E-ISSN : 2149-9330

TURKISH JOURNAL OF OBSTETRICS AND GYNECOLOGY

September 2024 Volume: 21 Issue: 3 www.tjoddergisi.org

TURKISH SOCIETY OF OBSTETRICS AND GYNECOLOGY

galenos yayınevi

TURKISH JOURNAL OF OBSTETRICS AND GYNECOLOGY

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Please refer to the journal's webpage **(https://tjoddergisi.org/)** for "Aims and Scope", "Instructions to Authors" and "Peer-Review and Ethics".

The editorial and publication process of the Turkish Journal of Obstetrics and Gynecology are shaped in accordance with the guidelines of ICMJE, WAME, CSE, COPE, EASE, and NISO. The journal is in conformity with the Principles of Transparency and Best Practice in Scholarly Publishing.

Turkish Journal of Obstetrics and Gynecology is indexed in **PubMed, PubMed Central (PMC), Web of Science-Emerging Sources Citation Index (ESCI), Tübitak/Ulakbim Turkish Medical Database, EBSCO, Embase, Scopus, ProQuest, British Library, Gale, CINAHL, Turk Medline, J-Gate, IdealOnline, CNKI, Hinari, GOALI, ARDI, OARE, AGORA** and **Turkiye Citation Index**.

The journal is published electronically.

Owner: İsmail Mete İtil on behalf of the Turkish Society of Obstetrics and Gynecology

Responsible Manager: Ercan Yılmaz

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Address: Molla Gürani Mah. Kaçamak Sk. No: 21/1 34093 İstanbul, Turkey
Phone: +90 (530) 177 30 97 / +90 (539) 307 32 03 **E-mail:** info@galenos.com.tr/yayin@galenos.com.tr **Web:** www.galenos.com.tr
Publisher Certificate Number:14521

Online Publication Date: September 2024 **E-ISSN:** 2149-9330
International scientific journal published quarterly.

TURKISH JOURNAL OF OBSTETRICS AND GYNECOLOGY

CONTENTS

Clinical Investigations

TURKISH JOURNAL OF OBSTETRICS AND GYNECOLOGY

CONTENTS

Reviews

TURKISH JOURNAL OF OBSTETRICS AND GYNECOLOGY

LETTER FROM THE PRESIDENT

Dear Turkish Society of Obstetrics and Gynecology,

I am pleased to be with you again with the September issue of our journal, which is our scientific publication and, a great source of pride for us. I would like to congratulate each and every one of our editors, section editors and judges who have put great effort into the preparation of our journal, published four times a year with an infrastructure consisting of knowledge, experience and meticulous work. I would also like to thank our authors and scientists who have sent their work in to be considered for publishing in one of the most prestigious journals in the obstetrics and gynecology community.

The Turkish Society of Obstetrics and Gynecology is not only a professional organization but also a social association that focuses on the vocational issues of obstetricians and gynecologists. In this sense, many of the problems experienced by our physicians have been expressed by the The Turkish Society of Obstetrics and Gynecology and, as always, the association has managed to become the most important source of trust for gynecologists. One of the most important of these issues is the work that is being done to solve the legal problems experienced by physicians who find themselves having to diagnose Down Syndrome. The statements made by The Turkish Society of Obstetrics and Gynecology in the national press and social media and the work done by our association's legal department have been instrumental in achieving significant results.

The Turkish Society of Obstetrics and Gynecology also ensured that the request for the Obstetrics and Gynecology TUKMOS Core Education Program to be revised was brought to the Medical Specialization Board Curriculum Creation and Standard Setting (TUKMOS) committee.

Additionally, The Turkish Society of Obstetrics and Gynecology has made the necessary press announcements in the national and social media to manage the decline in on-call income due to Specialization Students being absent the day after being on call by using leave after on-call duty as published and entered into force in the Official Gazette edition 31942 dated 03.09.2022.

In this context, the Turkish Society of Obstetrics and Gynecology is a scientific association that organizes congresses and meetings and publishes a very valued scientific journal; a social association addressing the important problems of our colleagues through the press bulletins we release, and of course a protective institution that weighs in on the problems of our young colleagues. I can confidently say that the Turkish Society of Obstetrics and Gynecology is present in every part of the Obstetrics and Gynecology field in Turkey.

My Best Regards
İsmail Mete İtil, Prof. MD
President of TJOD

TURKISH JOURNAL OF OBSTETRICS AND GYNECOLOGY

EDITORIAL

Dear Colleagues,

We are very pleased and honored to present our 3rd edition of 2024, the September edition, to you our valued colleagues. Our September edition was prepared over three months of hard work to be presented to our valued colleagues. We aimed to include original studies as well as compilations and meta-analyses in this issue. I would like to point out that the recognition of our journal is growing more with each passing day, its readership rate is increasing, and it is being cited by respected scientific journals in our field. I would like to thank our team who contributed to this success by using their scientific knowledge for our journal.

We believe that we will work tirelessly to make sure our journal, the scientific publication organ of the Turkish Society of Obstetrics and Gynecology, reaches the highest levels it deserves.

Ercan Yılmaz, Prof. MD
Fatih Şendağ, Prof. MD

Clinical Investigation / Araştırma

DOI: 10.4274/tjod.galenos.2024.80439

Turk J Obstet Gynecol 2024;21:135-41

Might retrigger with human chorionic gonadotropin be a solution for empty follicle syndrome after gonadotropin releasing hormone agonist trigger?

Gonadotropin salgılatıcı hormon agonisti ile tetikleme sonrası karşılaşılan boş folikül sendromu için insan koryonik gonadotropin ile yeniden tetikleme bir çözüm olabilir mi?

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Abstract

Objective: This study aimed to determine whether the use of human chorionic gonadotropin (hCG) as a trigger could offer a potential solution for addressing empty follicle syndrome following the administration of a trigger for gonadotropin-releasing hormone agonist.

Materials and Methods: A retrospective cohort analysis was conducted using data extracted from the hospital database pertaining to 415 patients who underwent in vitro fertilization (IVF) with an antagonist protocol triggered by a gonadotropin-releasing hormone (GnRH) agonist between December 2019 and January 2023 at the Acıbadem Maslak Hospital Assisted Reproductive Technologies Unit. All cases that failed to obtain oocytes and required rescue were analyzed.

Results: This study analyzed 415 women who underwent IVF using GnRH agonist-triggered antagonist protocols. Among them, 6 (1.4%) had empty folicle syndrome (EFS). Patients with EFS had lower luteinizing hormone levels and fewer oocytes, embryos, and blastocysts, resulting in lower oocyte utilization rate. However, pregnancy rates were similar, with no biochemical or ectopic pregnancies observed in the EFS group.

Conclusion: Use of an hCG retrigger in hyperresponders with no oocytes after GnRHa trigger may prevent cycle cancelation. Although the rates of egg utilization may decrease, oocyte maturity remains comparable. Frozen embryo transfer following hCG retrigger administration yields similar positive pregnancy test results and live birth rates.

Keywords: Empty follicle syndrome, rescue hCG trigger, failed agonist trigger

Öz

Amaç: Bu çalışma, gonadotropin salgılatıcı hormon agonisti tetiklemesi sonrası karşılaşılan boş folikül sendromuna potansiyel bir çözüm olarak insan koryonik gonadotropin (hCG) ile yeniden tetiklemenin sonuçlarını değerlendirmeyi amaçlamıştır.

Gereç ve Yöntemler: Bu çalışma, retrospektif bir kohort analizi olarak tasarlanmış olup, Acıbadem Maslak Hastanesi Yardımcı Üreme Teknikleri Birimi'nde Aralık 2019 ile Ocak 2023 tarihleri arasında gonadotropin salgılatıcı hormon (GnRH) agonisti tetiklemesi sonucu tüp bebek (IVF) uygulanan 415 hasta verilerini içermektedir. Oosit elde edilemeyen ve yeniden tetikleme gereken tüm olgular bu çalışmada değerlendirilmiştir.

Bulgular: GnRH agonisti ile tetiklemenin ardından IVF uygulanan 415 hastanın 6'sında (%1,4) boş folikül sendromu (BFS) ile karşılaşılmıştır. BFS'li hastalarda luteinize edici hormon seviyeleri daha düşük, elde edilen oosit, embriyo ve blastokist sayısı da daha azdı, bu da oosit kullanım oranlarının daha düşük olduğunu göstermektedir. Ancak, BFS grubunda biyokimyasal veya ektopik gebelik olmaksızın benzer gebelik oranları elde edilmiştir.

PRECIS: HCG retrigger in hyperresponder patients with no oocytes after GnRHa trigger is a potential strategy, avoiding cycle cancellation and yielding comparable IVF outcomes.

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Received/Geliş Tarihi: 04.02.2024 **Accepted/Kabul Tarihi:** 21.06.2024

Sonuç: GnRHa tetiklemesi sonrası oosit elde edilemeyen yüksek yanıtlı hastalarda hCG ile yeniden tetiklemenin siklus iptalini önleyebileceği görülmüştür. Bu yaklaşım oosit kullanım oranının azalmasına rağmen yeterli düzeyde olgun oosit elde edilmesini sağlamıştır. hCG ile yeniden tetiklemeyi takiben dondurulmuş embriyo transferi sonrası benzer pozitif gebelik testi sonuçları ve canlı doğum oranları elde edilebilir.

Anahtar Kelimeler: Boş folikül sendromu, hCG ile kurtarıcı tetikleme, başarısız agonist tetikleme

Introduction

In vitro fertilization (IVF) steps follow the same modalities that occur during normal menstrual cycles. During a normal menstrual cycle, preovulatory follicles grow to an adequate size and secrete estradiol (E2) through granulosa cells. When E2 levels rise, luteinizing hormone (LH) levels also increase, and once the LH level reaches a specific threshold, LH is triggered. This triggers a series of reactions, including loss of gap junction connections between oocytes and cumulus cells, expansion of cumulus cells, disintegration of the germinal vesicle, resumption of meiosis, and luteinization of granulosa cells[1]. During controlled ovarian stimulation in IVF treatment, human chorionic gonadotropin (hCG), which is similar to LH in terms of physiological activity, has been used to mimic the effects of LH trigger. From this perspective, hCG is the most common method for stimulating and luteinizing granulosa cells, final maturation of the oocyte before egg retrieval, and resumption of meiosis[2].

Women with multiple follicles, such as those with polycystic ovary syndrome, might be at increased risk of ovarian hyperstimulation syndrome (OHSS) following the administration of hCG for final maturation after ovarian stimulation[3]. OHSS during controlled ovarian stimulation is a rare but potentially life-threatening complication[4]. In recent years, the use of gonadotropin-releasing hormone agonist (GnRH-a) for oocyte maturation has gained popularity as a safer alternative to hCG, particularly in patients at risk of OHSS[3,5]. Triggering with GnRH-a to induce oocyte maturation is the most effective method for reducing the risk of OHSS[6]. GnRH-a can trigger oocyte maturation and ovulation by inducing the release of LH, and its activation effect called "flare-up".

The most common complication preventing the widespread adoption of this treatment protocol is that some patients may not adequately respond to GnRH agonists. There are reports in the literature on failed GnRH agonist trigger in which no oocytes were obtained from patients despite multiple appropriate follicle aspirations[7-9]. This condition, known as empty follicle syndrome (EFS), is extremely challenging for both clinicians and couples. The exact etiology of EFS is not clear, and its incidence has been reported to be approximately 1-3.5%[10,11]. EFS is characterized by the inability to retrieve oocytes from the follicles aspirated during the egg retrieval procedure, despite hCG or GnRH-a triggering, even when normal follicles develop during controlled ovarian stimulation with increasing serum E2 levels[8]. Two types of EFS have been defined in the literature. Genuine EFS is an inadequate response to hCG or GnRH-a and is probably related to intrinsic ovarian factors. Pseudo-EFS occurs mostly because of problems related to the structure of the medication or administration methods. Several hypotheses have been proposed regarding the causes of EFS after hCG stimulation. These include early oocyte atresia in functional folliculogenesis despite an apparently normal hormonal response[12], a biological abnormality of mature oocytes to be retrieved despite normal bioavailability[13], genetic factors such as LH/hCG receptor mutations[14], abnormalities in the *in vivo* biological activity of some commercially available batches of hCG or GnRHa[15], rapid clearance of hCG by the liver[15], pharmacological issues, and patient-related administration errors[13].

The aim of this study was to investigate whether retriggering with hCG might be a solution for EFS after triggering a gonadotropin-releasing hormone agonist.

Materials and Methods

Study Design

This study enrolled patients aged 20-42 years diagnosed with polycystic ovarian syndrome and underwent IVF treatment at the Acıbadem Maslak Hospital Assisted Reproductive Technologies Unit between December 2019 and January 2023. All patients were treated using the GnRH antagonist protocol, with the final follicular maturation being induced by GnRH-a. Additionally, patients who were unable to retrieve oocytes after aspirating up to four follicles during egg retrieval and who required rescue triggers were also included in the study. Patients with severe male factor infertility, those diagnosed with malignancy, those with endometriomas, and those diagnosed with hypogonadotropic hypogonadism were excluded from this study.

Ovarian Stimulation

Controlled Ovarian Hyperstimulation was initiated on the second to third days of the menstrual cycle, with the dose of recombinant FSH *(Gonal F; Merck or Fostimon; IBSA)* adjusted between 150 and 300 IU according to individual patient characteristics. When the dominant follicle reached 14 mm, Cetrorelix at a dosage of 0.25 mg per day subcutaneously (Cetrotide from Merck) was administered, and follicle maturation was triggered with triptorelin acetate at a concentration of 0.2 mg/mL (Gonapeptyl 0.1 mg/mL from Ferring) once the leading follicle reached 18 mm. Serum E2 and progesterone (P4) concentrations were measured on the day of trigger.

