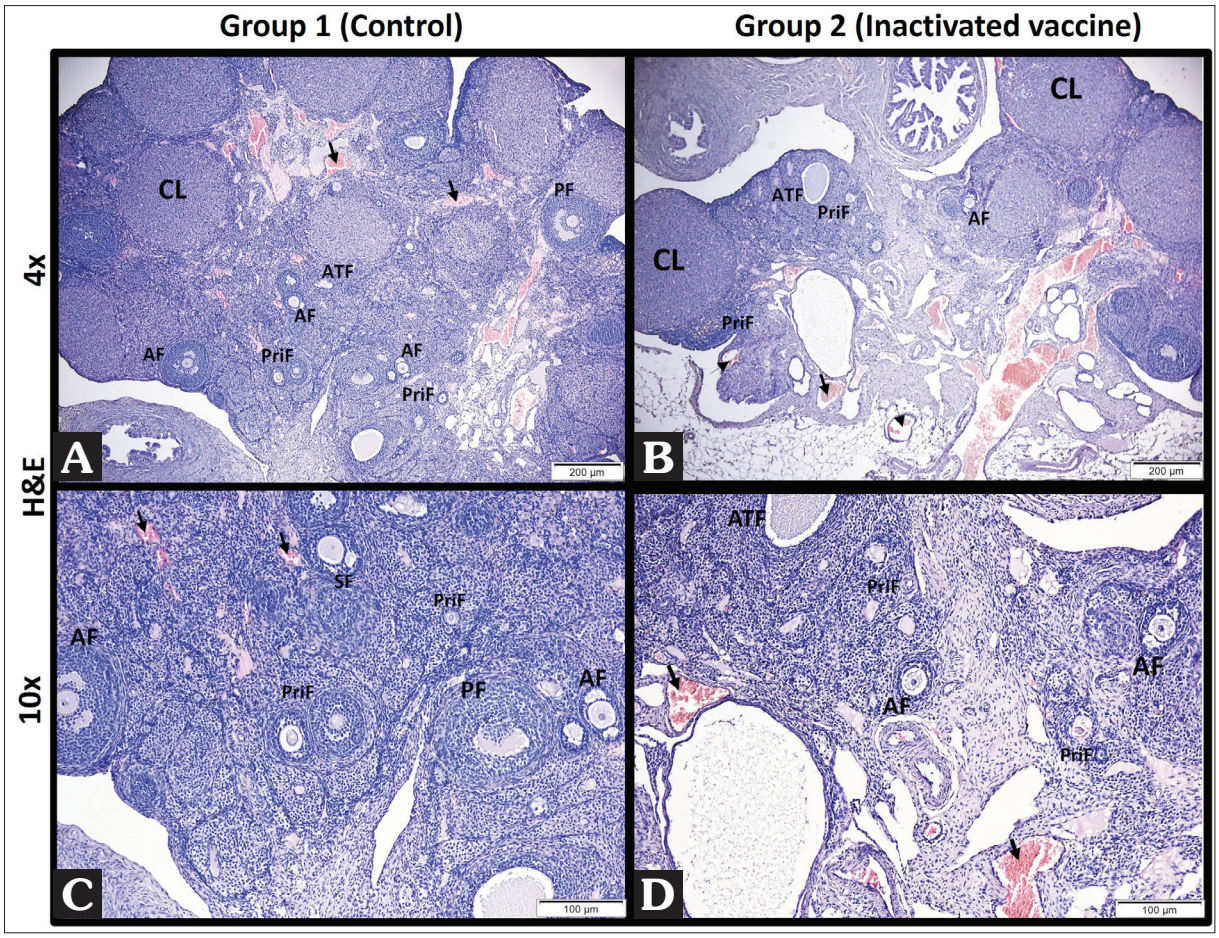

Figure 2. Light microscopic images of the control and inactivated vaccine groups (H&E, X4; H&E, X10).

H&E: Hematoxylin & Eosin, CL: Corpus luteum, PriF: Primary follicle, SF: Secondary follicle, AF: Antral follicle, PF: Preovulatory follicle, ATF: Atretic follicle, Arrow: Blood vessel, Arrowhead: Primordial follicle.