Oocyte pick-up was performed 36 hours after trigger injection under intravenous sedation. In cases in which no oocytes were retrieved from the initial follicles aspirated, the retrieval procedure was terminated, assuming that follicle maturation had failed. The retrieval attempt was terminated after aspirating

four follicles, with two follicles from each ovary. No oocytes were retrieved despite extensive aspiration and flushing of a minimum of four follicles with a diameter ≥17 mm, which was considered indicative of empty follicle syndrome. As a rescue procedure, 250 mcg recombinant hCG (rhCG) (250 µg/0.5 mL *Ovitrelle from Merck)* was administered subcutaneously. The second oocyte retrieval was scheduled 36 hours after hCG administration.

Retrieved oocytes were fertilized using intracytoplasmic sperm injection and subsequently cryopreserved at the blastocyst stage. Frozen-thawed embryo transfer was performed in hormonally primed cycles. For patients undergoing frozen-thawed embryo transfer, oral contraceptives were initiated on the 2-5th day of the preceding menstrual cycle. Following a 3.75 mg leuprolide acetate depot *(Lucrin; Abbott)* injection subcutaneously during the midluteal phase. Endometrial priming was initiated on the second day of the subsequent menstrual cycle. E2 (2 mg, *Estrofem; Novo Nordisk*) was administered orally, starting at 4 mg for 5 days and then sequentially increasing to 6 mg for an additional 4 days and to 8 mg for a subsequent 5 days. Fourteen days after E2 administration, the endometrium was assessed via transvaginal ultrasound, along with E2 and P4 level measurements. If the endometrial thickness exceeded 8 mm and the P4 level was less than 1.5 ng/mL, vaginal P4 (*Crinone* gel 8% *BID; Merck*) was administered twice daily, and frozen-thawed embryo transfer was scheduled. If the endometrial thickness remained below 8 mm, a 7.8 mg transdermal E2 patch (*Climara; Bayer*) was applied, and the cycle was reassessed 4 days later. If the thickness did not reach 8 mm, the cycle was canceled.

The oocyte utilization rate was calculated as the number of oocytes/number of follicles on the second egg retrieval after the hCG trigger for EFS and the number of oocytes/number of follicles on the first egg retrieval after the GnRHa trigger for non-EFS cases. The oocyte maturity index was calculated as the number of metaphase II (MII) oocytes/number of oocytes collected on the second egg retrieval after the hCG trigger for EFS and the number of MII oocytes/number of oocytes collected on the first egg retrieval after the GnRHa trigger for non-EFS cases. Clinical pregnancy was confirmed by elevated serum ß-hCG levels 12-14 days after embryo transfer, in conjunction with ultrasound verification of the gestational sac or fetal pole. Sustained implantation was defined as discharge from care at 12 weeks of pregnancy with a detectable fetal heartbeat.

Statistical Analysis

All data were analyzed using SPSS (SPSS-IBM 2.3, Inc., Chicago, IL, USA). Shapiro-Wilk test was used to assess data normality. Continuous variables were presented as mean values with accompanying standard deviations, whereas categorical variables were expressed as counts and percentages for both the EFS and non-EFS groups. Statistical significance between mean values was assessed using Student's t-test, and for categorical variables, the chi-squared test was employed. A significance level of $p<0.05$ was considered statistically significant.

Results

A total of 415 women [mean age ± standard deviation (minimum-maximum): 29.9±3.7 (25-42)] were analyzed in the study. Among the GnRHa trigger, the data of 6 (1.4% of total) women diagnosed with EFS were compared with 409 age-follicle number-matched women. Age, duration of infertility, body mass index (BMI), and serum follicle stimulating hormone (FSH) levels were similar between the groups (Table 1). Serum LH levels were compared between the groups and revealed a significantly lower value in the EFS group (6.5±0.9 vs. 8.7±2.2; p=0.02). Serum anti-Müllerian hormone (AMH) levels were also compared between the groups and revealed a significantly higher value in the non-EFS group (3.38±0.68 vs. 4.01±0.72; p=0.04).

The IVF stimulation cycles and laboratory outcomes were also compared between the two groups (Table 2). Variables affecting the oocyte output rate were also compared between the two groups. Among these factors, the duration of stimulation, gonadotrophin dose, number of antral follicles at the start of the cycle, on the trigger day, and the number of follicles ≥14 mm on the trigger day showed similar results between the two groups. Serum E2 and P4 levels were also similar on the trigger day among the two groups. The numbers of oocytes,

Table 1. Sociodemographic characteristics and basal hormonal levels of the patients compared in between the groups

Variables	EFS (n=6)	Non-EFS (n=409)	p
Age (years)	30.8±3.8	29.9±3.7	0.55
Infertility duration (years)	37.7±23.6	40.9±10.1	0.45
BMI	26.4±4.9	26.3±3.5	0.97
FSH (IU/mL)	7.5±1.2	7.4±1.3	0.94
LH (IU/mL)	6.5±0.9	8.7±2.2	0.02*
AMH (ng/mL)	3.38±0.68	4.01±0.72	0.04*

EFS: Empty follicle syndrome, BMI: Body mass index, LH: Luteinizing hormone, FSH: Follicle stimulating hormone, AMH: Anti-Müllerian hormone

MII oocytes, 2 pronuclei (2 pn) embryos, cleavage stage embryos, and blastocysts were significantly lower in the EFS group than in the non-EFS group. The oocyte utilization rate was compared between the groups, and the analysis revealed a statistically significantly lower rate in the EFS group compared with the non-EFS group (62.6±8.4 vs. 80.4±11.6; p<0.01). In contrast to the oocyte utilization rate, the oocyte maturity index was not significantly different between the groups (81.1±7.8 vs. 85.9±10.5; p=0.27).

The data of the six patients diagnosed with EFS are presented in Table 3. The mean age of the patients was 30.8±3.8 (minimum-maximum: 26-37). The mean serum FSH, LH, and AMH levels were 7.5±1.2, 6.5±0.9 and 3.4±0.7 (minimum-maximum: 5.5-9.1; 5.8-8.1; and 2.3-4.1, respectively). The mean serum E2 and P4 levels on the trigger day were 5641±1293 and 0.7±0.1 (minimum-maximum: 4370-7133; and 0.5-0.9, respectively). Analysis of antral follicle count and number of follicles on the trigger day revealed mean numbers of 36.8±8.4 and 30.5±7.2 (minimum-maximum: 26-50 and 24-42, respectively). Analysis of the number of follicles on the second egg retrieval day and the egg utilization rate revealed a mean number of 22.0±7.0 and 62.6±8.4% (minimum-maximum: 16-33; and 50.0-70.6%, respectively). The mean numbers of oocytes, MII oocytes, 2pn embryos, cleavage stage embryos, and number of blastocysts have been reported as 13.3±2.7, 10.8±2.5, 9.2±2.0, 6.5±0.5, and 3.3±0.8 respectively (minimum-maximum: 11-18, 8-15, 7-12, 6-7, and 2-4). Frozen embryo transfer was performed in all patients using hormone replacement therapy as part of the endometrial preparation protocol. Positive pregnancy test and live birth rates among the two groups revealed similar pregnancy test results between the two groups (50.0% vs. 58.4%; p=0.49; 33.3% vs. 49.4%; p=0.69). In the non-EFS group, while 14 patients (3.4%) had a biochemical pregnancy, 2 patients (0.5%) had an ectopic pregnancy, and none of the patients in the EFS group had either a biochemical or ectopic pregnancy. Among the 239 patients with a positive pregnancy test result, 21 (5.1%) experienced miscarriage. Among the three pregnant patients in the EFS group, one had a miscarriage at the 7th gestational week, and two had live births at the 38th and 39th gestational weeks. The mode of delivery was C-section in both patients, with healthy live births of 3460 and 3720 g.

Table 2. IVF outcomes and obstetrics results of the patients compared in between the groups

Variables	EFS (n=6)	Non-EFS (n=409)	p
Duration of stimulation (days)	10.0±1.3	9.1±1.2	0.56
Gonadotrophin dose (IU)	1858.3±205.9	2082.6±420.9	0.19
Antral follicle count	36.8±8.4	39.7±13.1	0.59
# Follicles on the trigger day	30.5±7.2	32.9±12.0	0.62
# Follicles ≥14 mm on the trigger day	25.2±5.9	30.9±11.1	0.21
E2 level on the trigger day (pg/mL)	5641.8±1293.3	5771.8±1756.7	0.86
P4 level on the trigger day (ng/mL)	0.69±0.13	0.77±0.15	0.23
# Oocyte	13.3±2.7	26.2±9.8	<0.01*
Oocyte utilization rate (%)[a]	62.6±8.4	80.4±11.6	<0.01*
# MII oocytes	10.8±2.5	22.9±9.7	<0.01*
Oocyte maturity index (%)[b]	81.1±7.8	85.9±10.5	0.27
# 2pn embryos	9.2±2.0	18.7±8.7	<0.01*
Cleavage embryos	6.5±0.5	17.4±8.1	<0.01*
# Blastocyst	3.3±0.8	5.3±1.4	<0.01*
Positive pregnancy test result (n)	50.0 (3)	58.4 (239)	0.49
Livebirth	33.3 (2)	49.4 (202)	0.69

*: Statistically significant
[a]Oocyte utilization rate: For EFS, number of oocytes/follicle on the second egg retrieval after hCG trigger and for non-EFS, number of oocytes/follicle on the first egg retrieval after GnRHa trigger.
[b]Oocyte maturity index: For EFS, #: MII oocytes/oocytes collected on the second egg retrieval after hCG trigger and for non-EFS, #: MII oocytes/oocytes collected on the first egg retrieval after GnRHa trigger.
Mean ± SD and percentage where appropriate
SD: Standard deviation, EFS: Empty follicle syndrome, MII: Metaphase II, GnRHa: Agonists gonadotropin-releasing hormone, hCG: Human chorionic gonadotropin, IVF: In vitro fertilization, E2: Estradiol, P4: Progesterone

Table 3. Sociodemographic characteristics, basal hormonal levels, IVF outcomes, and obstetrics results of the patients compared in between the groups

	Age	FSH	LH	AMH	E2	P4	AFC	First trigger	Rescue trigger	# Follicles on trigger day	# Follicles on 2nd egg retrieval day	# Oocytes	Oocyte utilization rate (%)	# MII oocytes	Oocyte maturity index (%)	#2pn embryos	- Clevage stage embryos	# Blastocysts	Outcome
1	28	7.2	6.3	3.8	7133	0.89	42	GnRHa	rhCG	32	21	14	66.7	12	85.7	11	7	4	Positive
2	32	7.2	5.8	2.9	6647	0.72	33	DCP	hCG	24	17	11	64.7	9	81.8	7	6	3	Negative
3	31	7.9	6.1	2.3	4491	0.68	50	DCP	hCG	42	33	18	54.5	15	83.3	12	7	3	Negative
4	26	9.1	6.9	4.1	6650	0.54	38	DCP	hCG	35	28	14	50	10	71.4	9	7	4	Negative
5	37	5.5	8.1	3.3	4370	0.78	26	DCP	hCG	24	17	12	70.6	11	91.7	9	6	2	Positive
6	31	8.0	6.0	3.9	4560	0.56	32	DCP	hCG	26	16	11	68.9	8	72.7	7	6	4	Positive

IVF: In vitro fertilization, LH: Luteinizing hormone, FSH: Follicle stimulating hormone, AMH: Anti-Müllerian hormone, GnRHa:: Gonadotropin-releasing hormone agonist, 0.2 mg; rhCG: recombinant hCG, 250 g, hCG: Human chorionic gonadotropin, E2: Estradiol, P4: Progesterone, AFC: Antral follicle count, MII: Metaphase II

Discussion

The introduction of the GnRH antagonist protocol enabled the use of GnRH agonists for oocyte maturation, dramatically reducing the risk of OHSS. However, GnRHa acts directly at the pituitary level; therefore, in cases of transient or permanent pituitary dysfunction, an increase in LH induced by the GnRHa trigger may be insufficient. This condition can lead to suboptimal maturation or empty follicle syndrome[16]. EFS is a rare but frustrating complication that can lead to cycle cancelation. Two types of EFS have been described in the literature. Genuine EFS is due to intrinsic factors, and false EFS is due to pharmacologic factors or patient practice[8]. According to a systematic review, 67% of EFS cases were false, that is, due to administration errors or pharmacological problems[17]. In this study, we analyzed data from 415 high-responder women. Our analysis revealed an incidence of 1.4% EFS among patients with no oocytes during the first egg retrieval after GnRHa trigger and a high utilization rate of oocytes within 36 hours after a second trigger (almost 72 hours after the first trigger).

Significant differences exist between the LH peak induced by GnRHa, the LH peak in the natural cycle, and continuous LH stimulation by hCG injection. The LH peak in the natural cycle is characterized by three phases with a total duration of 48 h[18]. The GnRHa-induced LH peak is characterized by a 2-phase rise lasting 28-32 hours[18,19]. However, with a long half-life, hCG produces LH-like activity that persists for 9-10 days[20,21]. Therefore, EFS caused by hCG and GnRH may not reflect the same pathology. The response to GnRH is independent of age, type of COS, and ovarian response[8]. In our study, there was no significant age difference among patients. Furthermore, the stimulation protocol was the same for all patients.

Retriggering with hCG is an option in patients who do not respond to GnRHa trigger. Chang et al.[22] reported one of the largest cohorts of patients, comprising 1.878 cycles using a GnRH agonist trigger. Within this cohort, 16 cycles exhibited inadequate response. These patients were subsequently triggered with hCG, leading to comparable outcomes in terms of the number of retrieved oocytes, number of normally fertilized (2pn) oocytes, pregnancy rates, and birth rates resulting from the first embryo transfer procedure. Additionally, the cumulative pregnancy and birth rates from all fresh and cryopreserved embryo transfers were similar[22]. In another report analyzing the use of hCG in poor responders to GnRH agonist trigger, the authors concluded that retriggering solely due to insufficient oocyte retrieval during the initial aspiration attempt might result in oocyte oversaturation, subsequently elevating the risk of abnormal fertilization[23]. Our study showed that after hCG trigger, >70% of the follicles persisted during the second egg retrieval. Although the oocyte utilization rate was lower in the EFS group than in the non-EFS group (almost 60% vs. 80%), the oocyte maturity index was similar between the two groups (80%). In addition, cleavage stage embryos and blastulation rates were statistically significantly lower in the EFS group, whereas positive pregnancy test results and miscarriage rates were not statistically significantly different (50% vs. 58% and 33.3% vs. 49.4%, respectively). In addition, no severe OHSS cases were reported after the second trigger with hCG, even though it has been reported as 0.5-5% in the literature[10].

Different potential factors that might increase the likelihood of a failed trigger with a GnRH agonist were examined, with a specific focus on the hypothesis that a lower BMI and baseline LH concentrations at the beginning of the cycle could be linked to a greater risk of GnRH failure[22]. In our study, the dose of GnRH was standard (0.2 mg independent of the BMI), which limits the effect of inadequate dosage of GnRHa administration. LH levels at the start of the cycle were statistically significantly lower in the EFS group than in the non-EFS group. Kitasaka et al.[23] showed that LH levels ≤0.35 IU/L at the start of the cycle might be attributed to an increased risk of EFS. However, the low LH level (6.5±0.9) was above the level of hypophysial hypogonadotrophism; therefore, the reason might not be attributed to hypo-hypo status. In addition, posttrigger LH levels have been suggested as a risk factor for EFS[24]. First, it is difficult to determine a cutoff level for the posttrigger LH threshold. LH reaches a peak level approximately 4 hours after the trigger and decreases gradually within 24 hours. In addition, since the LH has a pulsatile pattern, the measurement of one LH level might be higher than its exact level[25,26]. Kummer et al.[26] showed that LH level <15IU/L might be associated with an 18.8% increased risk of EFS. However, the optimal GnRHa dose might be affected by BMI, endogenous LH molecule, or LH receptor structure and should be investigated in future research. In a case series of 8 patients with failed GnRH agonist triggering following rescue hCG triggering, it was reported that seven patients successfully achieved ongoing pregnancies and delivered after frozen embryo transfer[9]. The first pregnancy after fresh embryo transfer following retriggeration with hCG was reported in 2015[10]. In our study, among the six frozen embryo transfer cycles, pregnancy was achieved in 3 patients with a similar pregnancy rate to the control group. Among the three pregnancies, one resulted in miscarriage at the 7th week of gestation. The other two patients had live births, reaching term (33% of total). From this point of view, we can say that hCG retrigger after GnRHa trigger might also have pregnancy and live birth results comparable to those of the control group.

Study Limitations

The strength of this study was that it showed comparable IVF outcomes in terms of oocytes and embryos, as well as pregnancy outcomes, to controls. A weakness of our study might be its retrospective nature and lack of serum LH levels at the first egg retrieval time with no oocytes. As an opportunity, this study will maintain data regarding a management modality in the case of no oocytes due to different etiologic factors.

Conclusion

Retrigger with hCG in hyperresponder patients with no oocytes after GnRHa trigger may be an alternative strategy without cycle cancelation. Although egg utilization rates might have been lower in the second procedure than in the controls, the oocyte maturity index might have been comparable to that of the controls. Fresh embryo transfer might be debatable, but frozen embryo transfer might result in comparable positive pregnancy test results and live birth rates with the hCG retrigger policy.

Acknowledgements: Authors thank the Acıbadem Embryology Laboratory members for their valuable contributions to the study.

Ethics

Ethics Committee Approval: Ethical approval was waived by the Acıbadem Mehmet Ali Aydınlar University Ethics Committee (ATADEK- 2023-07/231) in view of the retrospective nature of the study and all the procedures being performed were part of the routine care.

Informed Consent: Retrospective study.

Authorship Contributions

Surgical and Medical Practices: Z.E.U.K., A.Y., Ö.K., Y.Ç., B.T., Concept: Z.E.U.K., A.Y., Ö.K., Y.Ç., B.T., Design: Z.E.U.K., Y.Ç., B.T., Data Collection or Processing: Z.E.U.K., A.Y., Ö.K., Y.Ç., B.T., Analysis or Interpretation: Z.E.U.K., Y.Ç., B.T, Literature Search: Z.E.U.K., Y.Ç., Writing: Z.E.U.K., Y.Ç., B.T.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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Clinical Investigation / Araştırma

DOI: 10.4274/tjod.galenos.2024.84031

Turk J Obstet Gynecol 2024;21:142-52

True empty follicle syndrome is a subtype of oocyte maturation abnormalities

Gerçek boş folikül sendromu oosit olgunlaşma anomalilerinin bir alt türüdür

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Abstract

Objective: To review the outcomes of in vitro maturation (IVM) and in vitro fertilization (IVF) in women with empty follicle syndrome (EFS). The study evaluated the genetic underpinnings of EFS by analyzing mutations.

Materials and Methods: This retrospective case series involving 17 women with EFS over at least 2 IVF cycles was conducted. The study also employed whole-exome sequencing to analyze the genetic mutations. The treatment approaches included letrozole-primed IVM, follicle-stimulating hormone (FSH)-human chorionic gonadotrophin (hCG)-primed IVM, and conventional IVF.

Results: The average female age was 31.5±4.6 years, and the duration of infertility was 7.3±3.5 years. Four patients underwent IVF. IVM oocyte collections yielded oocytes in 12 of 13 subjects. Of these, 75% (9/12) yielded MII oocytes after 48 h of IVM media incubation. Six subjects had fertilized embryos, resulting in a 40.9% intracytoplasmic sperm injection (ICSI) fertilization rate (9 embryos/22 MII oocytes). Genetic analysis revealed mutations in seven patients. This study demonstrated the partial efficacy of letrozole-primed IVM plus growth hormone and FSH-hCG primed IVM protocols. No pregnancies or live births were recorded after IVM. One ongoing pregnancy post-IVF and one spontaneous live birth were observed.

Conclusion: Inter-cycle variabilities were observed in women with oocyte maturation abnormalities (OMAs). Almost all patients with EFS had oocytes collected during IVM following IVF. These oocytes have limited potential for maturation, fertilization, and live birth, as demonstrated by the low rates observed after IVM culture and ICSI. These conditions are observed in OMAs due to defects in the oocyte machinery. The proposed flowchart provides a comprehensive classification approach for various forms of EFS.

Keywords: Empty follicle syndrome, in vitro maturation, oocyte maturation abnormalities, oocyte maturation arrest

PRECIS: Empty follicle syndrome.

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Received/Geliş Tarihi: 27.05.2024 **Accepted/Kabul Tarihi:** 16.07.2024

Öz

Amaç: Bu çalışmada, boş folikül sendromu (BFS) olan kadınlarda in vitro matürasyon (IVM) ve in vitro fertilizasyon (IVF) tedavi sonuçlarını gözden geçirmeyi amaçladık. Çalışmada, BFS'nin genetik temelleri analiz edilerek mutasyonlar değerlendirildi.

Gereç ve Yöntemler: En az 2 IVF döngüsünde BFS olan 17 kadını içeren retrospektif bir olgu serisi gerçekleştirildi. Genetik mutasyonları analiz etmek için bütün ekzon dizilimi kullanıldı. Tedavi yaklaşımı, letrozol ile ön hazırlıklı IVM, folikül uyarıcı hormon (FSH)-insan koryonik gonadotropini (hCG) ile ön hazırlıklı IVM ve konvansiyonel IVF'yi içeriyordu.

Bulgular: Ortalama kadın yaşı 31,5±4,6 yıl ve infertilite süresi 7,3±3,5 yıl idi. Dört hastada IVF uygulandı. IVM ile, 13 olgunun 12'sinde oosit elde edildi. Bu toplamaların %75'inde (9/12) IVM medyumunda 48 saat inkübasyon sonrası MII oositler elde edildi. Altı hastada embriyo elde edildi ve fertilizasyon oranı %40,9 (9 embriyo/22 MII oosit) gözlendi. Genetik analiz ile yedi olguda mutasyon saptandı. Çalışma, letrozol ile ön hazırlıklı IVM artı büyüme hormonu ve FSH-hCG ile ön hazırlıklı IVM protokollerinin kısmi etkinliğini gösterdi. IVM sonrası hamilelik ve canlı doğum kaydedilmedi. IVF sonrası devam eden bir gebelik ve bir spontan canlı doğum gözlendi.

Sonuç: Oosit matürasyon anormallikleri (OMA) olan kadınlarda döngüler arası değişkenlikler gözlendi. IVF sonrası IVM sırasında BFS'li hastaların neredeyse tamamında oositler toplandı. Bu oositler, IVM kültürü ve intrasitoplazmik sperm enjeksiyonu sonrası düşük oranlarla gösterildiği üzere, sınırlı matürasyon, döllenme ve canlı doğum potansiyeline sahiptir. Bunlar, oosit makinelerindeki kusurlar nedeniyle OMA'da görülen durumlardır. Önerilen akış şeması, BFS'nin çeşitli formlarının sınıflandırılmasına yönelik kapsamlı bir yaklaşım sunmaktadır.

Anahtar Kelimeler: Boş folikül sendromu, in vitro matürasyon, oosit matürasyon anormallikleri, oosit matürasyon arresti

Introduction

Failure to yield oocytes from fully developed follicles at oocyte pick up (OPU) 35-37 hours after human chorionic gonadotropin (hCG) injection during an in vitro fertilization (IVF) cycle is defined as empty follicle syndrome (EFS)[1]. EFS has been a subject of intense debate regarding whether it is a true entity or failure of IVF cycle management. Before the implementation of gonadotropin-releasing hormone analogs (GnRHa) for triggering in antagonist IVF cycles, EFS was subdivided into two subtypes: The first is known as false-EFS (fEFS), which is due to the lack of hCG application before OPU or low efficacy of applied hCG; the second is referred to as genuine-EFS (gEFS), which is not related to errors in hCG but a repeated failure in at least two consecutive cycles to collect oocytes despite adequate dose and timing of hCG application[2].

The prevalence of gEFS ranges from 0.045% to 7% in the literature[3-6]. Hourvitz et al.[7] published the first successful pregnancies and live births via in vitro maturation (IVM) in women with gEFS, the cause of which was accepted as part of the oocyte maturation abnormalities (OMAS). Human genetic studies have revealed that mutations related to oocyte maturation and zona pellucida (ZP) play crucial roles in oocyte development[8]. Most cases of EFS were found to have mutations related to ZP proteins[9-11]. To date, more than 25 mutations in ZP-related proteins have been reported[12].

For follicular growth and oocyte developmental competence, the corona radiata projections and oocyte microvilli must be in contact with the ZP. Absence of, or deficiency of ZP formation impairs bidirectional communication between the oocyte and surrounding cumulus cells and diminishes oocyte development by destabilizing the cumulus, and affected subjects may develop female infertility[13-15]. Defects in ZP, especially those related to integrity rather than thickness, result in a lack of oocyte development, defective folliculogenesis, and early granulosa cell apoptosis, which may explain the pathophysiology of EFS[16]. Hatirnaz et al.[17] previously reported intracycle and intercycle variabilities in women with OMAs[17]. Among patients who had undergone 2-8 previous IVF attempts, some experienced EFS in at least two cycles and experienced oocyte maturation arrest and biochemical pregnancies in different cycles. This observation suggests that the follicles are not empty in women diagnosed with gEFS, but rather are due to oocyte failure. It is noteworthy that even in women with gEFS associated with genetic mutations, there may be potential for improvement in oocyte yield and maturation. EFS can be redefined as 1) EFS as part of OMAs, 2) Genuine EFS (gEFS) (altered gene expressions, or early apoptosis, etc.), 3) GnRHa use-related EFS, and 4) hCG-related EFS (fEFS). FEFS is a poorly researched condition that affects some women undergoing IVF treatment. HCG-treatment-related errors appear to be the main mechanism underlying fEFS. Improper hCG administration has often been the most common cause[3]. Some studies have suggested that increasing the dose of gonadotropins, administering a second (rescue) dose of hCG, or extending the duration from hCG injection to oocyte collection may improve the chances of retrieving oocytes.

The effectiveness of these approaches remains unclear and requires further investigation[18]. A potential preventative measure of serum hCG level the day after the trigger and a second bolus of hCG administration has been proposed to prevent against fEFS[5]. Clinicians can trigger GnRHa, especially in hyperresponder patients, because of the shorter surge effect of GnRHa compared with urinary or recombinant hCG[19-21]. The shorter duration of luteinizing hormone (LH) activity observed in GnRHa cells substantially limits the risk of OHSS but may also contribute to diminished oocyte performance, even leading to false EFS[22]. The mechanism of fEFS in this case was different from that observed with hCG, where GnRH agonists induce the release of LH and follicle-stimulating hormone (FSH) from the pituitary. Therefore, with any temporary or permanent factor leading to the dysfunction of the pituitary gland (i.e., hypogonadotropic-hypogonadism), the expected flare action of the analog can be altered and may be responsible for diminished

oocyte yield, maturation problems, and EFS[23]. For cases with a history of previous EFS, a dual trigger can be planned. During GnRH agonist-triggered cases, especially for patients with the predisposing factors listed subsequently, if no oocyte is yielded from an ovary, the procedure can be stopped, and a second trigger is planned, usually consisting of hCG. Here we performed a case series, identified 17 women with gEFS who had letrozole-primed IVM plus growth hormone (GH), FSH-hCG-primed IVM treatment, and IVF, and investigated oocyte maturation and embryo development, as well as pregnancy outcomes in women who had repetitive EFS after undergoing at least two IVF cycles. Additionally, in this paper, we introduce a flowchart for managing the multiple forms of gEFS. This study aimed to review IVM and IVF treatment outcomes in women with EFS and evaluate the genetic underpinnings of EFS by analyzing mutations.

Materials and Methods

In this case series study, 17 women who had gEFS in their previous (at least two) IVF cycles were detected. Only patients who had gEFS after hCG trigger were included in the study group. FEFS and EFS secondary to GnRH agonist triggering were not included in the study. Demographic characteristics and basal hormonal parameters were all evaluated and recorded. The 17 women were contacted, and written informed consent was obtained from the women studied. This study was approved by the Clinical Research Ethics Committee of Samsun University, Turkey (decision date: 26.04.2023, decision number: 2023/8/20).