Table 1. Comparison of the groups in terms of the mean follicle numbers

Follicle Forms	Group 1 (Control) (n= 10)	Group 2 (Inactivated vaccine) (n= 10)	p
Primordial follicle	50.0 (44.8-53.0)	39.5 (32.0-43.0)	0.001*
Primary follicle	41.0 (37.0-44.5)	32.5 (28.8-36.2)	0.001*
Secondary follicle	25.5 (22.2-29.2)	22.5 (19.8-23.8)	0.075
Antral follicle	13.0 (11.0-14.2)	13.5 (9.8-15.2)	0.796
Preovulatory follicle	11.5 (8.8-13.5)	10.0 (8.8-12.2)	0.529
Atretic follicle	7.0 (5.8-9.0)	6.0 (5.0-7.5)	0.280

Data are presented as mean ± standard deviation.
*p< 0.05.

In this research, rats vaccinated with the inactivated SARS-CoV-2 exhibited a significant decrease in the quantity of primordial and primary follicles compared to the control group. Nevertheless, there was no change in the number of other types of follicles, such as antral follicles. The initial phase of recruitment, when primordial follicles progress to primary follicles (referred to as the growing follicle pool), is influenced by several paracrine factors that are not dependent

on follicle-stimulating hormone (FSH). However, during cyclic recruitment, an increase in FSH levels in the bloodstream enables a group of antral follicles to evade apoptotic death[15]. Based on the previous findings, we propose that rats administered an inactivated SARS-CoV-2 vaccine successfully underwent a recruitment cycle and subsequent stages of follicle development under the influence of FSH without any impairment. This might explain the equivalent number of retrieved oocytes in ART cycles performed with FSH prior to vaccination, with no differences observed in women who received the SARS-CoV-2 virosomal vaccine.

Serum AMH levels were measured to evaluate the impact of SARS-CoV-2 infection and vaccination on ovarian reserve[16]. Hasdemir et al. measured serum AMH levels before vaccination and at the first, third, sixth, and ninth months afterward to evaluate the effect of the SARS-CoV-2 vaccination on serum AMH levels in women of reproductive age. They observed a gradual decrease in AMH levels from the beginning to the sixth month, followed by a significant increase in the ninth month compared to the sixth month. Furthermore, the same study revealed that inactivated vaccines resulted in significantly lower serum AMH levels compared to inactivated + mRNA-based vaccines.

The researchers suggested that SARS-CoV-2 vaccination led to a slight reduction in serum AMH levels[17]. In a systematic review and meta-analysis conducted by Ghaemi and colleagues, a pooled analysis of ten studies examining the potential impact of the SARS-CoV-2 vaccination on AMH levels revealed no statistically significant alteration in AMH levels after vaccination[16]. A recent systematic review evaluated the effect of SARS-CoV-2 vaccine on ovarian reserve and emphasized that it is safe to assume that COVID-19 vaccination does not exert any adverse effect on ovarian reserve parameters such as AMH, AFC, FSH, and estradiol[19]. In relation to prior research assessing the influence of SARS-CoV-2 vaccines on AMH levels in women, our results diverged from those presented by Hasdemir et al., yet they were consistent with the findings of Ghaemi et al. and Zhu et al.[16-18].

Successful reproduction in females results from numerous interactions, with the ovary and ovarian follicles playing a fundamental role[19]. The follicles, the functional unit of the ovaries, are composed of oocytes, granulosa and theca cells[20]. Follicular development, which serves as the physiological basis for female estrus and ovulation, commences with the formation of primordial follicles[21,22]. Germ cells in the embryonic mouse ovary follow a developmental pattern similar to that in humans. The concept of a non-renewable primordial follicle pool, assembled around birth in rodents and during gestation in humans, forms the basis of a finite reproductive lifespan[21]. Although some histomorphological and biochemical methods such as AF and ATF count and serum hormone concentrations have been described to demonstrate ovarian function and damage, AMH level and AF count are currently the simplest, most sensitive and specific measures of ovarian reserve[9]. AMH is expressed in the ovary after birth in the granulosa cells of healthy, small growing follicles, and serum AMH levels correlate strongly with the number of antral follicles in normo-ovulatory women. In humans, AMH expression follows a pattern similar to that observed in mice and rats. It is not detected in primordial follicles, while 74% of primary follicles exhibit at least a weak signal in the granulosa cells[23]. A study examining AMH immunoexpression in the ovaries of 40 patients with premature ovarian failure (POF) revealed that AMH immunolabeling was consistently absent in primordial follicles[24]. Notably, intermediate, primary, and secondary follicles exhibited comparable AMH expression in all cases, both normal and POF, except for two cases. The similarities between AMH in the pre-antral follicles of most POF patients and normal women indicate that the developing granulosa cells of the majority of POF patients are functionally efficient and capable of AMH production[25]. Rats administered an inactivated SARS-CoV-2 vaccine did not exhibit a statistically significant alteration in antral follicle counts or