Letrozole-priming IVM Plus Growth Hormone

Letrozole 2.5 mg tablets (PO) twice daily started on day 3 of the menstrual cycle for 5 days following the initial transvaginal ultrasound examination. On day 7, a second transvaginal ultrasound examination was performed to measure follicular growth and endometrial thickness. Recombinant GH (*Genotropin* 36 IU/12 mg) 2 mg/day subcutaneously was added to the treatment regimen on day 10 for 6 days. A third ultrasound examination was conducted to assess the antral follicles. Once at least 2-3 follicles reached a diameter of 10-12 mm and the endometrial thickness reached 8 mm, a 6.500 IU subcutaneous injection of hCG (*Ovitrelle, Merck Serono, Turkey*) was administered 38 h prior to oocyte collection. Oocyte retrieval was performed under an aspiration pressure of 80 mmHg using an 18-gauge aspiration needle with a double lumen for continuous flushing. Embryos obtained in this protocol were frozen either at the cleavage or blastocyst stages, and a frozen-thawed embryo transfer cycle was performed 1-2 months after the procedure.

FSH-hCG-priming IVM

Recombinant FSH (*Gonal-f, Serono*) was administered subcutaneously at a daily dose of 75-150 IU, starting on day 3 of the menstrual cycle and continuing for 3 days following the initial transvaginal ultrasound examination. On day 7, a second transvaginal ultrasound examination was performed to measure follicular growth and endometrial thickness. Around day 9-10, a third ultrasound examination was performed to measure the size of the antral follicles. When follicles reach 10-12 mm in diameter and the endometrial thickness reached 8 mm, recombinant hCG 6500 IU (*Ovitrelle, Merck Serono*) was administered 38 h before oocyte retrieval. Oocyte retrieval was performed with a 17-18-gauge aspiration needle with a double lumen. Embryos obtained in this protocol were transferred to the fresh cleavage stage, and the remaining embryos, if any, were frozen at the cleavage stage.

Luteal Phase Support

The luteal phase was supported with estradiol valerate at a dose of 6-8 mg per day until fetal heartbeat was detected during ultrasound examination. Progesterone 200 mg capsules (800 mg/day (*Koçak Farma, İstanbul, Turkey*) intravaginally were used routinely in both letrozole primed IVM plus GH and in FSH-hCG IVM cycles until the 12th week of gestation.

Laboratory Procedures

Laboratory procedures for IVM were performed using both methods according to a modified protocol, as previously described[24]. The authors have been using MediCult IVM culture systems (CooperSurgical, USA) since 2007. A SAGE Vitrification Kit (Cooper Surgical, USA) was used for the vitrification and warming procedures.

The maturation process was assessed at 26 and 48 h. Oocytes that had matured at 26 h underwent intracytoplasmic sperm injection (ICSI) as further culture was deemed unnecessary. Any remaining immature oocytes were maintained in IVM culture until 48 h. Oocytes that matured at 48 h were then subjected to ICSI. The cleavage-stage embryos were graded according to the system reported by Hsu et al.[25]. Blastocysts were graded according to a previously reported system by Neuber et al.[26].

A pregnancy test was administered on either day 10 post-blastocyst transfer or day 12 following cleavage-stage embryo transfer. Serum hCG levels exceeding 5 IU/L during the initial assessment indicated a positive pregnancy. Clinical pregnancy was defined as the detection of an intrauterine fetus with a heartbeat on ultrasound. Ongoing pregnancy was defined as the pregnancies over 12th weeks of gestation. A spontaneous abortion is defined as the presence of an empty gestational sac or a gestational sac containing an embryo or fetus without fetal heart activity within the first 12 weeks of gestation. Live birth was defined as the birth of a living child born after 28 gestational weeks. Falling beta hCG levels without visualization of pregnancy on ultrasound were considered as biochemical pregnancy loss.

Whole-exome Sequencing

Genetic mutations in 9 out of 17 patients with gEFS were analyzed by whole-exome sequencing (WES). Briefly, DNA

was extracted from whole blood samples using a QIAmp kit (Hilden, Germany) in a QIAcube HT system (Hilden, Germany) following the manufacturer's instructions (Richards et al. 2015). Library preparation/comparison was conducted using a QIAseq Index I set A kit (Hilden, Germany) in combination with a custom-designed targeted NGS panel (CDHS-15607Z-1008) for female infertility-associated genes.

Statistical Analysis

Statistical analyses were performed using SPSS software (IBM SPSS Statistics, Version 21.0, USA). Descriptive statistics, including means and standard deviations, were calculated to summarize the demographic and baseline clinical characteristics of the study participants.

Results

The average age of the 17 female participants was 31.5±4.6 years (range: 24-38), and the average duration of infertility averaged 7.3±3.5 years (range: 1.5-15.0). Basal serum measurements revealed FSH levels at 8.24±3.56 IU/L, LH levels at 8.93±6.40 IU/L, estradiol levels at 59.67±44.48 pg/mL, progesterone levels at 0.58±0.29 ng/mL, thyroid stimulating hormone levels at 1.93±0.99 mIU/mL, T3 levels at 2.96±0.40 pg/mL, T4 levels at 1.16±0.25 ng/mL, anti-Mullerian hormone levels at 2.96±2.03 ng/mL, PRL levels at 21.17±9.74 ng/mL, and antral follicle count of 17.4±8.9. Notably, four (out of the 17) patients did not undergo the IVM procedure. IVM procedures resulted in successful oocyte collection in 12 of 13 subjects. Among these, 9 out of 12 subjects (75%) yielded MII oocytes after 48 h of incubation with IVM medium (with a range of mature oocytes in those who matured being 2-8). Only 6 subjects ended up with fertilized embryos (9 of 22 MII oocytes total. This represents a fertilization rate of 40.9% with ICSI. None of the subjects in this study had experienced any IVM pregnancies. One patient achieved an ongoing pregnancy following an IVF cycle (Case 5), while another case (Case 2) resulted in a spontaneous live birth.

The detailed demographic, clinical, and laboratory characteristics of each patient are presented in Table 1.

The application of IVF and IVM treatments and outcomes are outlined in Table 2.

Wes analysis was performed on 9 subjects. Genetic mutations (FSHR mutations, TACR3 mutation, LHX4, mutation, STAG3 mutation, ZP1 mutation, ZP3 mutation, PATL2 mutation and LHCGR mutation) were detected in 7 women. Cases 7 and 8 had no mutation in WES analysis. In eight patients (Case 1, 3 9, 10, 14-17), WES analysis was not performed, as indicated in Table 3.

- In Case 2, a heterozygous mutation in STAG3 was identified on Exon 4, specifically at nucleotide position c.319G>A. This mutation follows an autosomal recessive (AR) inheritance pattern.
- Case 4 exhibited a homozygous mutation in the *LHCGR* gene located in Exon 11 at nucleotide position c.970_971del A>G, consistent with AR inheritance.
- Case 5 was found to have a novel homozygous mutation in the *ZP1* gene in Intron 11, denoted as c.1775.3C>G, which also followed an AR inheritance pattern.
- A more complex genetic profile was observed in Case 6, which exhibited three mutations: a homozygous mutation in the *FSHR* gene on Exon 10 (p. S680N), a heterozygous mutation in the same gene but at a different site (Exon 10, p. A307T), and additional heterozygous mutations in the *TACR3* gene (Exon 2, c.737c>t) and *ZP3* gene (Exon 2, c.382A>C). The FSHR and TACR3 mutations follow AR inheritance, whereas the ZP3 mutation is autosomal dominant.
- Case 11 showed a heterozygous mutation in LHX4 located at c.1158T>A, which followed an AR inheritance pattern.
- In Case 12, two homozygous mutations were detected in PATL2: one in Exon 7 (c.320C>T) and the other in Intron 7 (c.446+1G>C), both consistent with AR inheritance.
- Case 13 was identified with a homozygous mutation in ZP1 on Exon 12, which was noted as C1775-3C>A, following an AR pattern.

Detailed data are presented in Table 3.

Discussion

Our paper introduces several novel insights into the study of EFS. First, we present EFS as a subtype of OMAs that is determined by genetic mutations through a comparative study. Second, it explores the role of a letrozole-primed plus GH IVM protocol in women with gEFS. OMAs often result in the collection of 100% immature oocytes, even after triggering with hCG, GnRH agonist, or both[27,28]. IVM is performed to collect oocytes from follicles measuring 2-10 mm, smaller than the size in IVF cycles. IVM, being independent of the development of LH receptors in the follicle, as required in IVF, could serve as a treatment for EFS. The study investigated 17 women diagnosed with gEFS. Out of the 9 women analyzed by WES, seven had various mutations noted (FSHR mutations, TACR3 mutation, LHX4, mutation, STAG3 mutation, ZP1 mutation, ZP3 mutation, PATL2 mutation and LHCGR mutation). Two women did not present with mutations in their WES analysis. The results of this study indicated that IVM for gEFS, whether using the letrozole-primed IVM + GH protocol or FSH-hCG primed IVM, may result in oocytes and embryos in rare cases but is not likely to result in a live birth. This study also demonstrated for the first time that EFS is a subtype of OMA and that genetic testing for mutations is highly recommended if available.

Since its first report, EFS has been a hot topic of debate in the field of assisted reproductive technology[1]. EFS is classified as g EFS, in which repeated failure to retrieve oocytes with appropriate blood hCG levels is observed, and fEFS, in which a low blood level of hCG (<40 IU/L) is detected either due to misuse of hCG for triggering or low bioavailability of the HCG used[29,30]. Filtrates of follicular fluids collected from patients diagnosed with gEFS during a stimulated IVF cycle were investigated, and it was demonstrated that immature

Table 1. Demographic and clinical variables

Patients	Age (years)	Time to infertility (years)	Basal serum FSH (IU/L)	Basal serum LH (IU/L)	Basal serum E2 (pg/mL)	Basal serum P4 (ng/mL)	Basal serum TSH (mU/mL)	Basal serum T3 (pg/mL)	Basal serum T4 (ng/mL)	Basal serum AMH (ng/mL)	Basal serum PRL (ng/mL)	Total AFC (number)
Case 1	24	4	11.10	5.70	21.20	0.40	3.53	3.50	1.12	2.10	22.90	9
Case 2	29	2	11.20	2.53	125.00	0.20	1.10	3.60	0.90	0.43	18.70	8
Case 3	33	15	4.70	3.02	40.00	0.30	1.25	2.50	1.40	4.60	47.60	20
Case 4	37	11	18.40	27.2	38.00	0.30	1.98	2.85	1.00	0.50	21.70	5
Case 5	32	5	8.05	9.00	35.25	0.60	1.52	2.78	1.05	6.44	34.78	26
Case 6	31	7	10.70	6.50	10.00	0.45	2.31	3.45	1.24	1.04	24.10	6
Case 7	29	8	10.08	4.68	35.83	0.46	2.48	3.00	1.27	0.43	6.16	5
Case 8	29	1.5	7.35	6.40	68.56	0.28	3.19	2.89	1.24	4.60	12.68	32
Case 9	32	12	6.60	7.00	15.00	0.76	4.40	2.64	0.99	3.61	20.09	20
Case 10	35	6	6.45	20.61	53.10	0.36	2.06	2.86	0.93	5.78	24.14	18
Case 11	28	7	3.04	5.97	143.13	0.89	1.08	2.45	1.22	3.08	8.99	28
Case 12	25	7	8.46	7.80	62.01	0.97	1.05	2.45	0.89	0.97	17.50	22
Case 13	38	10	6.86	4.98	42.08	0.78	2.13	3.04	1.45	1.64	14.27	12
Case 14	37	10	8.79	12.40	49.32	0.97	1.05	3.04	0.96	5.45	19.12	28
Case 15	38	4	6.45	11.46	46.02	0.98	1.45	3.45	1.42	3.05	20.05	23
Case 16	25	7	8.46	11.56	62.01	0.97	1.05	2.45	0.89	4.98	17.05	22
Case 17	33	7	3.50	5.040	168.00	0.20	1.22	3.45	1.800	1.76	30.10	11
Total (mean ± SD)	31.5±4.6	7.3±3.5	8.24±3.56	8.93±6.40	59.67±44.48	0.58±0.29	1.93±0.99	2.96±0.40	1.16±0.25	2.96±2.04	21.17±9.74	17.35±8.89

AFC: Antral folicle count, AMH: Anti-Müllerian hormone, E2: Estradiol, FSH: Folicle stimulating hormone, hCG: Human chorionic gonadotropin, LH: Luteinizing hormone, P4: Progesterone, PRL: Prolactin, SD: Standard deviation, TSH: Thyroid stimulating hormone

Table 2. Treatments administered to patients and their outcomes

Patients	OMA diagnosis	IVF treatments			IVM treatments				Additional information
		IVF cycle numbers	Collected oocytes	Outcome	IVM cycle numbers	Collected oocytes	Matured oocytes	Outcome	
Case 1	EFS/OMA TYPE V	4	0, 0, 4, 0	1 Spontaneous abort (from a fresh transferred day 3 and grade 2 embryo), others no fertilization	3	4, 1, 4	3, 0, 2	Two ET (each fresh transferred day 3 and grade 2 embryos), negative hCG test	Severe OAT, spontaneous abortion, and EFS as part of OMA
Case 2	EFS/POI/ POF	2	0, 0	None	4	0, 5, 3, 3	0, 2, 0, 2	Two ET (each fresh transferred day 3 and grade 2 embryos), negative hCG test/ Frozen embryos	Three cases of spontaneous biochemical pregnancy loss, one case of spontaneous livebirth, EFS as part of OMAs
Case 3	EFS	3	0, 0, 0	None	2	2, 4	0, 0	None	True EFS
Case 4	EFS/POI/ POF	3	0, 0, 0	None	2	2, 5	2, 0	One ET (fresh transferred day 3 and grade 2 embryo), negative hCG test	True EFS
Case 5	EFS/Zona free	4	0, 0, 1, 2	3rd attempt: Zone-free oocyte ICSI and embryo frozen (grade 2). 4th attempt: 2 grade 2 embryos. One transferred frozen, pregnancy	No IVM	-	-	-	EFS as part of the OMAs, oocytes with zona free formation were also obtained.
Case 6	EFS/POI/ POF	4	0, 0, 0, 1	Degenerated oocyte	2	1, 1	0, 0	None	True EFS and zona-free degenerated
Case 7	EFS/POI/ POF	4	0, 0, 0	None	1	0	0	None	True EFS
Case 8	EFS	2	0, 0	None	1	7	3	One ET (frozen/thawed transferred day 3 and grade 2 embryo), negative hCG test	True EFS
Case 9	EFS	2	0, 0	None	No IVM	-	-	-	True EFS
Case 10	EFS	3	0, 0, 0	None	1	3	0	None	True EFS

Table 2. Continued

Patients	OMA diagnosis	IVF treatments			IVM treatments				Additional information
		IVF cycle numbers	Collected oocytes	Outcome	IVM cycle numbers	Collected oocytes	Matured oocytes	Outcome	
Case 11	EFS	2	0, 0	None	1	6	3	One ET (fresh transferred day 3 and grade 2 embryo), negative hCG test	Spontaneous biochemical pregnancy loss and EFS as OMAs
Case 12	EFS	5	0, 0, 0, 0	None	No IVM	-	-	-	EFS as an OMA component One of three sisters with ZP1 mutation in a consanguineous family
Case 13	EFS	3	0, 0, 0	None	No IVM	-	-	-	EFS as an OMA component
Case 14	EFS	2	0, 0	None	1	12	0	None	Male azoospermia, true EFS
Case 15	EFS	2	0, 0	None	1	14	0	None	True EFS
Case 16	EFS	2	0, 0	None	1	8	0	None	Ture EFS
Case 17	EFS/OMA TYPE V	3	0, 0, 1	Biochemical pregnancy loss (from a fresh transferred day 3 and grade 2 embryo)	2	5, 2	5, 0	Two ET (each fresh transferred day 3 and grade 2 embryos), negative hCG test	EFS as an OMA component

EFS: Empty folicle syndrome, ET: Embryo transfer, hCG: Human chorionic gonadotropin, IVF: In vitro fertilization, IVM: In vitro maturation, OAT: Oligoasthenoteratozoospermia, OMA: Oocyte maturation arrest, OMAs: Oocyte maturation abnormalities, POF: Premature ovarian failure, POI: Premature ovarian insufficiency, ZP1: Zona pellucida glycoprotein 1

oocytes were present in the filtrates and that these could mature *in vitro* using IVM culture media[31]. This finding, though it is only a case report, showed that the follicles were not empty. Inan et al.[32] reported the whole gene expression profile of granulosa cells from the follicular fluid of a woman who underwent three repeated gEFS and showed that around 160 genes were differentially expressed. This study is the first to demonstrate the role of gene expressions that trigger early apoptosis in healthy oocytes and fail maturation. Thin ZP was associated with early apoptosis of oocytes present in 200 preantral follicles. Ovarian aging or low ovarian reserve is also a cause of gEFS[32,33]. A possible genetic cause of gEFS was reported by Onalan et al.[34], in which two sisters with moderate sensorineural deafness were diagnosed with gEFS due to alterations in the transient and sequential expression of epidermal growth factor, which is essential in the meiotic resumption process. The recurrence rate of EFS was reported to be 15.8%[35]. Baum et al.[35] found that patients with recurrent EFS exhibited significantly prolonged infertility and lower estrogen levels compared with those with sporadic EFS. However, their study did not include hCG hormone analysis; thus, the differentiation of EFS subtypes was not performed. Additionally, no genetic screening was performed for either sporadic or recurrent cases. Therefore, elucidating the underlying cause is challenging based on these data.

Mutation studies related to female infertility have revealed the involvement of ZP1-4, LHCGR, STAG3, and mutations related to premature ovarian failure (POF) that play a role in the pathogenesis of EFS. Thus, EFS, POF, and resistant ovary syndrome were included as OMA subtypes.

The significance of EFS in relation to mutations in the *LHCGR* gene[36,37]. We identified an LHCGR mutation in one of our EFS cases (Case 4) in which IVM resulted in oocyte retrieval and embryo transfer, but pregnancy was not achieved.

Furthermore, the relevance of EFS has been emphasized in connection with mutations in the *ZP1* gene[38,39]. In our study, a noteworthy observation was made regarding the ZP1 mutation within a consanguineous family, specifically involving three sisters. It exhibited distinct manifestations in the three sisters, particularly in the eldest sister (Case

Table 3. Mutation screening in patients

Patients	Mutation 1 (zygosity/ inheritance)	Gene 1	Nucleotide 1	Mutation 2 (zygosity/ inheritance)	Gene 2	Nucleotide 2	Mutation 3 (zygosity/ inheritance)	Gene 3	Nucleotide 3	Mutation 4 (zygosity/ inheritance)	Gene 4	Nucleotide 4
Case 1	Not evaluated	-	-	-	-	-	-	-	-	-	-	-
Case 2	STAG3 (Het./AR)	Exon 4	c.319G>A	-	-	-	-	-	-	-	-	-
Case 3	Not evaluated	-	-	-	-	-	-	-	-	-	-	-
Case 4	LHCGR (Hom./AR)	Exon 11	c.970_971 DEL A>G	-	-	-	-	-	-	-	-	-
Case 5	ZP1 (Hom./AR)	Intron 11	c.1775.3C>G (NOVEL)	-	-	-	-	-	-	-	-	-
Case 6	ZP3 (Het./AD)	Exon 10	p. S680N	FSHR (Het./AR)	Exon 10	p. A307T	TACR3 (Het./AR)	Exon 2	c.737c>t	FSHR (Hom./AR)	Exon 2	c.382A>C
Case 7	No mutation was detected	-	-	-	-	-	-	-	-	-	-	-
Case 8	No mutation was detected	-	-	-	-	-	-	-	-	-	-	-
Case 9	Not evaluated	-	-	-	-	-	-	-	-	-	-	-
Case 10	Not evaluated	-	-	-	-	-	-	-	-	-	-	-
Case 11	LHX4 (Het./AR)	Exon 6	c.1158T>A	-	-	-	-	-	-	-	-	-
Case 12	PATL2 (Hom./AR)	Exon 7	c.320C>T	PATL2 (Hom./AR)	Intron 7	c.446+1G>C	-	-	-	-	-	-
Case 13	ZP1 (Hom./AR)	Exon 12	c.1775-3C>A	-	-	-	-	-	-	-	-	-
Case 14	Not evaluated	-	-	-	-	-	-	-	-	-	-	-
Case 15	Not evaluated	-	-	-	-	-	-	-	-	-	-	-
Case 16	Not evaluated	-	-	-	-	-	-	-	-	-	-	-
Case 17	Not evaluated	-	-	-	-	-	-	-	-	-	-	-

FSHR: Follicle stimulating hormone receptor, LHCGR: Luteinizing hormone/chooriogonadotropin receptor, LHX4: LIM homeobox 4, PATL2: PAT1-like protein 2, STAG3: Stromal antigen 3, TACR3: Tachykinin receptor 3, ZP1: Zona pellucida glycoprotein 1, ZP3: Zona pellucida glycoprotein 3

12), which was included in our study. These manifestations included EFS, oocyte maturation arrest, and the production of mature oocytes with successful embryo transfer in one cycle. Additionally, her IVF cycles produced zona-free oocytes. The genetic analysis of this case was performed at the outer center. Although we have a report in our archives indicating the presence of a ZP1 mutation, we do not have the details. Therefore, it could not be included in Table 3. The genetic profile of the family has been published[40]. Additionally, in our WES analysis, we identified two distinct PATL2 mutations in this case. We also observed patients with gEFS during IVF cycles while having spontaneous biochemical pregnancies and sometimes mature oocytes during other IVF cycles. This finding is novel, and it is difficult to determine whether these patients have EFS or not, but we can accept these cases as subtypes of OMAs.

According to our WES analysis, two distinct mutations in ZP1 were identified in cases 5 and 13. The mutation detected in Case 5 is novel; however, it is located within Intron 11 of the *ZP1* gene, suggesting no phenotypic impact. Case 5 was diagnosed with EFS as part of OMAs. In the initial two IVF attempts, no oocytes were obtained. In the third IVF attempt, only one zona-free oocyte was retrieved, and ICSI was performed, resulting in embryo freezing. In the fourth IVF attempt, two normal oocytes were retrieved. Successful ongoing pregnancy was achieved through embryo transfer. Case 13, who was also diagnosed with EFS as part of OMAs, experienced this condition in two IVF attempts. In another IVF attempt, a germinal vesicle arrest. Both cases did not receive IVM treatment.

Moreover, the emphasis on EFS has been placed in the context of mutations in the *ZP3* gene[41,42]. Within our study, Case 6 was diagnosed with premature ovarian ınsufficiency (POI) and true EFS. Additionally, zona-free oocytes were observed in some cycles. Intriguingly, based on WES analysis, one mutation in ZP3 and two mutations in FSHR. The patient underwent four IVF attempts, with oocytes failing to be retrieved in three of the cases. In the fourth attempt, an oocyte was obtained, but it degenerated. Two attempts of IVM resulted in the retrieval of one oocyte each time; however, subsequent observations revealed zona-free oocytes and subsequent degeneration in the germinal vesicle stage.

In one of our cases (Case 2) with g-EFS in her previous two consecutive IVF cycles, we determined a STAG3 mutation in her WES analysis (this mutation was previously a variant of uncertain significance but likely benign according to Association for Clinical Genomic Sciences classification). She had a history of biochemical pregnancy prior to IVF attempts and was diagnosed with POI. We performed IVM cycles, obtained embryos, and transferred them twice but failed to achieve pregnancy. However, the patient had three consecutive spontaneous biochemical pregnancies while waiting for another frozen-thawed embryo transfer. The last result resulted in the delivery of a healthy baby. The clinical course of the patient was interesting, beginning with POI and EFS and ending with spontaneous pregnancy.

When we looked at the cases studied, apart from the FSH-hCG primed IVM group, in which all cases were gEFS, the remaining nine women treated with letrozole-primed IVM + GH had intercycle variabilities either in their previous IVF cycles or in their IVM cycles. This finding is important and explains why EFS has follicles that are not empty, providing new evidence that EFS is a subtype of OMAs[17,27].

We propose a novel flowchart for the clinical management of all types of EFS (Figure 1). Apart from gEFS, Functional EFS, and GnRH-related EFS, we describe a novel subtype: EFS as a subtype of OMAs. In managing this entity, repeating the cycle for accurate diagnosis or directly investigating mutations via WES may be recommended. Because of the importance of intercycle variability, mutation analysis before a second attempt is preferable.

Study Limitations

Our study has some limitations. The small sample size and retrospective design may limit the generalizability of our findings. Therefore, studies with larger series should be scheduled to draw proper conclusions regarding the use of letrozole IVM plus GH therapy.

Conclusion

Almost all patients diagnosed with EFS after IVF had oocytes collected during IVM, indicating that the follicles were not truly empty. These oocytes have limited potential for maturation and fertilization, as evidenced by the low success rates following IVM culture and ICSI. The conditions observed in these OMAs stem from defects in the oocyte machinery, resulting in oocyte and cytoplasmic immaturity. Despite these challenges, pregnancies did occur in some subjects, suggesting that EFS can occasionally be overcome. The current evidence does not support the success of IVM in patients with g-EFS; however, the number of cases studied is limited, and further research is needed.

Ethics

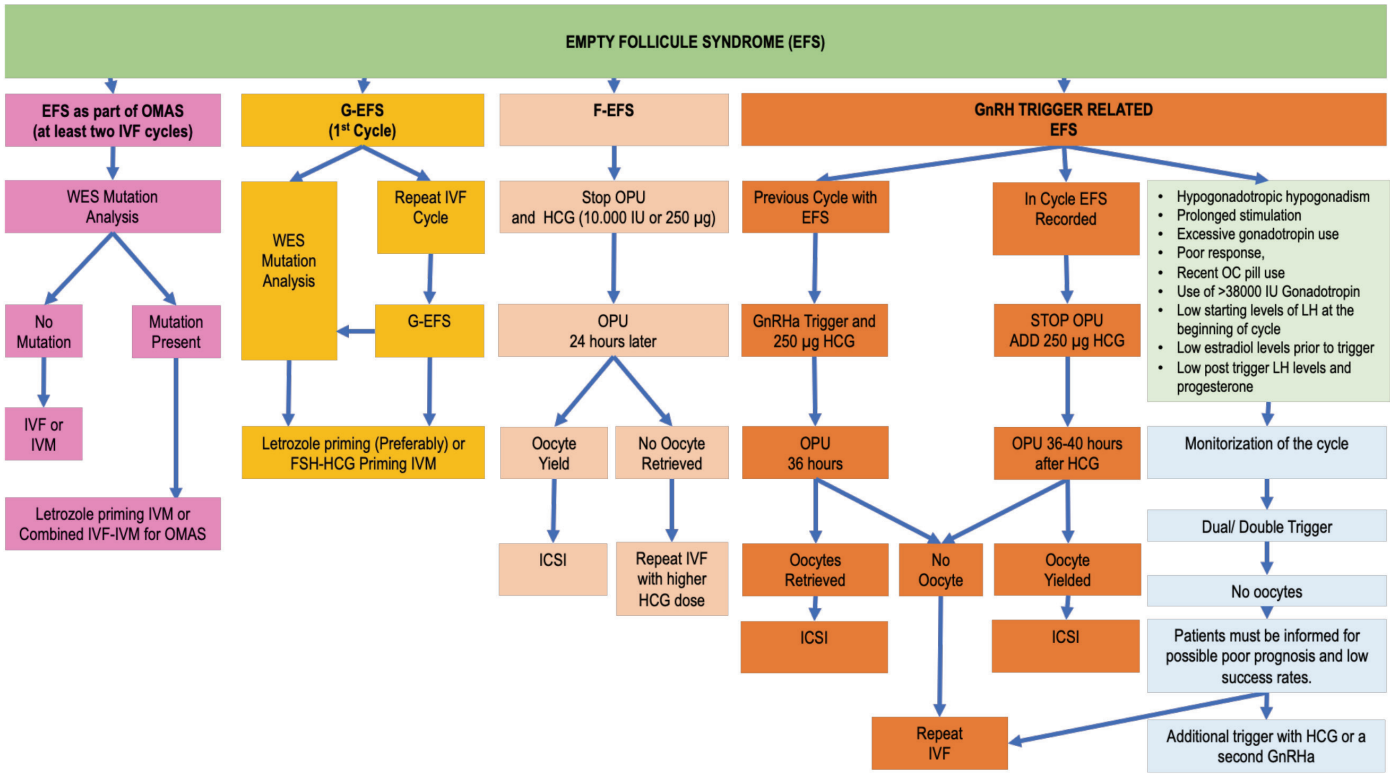

Figure 1. Empty follicule syndrome

EFS: Empty follicle syndrome, OMAS: Oocyte maturation abnormalities, IVF: In vitro fertilization, G-EFS: Genuine-EFS, F-EFS: False-EFS, OPU: Oocyte pick up, WES: Whole exome sequencing, IVM: In vitro maturation, FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, HCG: Human chorionic gonadotropin, ICSI: Intracytoplasmic sperm injection, GnRHa: Gonadotrophin-releasing hormone analogues, OC: Oral contraceptive

Ethics Committee Approval: This study was approved by the Clinical Research Ethics Committee of Samsun University, Turkey (decision date: 26.04.2023, decision number: 2023/8/20).