serum AMH levels compared to the control group. However, a comparison between the vaccine group and the control group revealed a statistically significant decrease in the quantity of primordial and primary follicles. Moreover, the study evaluated the relationships within the groups regarding the number of follicular forms and the connection between the number of follicular forms and serum AMH levels. A negative relationship was established between primordial and atretic follicles in the control group, while no additional relationships were found in either group. It can thus be proposed that the normal serum AMH levels and antral follicle counts observed in rats administered an inactivated SARS-CoV-2 vaccine may not accurately reflect ovarian reserve, given the reduction in the primordial follicle pool.

Although a systematic review and meta-analysis of controlled and randomized clinical trials found that all approved vaccines were safe and efficacious, the rapid development of SARS-CoV-2 vaccines, compared to traditional vaccine development methods, has raised questions about the safety and efficacy of the approved vaccines[26]. The estrous cycle of the rat is relatively brief, with a duration of approximately four days, making it ideal for investigating changes that occur during the reproductive cycle[27]. The results observed in rats in this study may provide a basis for human studies. In addition, since the number of studies evaluating follicular forms separately while assessing ovarian reserve is limited in the literature, this current study may also contribute to filling this gap. Finally, the fact that experimental animal studies are more homogeneous than human studies is very important in terms of reducing the bias in the study. While these aspects represent the strengths of our study, there are also some limitations. Given the significant differences between the human and animal reproductive systems, direct generalization of the findings to humans should be made with caution.

Furthermore, since this study focused only on the inactivated COVID-19 vaccine, the potential effects of other types of vaccines on ovarian reserve were not considered. One of the limitations of our study is that long-term effects were not observed.

CONCLUSION

In conclusion, the administration of an inactivated SARS-CoV-2 vaccine to rats was observed to reduce the number of primordial and primary follicles, while serum AMH levels and counts of other follicle types, including antral follicles, remained unchanged. These findings suggest that the vaccine may impact the primordial follicle pool, a critical determinant of ovarian reserve. However, the lack of change in AMH levels and antral follicle counts highlights a potential limitation in relying solely on these markers to assess ovarian reserve accurately.

It is important to note that this study was conducted in rats, and the direct applicability of these findings to humans remains uncertain. Differences in reproductive physiology between species, as well as variations in vaccine formulation and administration, may influence outcomes. Additionally, the study focuses on short-term effects, leaving the long-term impact of SARS-CoV-2 vaccination on ovarian function unexplored. Given the global importance of vaccination in combating the COVID-19 pandemic, further research is essential to understand the broader reproductive implications in humans. Specifically, studies with longer follow-up periods and human participants are needed to determine whether these findings translate to clinical significance in women. Such investigations will help ensure that vaccination strategies are both safe and effective across all populations.

ETHICS COMMITTEE APPROVAL

The study was approved by the Niğde Ömer Halisdemir University (OHU) Animal Experiments Local Ethics Committee (Decision no: 2024/09, Date: 31.05.2024).

CONFLICT of INTEREST

The authors have no conflicts of interest to declare that are relevant to the content of this article.

AUTHORSHIP CONTRIBUTIONS

Concept and Design: EK, AY, İT, İS, YD, MEA

Analysis/Interpretation: EK, AY, DA, EB, FY, ES, YD, OÇ

Data Collection or Processing: EK, DA, ES, İT, İS, YY, MEA, YD

Writing: All of authors

Review and Correction: EK, DA, MEA, YY

Final Approval: EK, AY, DA

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