Informed Consent: Written informed consent was obtained from the women studied.

Authorship Contributions

Surgical and Medical Practices: Ş.H., A.Baş., M.B., N.D.G., G.A., M.H.D., S.S.Ü., Concept: E.H., S.Ç., A.B., M.B., H.Ç., A.E.K., Design: J.T., S.L.T., Data Collection or Processing: C.S.Ç., M.C., Analysis or Interpretation: A.Baş., Literature Search: A.Baş., Writing: C.S.Ç.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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Clinical Investigation / Araştırma

DOI: 10.4274/tjod.galenos.2024.38364

Turk J Obstet Gynecol 2024;21:153-7

High serum progesterone levels on the day of embryo transfer in patients undergoing artificial frozen-thawed blastocyst transfer: Is there a ceiling effect?

Yapay dondurulmuş-çözdürülmüş blastokist transferi yapılan hastalarda embriyo transferi günündeki yüksek serum progesteron düzeyleri: Tavan etkisi var mı?

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Abstract

Objective: To evaluate the potential ceiling effect of high serum progesterone levels on the day of embryo transfer for pregnancy outcomes in patients undergoing artificial frozen-thawed blastocyst transfer (FET) cycles.

Materials and Methods: This retrospective cohort study included 595 patients who underwent artificial FET cycles. We evaluated progesterone levels and found that 40.6 ng/mL corresponded to the 90th percentile and 23.9 ng/mL corresponded to the 50th percentile. Based on these findings, we categorized progesterone levels as <20 ng/mL (n=220, 37.0%), 20-40 ng/mL (n=312, 52.4%), and ≥40 ng/mL (n=63, 10.6%). The primary outcome measures were the clinical pregnancy rate (CPR) and live birth rate (LBR).

Results: Blastocyst morphology grades, including expansion, trophectoderm, and inner cell mass grades, were significantly associated with clinical pregnancy (p<0.001 for all). Progesterone levels between 20 and 40 ng/mL were associated with higher CPR (p=0.043). In the multivariate analysis, only blastocyst expansion and inner cell mass grades were independently and significantly associated with CPR [p=0.011, odds ratio (OR)=1.6, (confidence interval) CI 95%=1.13-2.39, and p=0.007, OR=1.65, CI 95%=1.14-2.39, respectively]. The progesterone level and trophectoderm grade were not statistically significant. Regarding LBR, only blastocyst expansion grades 4 and trophectoderm grades A or B were significantly associated.

Conclusion: Based on these data, we speculate that if serum progesterone levels exceed 40 ng/mL on the day of embryo transfer in patients undergoing artificial FET cycles, there is no need to reduce the progesterone dose.

Keywords: Progesterone, frozen-thawed blastocyst transfer, ceiling effect

Öz

Amaç: Yapay dondurulmuş-çözünmüş blastosist transferi (FET) siklusu uygulanan hastalarda embriyo transfer gününde yüksek serum progesteron düzeylerinin gebelik sonuçları üzerindeki tavan etkisi olup olmadığını değerlendirmeyi amaçladık.

Gereç ve Yöntemler: Bu çalışma yapay FET döngüsü uygulanan 595 hastayı içeren retrospektif bir kohort çalışmasıydı. Progesteron düzeylerine göre yüzdelik dilimleri değerlendirdiğimizde 40,6 ng/mL 90. yüzdeliğe, 23,9 ng/mL ise 50. yüzdeliğe karşılık geliyordu. Bu bulguya dayanarak progesteron düzeyi kesme noktasını <20 ng/mL, n=220 (%37,0); 20-40 ng/mL, n=312 (%52,4) ve ≥40 ng/mL, n=63 (%10,6) olarak belirledik. Birincil sonuç ölçüsü, klinik gebelik (CPR) ve canlı doğum oranı (LBR) olarak belirlendi.

PRECIS: There is no ceiling effect of high serum progesterone levels on day of embryo transfer (>40 ng/mL) for pregnancy outcomes in patients undergoing artificial frozen-thawed blastocyst transfer.

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Received/Geliş Tarihi: 11.06.2024 **Accepted/Kabul Tarihi:** 29.07.2024

Bulgular: Blastokist morfolojisi olarak genişleme derecesi, trofoektoderm ve iç hücre kitle derecesi klinik gebelik ile istatistiksel olarak anlamlı düzeyde ilişkili olduğunu bulduk (hepsi için p<0,001) ve 20-40 ng/mL arasındaki progesteron düzeyinin ise daha yüksek CPR ile ilişkili olduğunu bulduk (p=0,043). Çok değişkenli analizde; CPR ile ilişkili faktörler yalnızca blastosist genişlemesi ve iç hücre kütle derecesi bağımsız ve anlamlı faktörlerdi [p=0,011, (odds oranı) OO=1,6, güven aralığı (GA) 95%=1,13-2,39 ve p=0,007, OO=1,65, GA 95%=1,14-2,39, sırasıyla]. Progesteron düzeyi ve trofoektoderm derecesi istatistiksel olarak anlamlı bulunmadı. Faktörler ile LBR arasındaki ilişkinin değerlendirilmesinde sadece blastokist genişleme derecesi 4'e eşit veya üzerinde ve trofektoderm derecesi A veya B istatistiksel olarak anlamlı LBR ile ilişkiliydi.

Sonuç: Bu verilere göre yapay FET siklusu yapılan hastalarda embriyo transferi gününde serum P4 düzeyi 40 ng/mL'nin üzerinde ise kullanılan progesteron dozunun azaltılmasına gerek olmadığını düşündük.

Anahtar Kelimeler: Progesteron, dondurulmuş-çözülmüş blastokist transferi, tavan etkisi

Introduction

Progesterone increases during the luteal phase of the menstrual cycle with the occurrence of ovulation and prepares the endometrium for embryo implantation[1-3]. In natural conception, progesterone is synthesized by the corpus luteum, whereas exogenous progesterone is obtained in artificial frozen embryo transfer (FET) cycles in which there is no corpus luteum[4]. This exogenous progesterone is required for synchronization between the embryo and endometrium[5]. However, an important question arises in artificial FET cycles: What should be the lower and upper thresholds for serum progesterone levels on the day of embryo transfer for synchronization?

In recent studies, values in the range of 8.8-9.2 ng/mL were usually used as lower threshold values for serum progesterone (P4) levels on the day of embryo transfer in patients undergoing artificial FET cycles, and pregnancy outcomes were compared between patients with P4 levels above and below those thresholds[6-8]. The main reason for choosing these thresholds in such studies is that they reflect the minimal mid-luteal progesterone level that a healthy corpus luteum should secrete to prevent luteal phase defects[2]. Most previous studies have evaluated optimal serum progesterone concentrations following vaginal administration. Serum progesterone concentrations and pregnancy outcomes have been evaluated in a limited number of studies[9,10].

Although many studies have investigated lower threshold values, there is a paucity of data in the literature on higher threshold values, indicating the possible ceiling effect of P4. Therefore, in this study, we examined whether high serum progesterone levels on the day of embryo transfer have a ceiling effect on pregnancy outcomes in patients undergoing artificial FET cycles.

Materials and Methods

This retrospective cohort study included 595 patients who underwent artificial FET cycles at our fertility center between 2017 and 2021. The study was conducted in accordance with the ethical standards established in the 1964 Declaration of Helsinki.

Patients younger than 35 years of age for whom the transfer of one top-quality embryo was performed and, to eliminate age-related bias, patients older than 35 years of age for whom euploid embryo transfer was performed were included in the study. In our clinic, blastocyst morphology was evaluated using the Gardner-Schoolcraft classification system[11].

Patients with uterine diseases and changes in the progesterone dose or route according to serum progesterone levels on the day of embryo transfer were excluded from the study.

Oral estrogen (Estrofem, Novo Nordisk, Istanbul, Turkey) was administered on day 2-3 of the menstrual period with an ascending protocol: 4 mg/day for the first 4 days, 6 mg/day for the next 4 days, and 8 mg/day for the last 4 days. The day after the procedure, endometrial thickness was evaluated by transvaginal ultrasonography. Endometrial preparation was considered sufficient if the endometrial thickness was 7 mm. After endometrial preparation, 50 mg of progesterone (Koçak Farma İlaç ve Kimya Sanayi A.Ş., Istanbul, Turkey) was administered intramuscularly for 5 days. The serum progesterone levels were measured 2 h before the embryo transfer and approximately 16 h after the last dose of progesterone was administered.

In our evaluation of progesterone levels according to percentiles, 40.6 ng/mL corresponded to the 90th percentile and 23.9 ng/mL corresponded to the 50th percentile. Based on these findings and rounding up the values, we established progesterone level thresholds as <20 ng/mL (n=220, 37.0%), 20-40 ng/mL (n=312, 52.4%), and ≥40 ng/mL (n=63, 10.6%). The primary outcome measures were clinical pregnancy and live birth rates. The blastocyst grading was based on the assessment of inner cell mass and trophectoderm appearance, as described by Gardner and Schoolcraft[11].

We compared pregnancy outcomes according to age, body mass index (BMI), blastocyst expansion, trophectoderm and inner cell mass grades, and progesterone threshold levels. Data were analyzed using IBM SPSS Statistics 25 for Windows (IBM Corp., Armonk, NY, USA). All procedures performed in the study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Since our study was designed retrospectively, IRB, informed consent and ethical approval has not been obtained.

Results

The median age of the patients was 31 years (range: 20-46), and the mean BMI was 24.5±4.0 kg/m^2. The overall clinical pregnancy rate was 61.8% (368/595), and the live birth rate was 52.9% (315/595). Patients were subdivided into two groups based on

clinical pregnancy and live birth outcomes. The ages and BMIs of the patients were comparable between the two groups (Table 1). Blastocyst morphology, as indicated by the expansion grade and the grades of the trophectoderm and inner cell mass, was significantly associated with clinical pregnancy (p<0.001 for all). Progesterone levels of 20-40 ng/mL were associated with higher clinical pregnancy rates (p=0.043). In the multivariate analysis, only blastocyst expansion and inner cell mass grades were found to be independent significant factors associated with the clinical pregnancy rate [p=0.011, odds ratio (OR): 1.6, 95% confidence interval (CI): 1.13-2.39, and p=0.007, OR: 1.65, 95% CI: 1.14-2.39, respectively]. The progesterone level and trophectoderm grade were not statistically significant (p=0.310 and p=0.489, respectively) (Table 2). Regarding the live birth rate, only a blastocyst expansion grade of ≥4 and trophectoderm grades A and B were significantly associated with a difference (Table 3).

Discussion

In the present study, we demonstrated that high serum progesterone levels on the day of embryo transfer may not have a ceiling effect on pregnancy outcomes in patients undergoing artificial FET cycles. Our findings are important because there is a paucity of data on the possible ceiling effect of P4 in the literature. Thus, our data offer guidance for future prospective studies on this topic.

The receptive endometrium for embryo implantation is mainly coordinated by estrogen and progesterone, and increased estrogen levels during the implantation period can disrupt implantation[12,13]. However, limited data are available on the association between increased progesterone levels and embryo implantation. In an experimental study by Liang et al.[14], increased progesterone levels during the implantation period had a deleterious effect on endometrial receptivity and decidualization. However, their study was conducted in rats, and we cannot extrapolate the extent to which progesterone levels have similar negative effects on endometrial receptivity in humans or whether we could reach the theoretical progesterone levels with the doses of exogenous progesterone applied during FET cycles.

According to the literature, excessive progesterone may accelerate endometrial development and cause the implantation window to open and close earlier. As a result, it is speculated that this may cause asynchrony between the embryo and the endometrium[15-17]. Based on this information, previous studies have been conducted with the aim of determining the

Table 1. General characteristics

	Mean ± standard deviation	Median (range)
Age	31.3±4.7	31 (20-46)
BMI	24.5±4.0	23.7 (17.2-41.0)
	n	**%**
Age		
<35	471	79.2
≥35	124	20.8
BMI		
<25	328	55.1
≥25	197	33.1
Missing	70	11.8
Clinical pregnancy		
Biochemical	57	9.6
Positive	368	61.8
Negative	170	28.6

BMI: Body mass index

Table 2. Association between clinical and laboratory markers and clinical pregnancy

	Clinical pregnancy				
	Positive		Negative + biochemical		
	n	%	n	%	p-value
Age					
<35	299	63.5	172	36.5	**0.110**
≥35	69	55.6	55	44.4	
BMI					
<25	199	60.7	129	39.3	**0.864**
≥25	121	61.4	76	38.6	
Progesterone level					
<20.0	130	59.1	90	40.9	**0.43**
20.0-40.0	206	66.0	106	34.0	
≥40.0	32	50.8	31	49.2	
Blastocyst expansion grade					
3	26	43.3	34	56.7	**<0.001**
4	229	70.7	95	29.3	
5 and 6	113	53.6	98	46.4	
Trophectoderm grade					
A	159	74.3	55	25.7	**<0.001**
B	197	55.8	156	44.2	
C	12	42.9	16	57.1	
Inner cell mass grade					
A	87	77.0	26	23.0	**<0.001**
B	216	64.5	119	35.5	
C	65	44.2	82	55.8	

BMI: Body mass index

Table 3. Evaluation of the relationship between factors and the live birth rate

	Pregnancy				
	Live birth		Abortus		
	N	%	N	%	p-value
Age					
<35	254	88.5	33	11.5	**0.752**
≥35	61	87.1	9	12.9	
BMI					
<25	171	88.1	23	11.9	**0.389**
≥25	100	84.7	18	15.3	
Progesterone level					
<20.0	111	87.4	16	12.6	**0.686**
20.0-40.0	177	88.1	24	11.9	
≥40.0	27	93.1	2	6.9	
Blastocyst expansion grade					
3	19	70.4	8	29.6	**0.9**
4	200	90.5	21	9.5	
5 and 6	96	88.1	13	11.9	
Trophectoderm grade					
A	138	90.8	14	9.2	**0.13**
B	171	87.7	24	12.3	
C	6	60.0	4	40.0	
Inner cell mass grade					
A	76	90.5	8	9.5	**0.337**
B	185	88.9	23	11.1	
C	54	83.1	11	16.9	

BMI: Body mass index

upper level of progesterone at which pregnancy rates begin to decrease during FET cycles[9,15,18,19]. However, the first point of concern regarding these studies is that the effects of different progesterone administration routes on serum progesterone levels will vary, and thus, the cutoff values may also vary according to whether the administration route was vaginal, intramuscular, or subcutaneous. Yovich et al.[15] used only the vaginal route for luteal support and reported a cutoff point of >31.45 ng/mL, whereas Kofinas et al.[9] used only the intramuscular route and reported ≥20 ng/mL. Interestingly, the cutoff value was thus found to be higher in the study using the vaginal route. On the other hand, Alyasin et al.[18] used both vaginal and intramuscular routes and found that serum progesterone levels >32.5 ng/mL on the day of embryo transfer were associated with lower live birth rates. The differences in the administration routes used in these studies make it difficult to compare the results. In contrast, our study showed that values >40 ng/mL did not have a negative effect on pregnancy outcomes. Therefore, we speculate that excessive progesterone use may have a ceiling effect on the endometrium; however, the dosages or routes of progesterone that we use for luteal support may not be able to cause this ceiling effect.

A retrospective cohort study conducted by González-Foruria et al.[19], which included 3,183 FET cycles, supports our data. The authors showed that high serum progesterone levels before embryo transfer did not impair reproductive outcomes in patients undergoing artificial FET cycles. Based on these data, authors speculated that when adequate endometrial progesterone impregnation is achieved, serum progesterone levels are not related to reproductive outcomes.

It should be kept in mind that vaginal and intramuscular progesterone administration significantly differ regarding tissue and serum concentrations. Although we do not know the exact adverse effects of high tissue concentrations, there may be possible relaxin inhibition in the tissue, which may cause adverse effects on both implantation and perinatal outcomes[20]. In addition, systemic progesterone concentrations are critical for the immunological mechanisms during implantation and pregnancy.

Study Limitations

There are two limitations to this study. First, this was a retrospective study, and the number of patients with serum progesterone levels ≥40 ng/mL. Despite these limitations, a key strength of this study was that it was conducted at a single center using the same luteal-phase support protocol for all patients.

Conclusion

In conclusion, based on the current study data, we suggest that if serum P4 level is >40 ng/mL on the day of embryo transfer in patients undergoing artificial frozen-thawed blastocyst transfers, there is no need to reduce the dose of progesterone used.

Ethics

Ethics Committee Approval: All procedures performed in the study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Since our study was designed retrospectively, IRB, informed consent and ethical approval has not been obtained.

Informed Consent: Informed consent was obtained from all participants included in the study.

Authorship Contributions

Concept: Y.A.T., B.D., M.B., G.B., Design: Y.A.T., B.D., B.Dü., M.B., G.B., Data Collection or Processing: Y.A.T., F.K.B., N.F., Analysis or Interpretation: Y.A.T., B.Dü., F.K.B., N.F., M.B., G.B., Literature Search: Y.A.T., B.D., B.Dü., N.F., M.B., Writing: Y.A.T., B.D., F.K.B., G.B.

Conflict of Interest: No conflict of interest was declared by the authors.
Financial Disclosure: The authors declared that this study received no financial support.

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Clinical Investigation / Araştırma

DOI: 10.4274/tjod.galenos.2024.74419

Turk J Obstet Gynecol 2024;21:158-65

Association between Mir-499, Mir-27a, and Mir-146a polymorphisms and their susceptibility to recurrent spontaneous abortion; *in silico* analysis

Mir-499, Mir-27a ve Mir-146a polimorfizmlerinin tekrarlayan spontan düşüklere yatkınlıkla ilişkisi; in silico analizi

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Abstract

Objective: Recurrent spontaneous abortion (RSA) is defined as two or more pregnancy losses before 24 gestational weeks, accounting for 1-3% of fertile couples. A vast majority of single-nucleotide polymorphisms (SNPs) in some *microRNA (miRNA)* genes can change the miRNA-mRNA interaction and are associated with the risk of RSA. This study was designed to better elucidate the association between miR-27a, miR-499, and miR-146a polymorphisms and RSA risk.

Materials and Methods: SNP genotyping of miR-27a (rs895819), miR-499 (rs3746444), and miR-146a (rs2910164) was performed using polymerase chain reaction (PCR)-restriction fragment length polymorphism and tetra amplification-refractory mutation system PCR in 98 patients with RSA and 105 healthy subjects.

Results: Our results showed that the miR-499 rs3746444 and miR-27a rs895819 polymorphisms were significantly associated with RSA risk, whereas no significant differences were observed between the rs2910164 polymorphism and RSA susceptibility.

Conclusion: We proposed that the miR-499 rs3746444 and miR-27a rs895819 polymorphisms were correlated with RSA in our population, but the miR-146a rs2910164 variant was not associated with the risk of RSA.

Keywords: MiR-499 rs3746444, miR-27a rs895819, miR-146a rs2910164, RSA risk

Öz

Amaç: Tekrarlayan spontan düşük (RSA), 24 gebelik haftasından önce iki veya daha fazla kez gebelik kaybı olarak tanımlanır ve doğurgan çiftlerin %1-3'ünü etkiler. Bazı mikroRNA (miRNA) genlerindeki tek nükleotid polimorfizmlerinin (SNP'lerin) büyük çoğunluğu miRNA-mRNA etkileşimini değiştirebilir ve RSA'nın ortaya çıkma riskiyle ilişkilidir. Bu çalışma, miR-27a, miR-499 ve miR-146a polimorfizmleri ile RSA riski arasındaki ilişkiyi daha iyi açıklamak için tasarlanmıştır.

Gereç ve Yöntemler: Doksan sekiz RSA'lı hastada ve 105 sağlıklı bireyde polimeraz zincir reaksiyonu (PCR)- restriksiyon fragment uzunluk polimorfizmi ve tetra amplifikasyona dirençli mutasyon sistemi PCR yöntemleri kullanılarak miR-27a (rs895819), miR-499 (rs3746444) ve miR-146a rs2910164'ün SNP genotiplemesi gerçekleştirildi.

PRECIS: The current study has investigated the association of three miRNA variations with the susceptibility of RSA in a fraction of the Iranian population. All polymorphisms except miR-146a C>G polymorphism significantly increased the RSA risk in the dominant inheritance model.

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Received/Geliş Tarihi: 06.06.2024 **Accepted/Kabul Tarihi:** 28.07.2024

Bulgular: Sonuçlarımız miR-499 rs3746444 ve miR-27a rs895819 polimorfizmlerinin RSA riskiyle anlamlı şekilde ilişkili olduğunu, rs2910164 polimorfizmi ile RSA duyarlılığı arasında anlamlı bir fark gözlenmediğini gösterdi.

Sonuç: MiR-499 rs3746444 ve miR-27a rs895819 polimorfizmlerinin popülasyonumuzda RSA ile ilişkili olduğunu, ancak miR-146a rs2910164 varyantının RSA'nın ortaya çıkma riski ile ilişkili olmadığını düşünmekteyiz.

Anahtar Kelimeler: MiR-499 rs3746444, miR-27a rs895819, miR-146a rs2910164, RSA riski

Introduction

Recurrent spontaneous abortion (RSA) is a common pregnancy complication that occurs in 1-3% of fertile couples. The disease is described as two or more times early pregnancy loss before 24 gestational weeks and accounts for about 10-15% of clinically recognized pregnancies. Although several etiologic factors, including infectious, uterine abnormalities, hormonal disorders, chromosomal abnormalities, and gene polymorphisms, have been reported as pathophysiological mechanisms of RSA, the etiology of 50% of pregnant women with RSA still cannot be explained[1-3]. Therefore, future research is required to elucidate the pathogenesis of RSA.

MicroRNAs (miRNAs) are small endogenous RNAs that modulate the translation and stability of mRNAs through the recruitment of regulatory proteins[4]. miRNAs have important regulatory roles in various biological processes, such as cell growth, apoptosis, and differentiation[5]. Recent studies have reported that single-nucleotide polymorphisms (SNPs) within miRNA sequences may alter miRNA processing and target selection associated with the risk of RSA. In a study by Santamaria and Taylor[6] examined the possible relationship of miRNA polymorphisms, including miR-146aC>G, miR-149T>C, miR-196a2T>C, and miR-499A>G, in patients with RSA was examined. Their result showed that all these polymorphisms were significantly associated with idiopathic RSA. Concomitant mutations have a synergistic effect[6].

Moreover, two SNPs (rs41275794, rs12976445) residing within the pri-miR-125a sequence were associated with increased RSA risk via decreased miR-125a expression[7]. Therefore, this study aimed to assess the significant differences in miR-499, miR-27a, and miR-146a polymorphisms and RSA susceptibility in the southeast Iranian population.

Materials and Methods

Demographic Characteristics

This case-control study enrolled 98 women with a history of two or more early pregnancy losses and 105 control subjects who had no history of abortion and at least one normal birth. In addition, participants with known causes, including anatomical, autoimmune, endocrine, and chromosomal abnormalities, were excluded from this study. The local Ethics Committee of Zahedan University of Medical Sciences (decision no: IR.ZAUMS.REC.1396.218, date: 20.01.2024) approved the study and provided informed consent. Total blood samples were obtained from all participants. The salting-out method was used to extract genomic DNA as described previously[8] and stored at 20°C until use.

Genotyping

Polymerase chainreaction-restriction fragment length polymorphism (PCR-RFLP) method was used to genotype miR-27a (rs895819) and miR-499 (rs3746444) polymorphisms. Genotyping of the miR-146a rs2910164 polymorphism was determined using the Tetra-amplification-refractory mutation system method. Table 1 lists the primers used in this study. In the PCR-RFLP method, each PCR reaction was performed in a final volume of 20 µL including one microliter of extracted DNA, 10 pmol of each primer, 10 L of master mix, and 7 L of DNase-Free Distilled water. The PCR conditions for miR-27a rs895819 consisted of pre-denaturation at 95°C for 6 min, followed by 35 cycles of 95°C for 30 s, 65°C for 35 s, and 72°C for 35 s, and a final extension step of 72°C for 10 min. For miR-499 rs3746444, the PCR conditions were initial denaturation at 95°C for 5 min; 30 cycles of denaturation (95°C, 30 seconds), annealing (63°C 30 s), and extension (72°c, 30 s). After the final extension step, an appropriate restriction enzyme was

Table 1. The primers used to detect the miR-27a, miR-499 and miR-146a polymorphisms

Polymorphism	PCR Primers (5' →3')	Restriction enzyme	Fragment, bp
MiR-27a (rs895819)	F: GAACTTAGCCACTGTGAACACGACTTCG R: GGGTTCCTGGGGATGGGATTTG	BstUI	T allele: 201 C allele: 173+28
MiR-499 (rs3746444)	F: CAAAGTCTTCACTTCCCTGCCA R: GATGTTTAACTCCTCTCCACGTGATC	BclI	C allele: 146 T alleles: 122.24
MiR-146a (rs2910164)	FO: GGCCTGGTCTCCTCCAGATGTTTAT RO: ATACCTTCAGAGCCTGAGACTCTGCC FI (C allele): ATGGGTTGTGTCAGTGTCAGACGTC, RI (G allele): GATATCCCAGCTGAAGAACTGAATTTGAC	-	Control: 364 G allele: 249 C allele: 169

PCR: Polymerase chain reaction

used to digest the PCR products of the miR-27a and miR-499 polymorphisms (according to Table 1). For genotyping miR-146a rs2910164, 10 µL of the master mix was combined with 5 µL of double-distilled water and 1 µL of each primer in each reaction mixture. The amplification parameters for the SNP were similar to the PCR conditions of miR-499 rs3746444, except for the annealing step (61°C, 25 s). Electrophoresis using 2% agarose gel containing 0.5 µg/mL ethidium bromide was performed to visualize the PCR products under ultraviolet light (Figures 1-3). Genotyping quality was confirmed by re-checking approximately 20% of all samples, resulting in a concordance of 100%.

Using SPSS software (version 20, USA), an independent sample t-test and $\chi 2$ test were used to analyze the comparison of differences between the two groups. The odds ratios (ORs) with 95% confidence intervals (CIs) were employed to calculate any possible association between these polymorphisms and RSA risk. The significance probability was <0.05.

Statistical Analysis

In this study, we used "miRDB"[9] and miRWalk[10] software to demonstrate the interaction of miR-27a, miR-499a, and miR-146a with putative target genes. In miRDB, an online database was developed to predict miRNA target sites and generate functional data from large-scale RNA sequencing experiments. The four common features assessed by the program were free energy, seed match, conservation, and site accessibility[9]. In the output of this server, we only included the top 10 results in terms of the target score. Then, we examined the relationship between RSA and possible target genes. Then, the genes were presented in bold. In this analysis, we separately applied both strands (designated 5p- or 3p-) of the miRNA stem-loop structure for gene target prediction. The miRWalk database lists predicted and validated miRNA binding sites from human, dog, cow, and rat genes[10]. First, we used the miRWalk database to download the introduced targets for each miRNA; then we used Cytoscape software to draw the interaction network related to these targets. Cytoscape is also an open-source software for the visualization and analysis of bio-molecular interaction networks, with several plug-ins, including the investigation of biological pathways for further analysis[11]. Finally, using the String App[12] tool, the enrichment of miRNA targets was performed only for confirmed interactions, and in this way, the indirect effects of miRNAs on various processes were investigated (Figure 4).

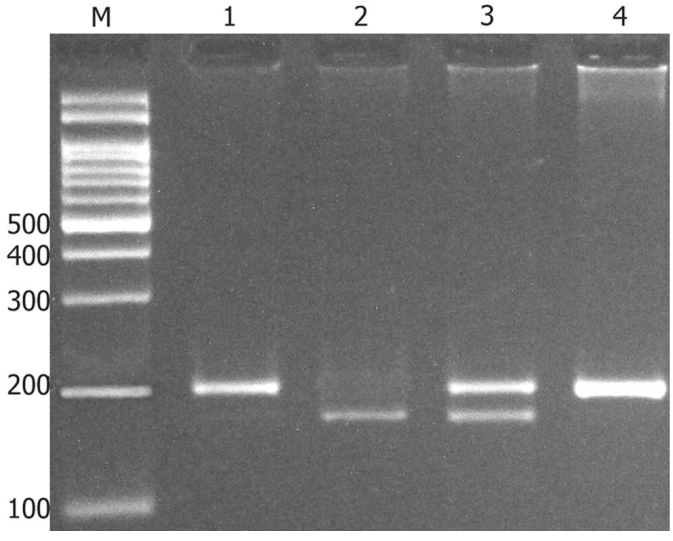

Figure 1. Photograph of the PCR-RFLP method for the detection of miR-27a rs895819 polymorphism

M: DNA marker; Lanes 1 and 4: TT; Lane 2: CC; Lane 3: TC, PCR: Polymerase chain reaction, RFLP: Restriction fragment length polymorphism

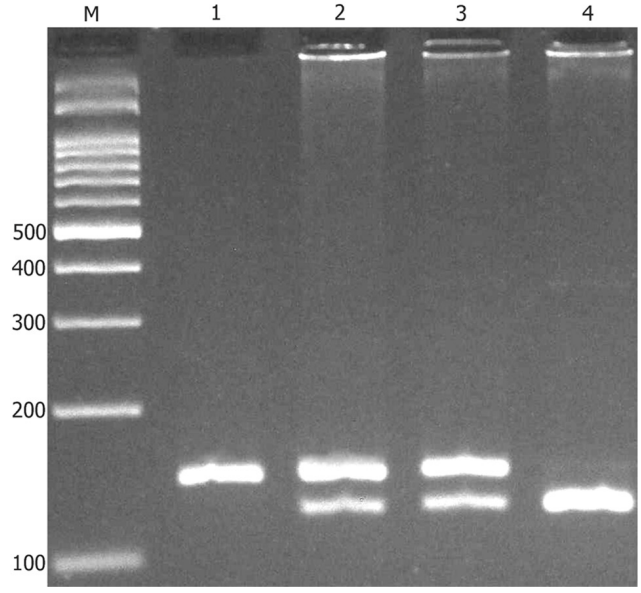

Figure 2. Photograph of the PCR-RFLP method for the detection of the miR-499 rs3746444 polymorphism.

M: DNA marker; Lane 1: CC; Lanes 2 and 3: CT; and Lane 4: TT, PCR: Polymerase chain reaction, RFLP: Restriction fragment length polymorphism

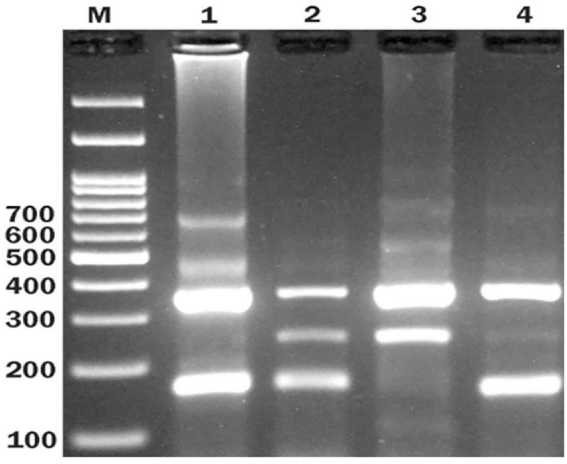

Figure 3. Photograph of the T-ARMS Method for the detection of miR-146a rs2910164

T-ARMS: Tetra amplification-refractory mutation system

Results

In the case-control study, 98 patients with >2 miscarriages, age 28.79±5.00 years, and 105 non-consanguineous healthy women (29.50±4.85 years) were recruited. There was no significant difference in age between the case and control subjects (p=0.306). Table 2 shows the frequency of genotypes and alleles of the miR-27a (rs895819), miR-499 (rs3746444), and miR-146a (rs2910164) polymorphisms in the groups. The miR-27a rs895819 polymorphism increased the susceptibility to RSA in co-dominant (OR=1.98, 95% CI=1.05-3.76, p=0.035 TC vs. TT) models. The miR-499 rs3746444 polymorphism increased the predisposition to RSA in the codominant (OR=2.49, 95% CI=1.36-4.56, p=0.005 TC vs. TT; OR=2.92, 95% CI=1.16-7.34, p=0.036 CC vs. TT) and dominant (OR=2.58, 95% CI=1.47-4.55, p=0.001 TC+CC vs. TT) models. The C allele of miR-499 rs3746444 polymorphism was significantly higher in the patients (OR=2.02, 95% CI=1.32-3.09, p=0.002) than in control subjects. There were no significant differences between cases and controls regarding the miR-146a variants in any inheritance model (Table 2). The genotypes of the miR-27a, miR-499, and miR-146a variants in controls and cases were classified under Hardy-Weinberg equilibrium. (X^2=0.055, 0.136, and 0.337 and X^2=0.078, 0.958, and 0.947 respectively).

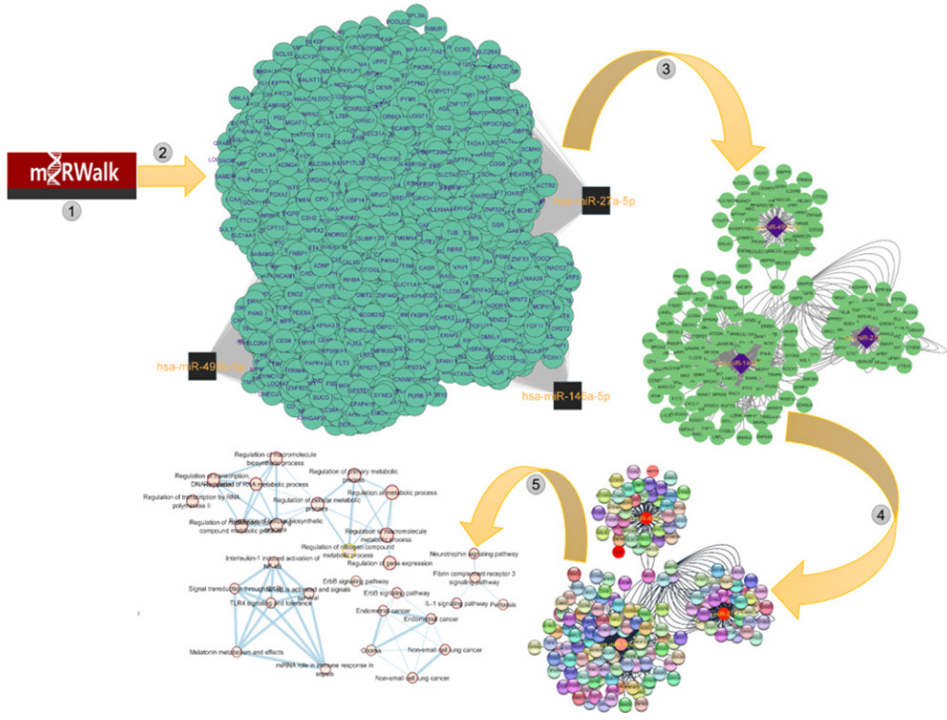

Figure 4. Approach was performed using miRWalk and Cytoscape software. Step 1: search and download process for hsa-miR-27a, hsa-miR-499, and hsa-miR-146a. Step 2: Draw the miRNA target interaction network using the Cytoscape tool. Step 3: Select validated targets and remove other targets from network. Steps 4 and 5: network drawing and enrichment by StringApp

Table 2. Association between *miR-27a, miR-499, and miR-146a* gene polymorphisms and the risk of recurrent spontaneous abortion

Polymorphism	Case number (%)	Control n (%)	OR (95% CI)	p-value
MiR-27a rs895819				
Codominant				
TT	26 (26.5)	38 (36.2)	1	-
TC	57 (58.2)	42 (40.0)	1.98 (1.05-3.76)	**0.050**
CC	15 (15.3)	25 (23.8)	0.88 (0.39-1.97)	0.911
Dominant				
TT	26 (26.5)	38 (36.2)	1	-
TC+CC	72 (73.5)	77 (63.8)	1.37 (0.76-2.47)	0.377
Recessive				
TT+TC	83 (84.7)	80 (76.2)	1	-

Table 2. Continued				
Polymorphism	Case number (%)	Control n (%)	OR (95% CI)	p-value
CC	15 (15.3)	25 (23.8)	0.58 (0.28-1.18)	0.178
Allele				
T	109 (55.6)	118 (56.2)	1	-
C	87 (44.4)	92 (43.8)	1.02 (0.69-1.52)	0.986
MiR-499 rs3746444				
Codominant				
TT	36 (36.7)	63 (60.0)	1	-
TC	47 (48.0)	33 (31.4)	2.49 (1.36-4.56)	**0.005**
CC	15 (15.3)	9 (8.6)	2.92 (1.16-7.34)	**0.036**
Dominant				
TT	36 (36.7)	63 (60.0)	1	-
TC+CC	62 (63.3)	42 (40.0)	2.58 (1.47-4.55)	0.001
Recessive				
TT+TC	83 (84.7)	96 (91.4)	1	-
CC	15 (15.3)	9 (8.6)	1.93 (0.80-4.63)	**0.205**
Allele				
T	119 (60.7)	159 (75.7)	1	-
C	77 (39.3)	51 (24.3)	2.02 (1.32-3.09)	**0.002**
MiR-146a rs2910164				
Codominant				
GG	56 (57.1)	62 (59.1)	1	-
GC	36 (36.7)	35 (33.3)	1.14 (0.63-2.05)	0.778
CC	6 (6.2)	8 (7.6)	0.83 (0.27-2.54)	0.966
Dominant				
GG	56 (57.1)	62 (59.1)	1	-
GC+CC	42 (42.9)	43 (40.9)	1.08 (0.619-1.89)	0.895
Recessive				
GG+GC	92 (93.8)	97 (92.4)	1	-
CC	6 (6.2)	8 (7.6)	0.79 (0.26-2.37)	0.886
Allele				
G	148 (75.5)	159 (75.7)	1	-
C	48 (24.5)	51 (24.3)	1.01 (0.64-1.59)	1.000

OR: Odds ratio, CI: Confidence interval

Bioinformatics Findings

According to Table 3, our analysis illustrated that the *IL2* gene is affected by hsa-miR-27a-5p with a target score of 94. The *SOX6 (SRY-box containing gene 6)* gene with a target score of 100 and the *LEPR* gene with a target score of 96 were affected by hsa-miR-499a-5p and hsa-miR-499a-3p, respectively. The *TRAF6* gene with a target score of 100 and the *TXNIP* gene with a target score of 96 were affected by hsa-miR-499a-5p and hsa-miR-499a-3p, respectively.

Furthermore, functional enrichment analysis using StringApp with a false discovery rate (FDR) threshold of 5% showed that in the KEGG Pathways category, most of the studied target genes of miRNAs are involved in the pathways of cancer, bacterial and viral infections, and hepatitis C.

Discussion

Increasing evidence has confirmed that miRNAs play a vital role in the pathophysiology of RSA and may be potential diagnostic

or prognostic markers for this disease[13]. Moreover, SNPs present both in *miRNA* genes or within miRNA-mRNA binding sites may contribute to susceptibility to RSA by affecting the expression and function of the miRNA target[14]. In this study, we investigated the association between three miRNA polymorphisms (miR-27a, miR-499, and miR-146a) and the risk of RSA in Iranian women.

Our results showed that the mir-499 rs3746444 polymorphisms were statistically associated with an increased risk of RSA in the co-dominant inheritance model. Additionally, we found that the C allele of miR-499 rs3746444 C/T enhanced the risk of RSA compared with the healthy group. The association between miR-499 rs3746444 and RSA susceptibility has been well studied in some populations. The following are some important points of them. In a study conducted on north Indian women affected by RSA, there was a possible correlation between polymorphism and SRA risk[15], which was consistent with our findings. In another study, Fazli and Ghorbian[16] suggested that the miR-499a polymorphism was significantly correlated with susceptibility to idiopathic RSA in the torque ethnic group. Conversely, the bioinformatics analysis predicted that SOX6 and LEPR are affected by miR-499a-5p and miR-499a-3p. Recently published articles have demonstrated that the ***SOX6*** gene (as a direct target of miR-499) can effectively modulate differentiation and cell proliferation during embryonic development via the repression of FGF-3 transcription. It was hypothesized that the deregulation and dysfunction of miR-499 caused by genetic material alteration are likely to influence female reproductive and fertility[17].

Furthermore, the present study assessed the association between rs895819 alleles and genotypes and RSA susceptibility. Our results showed that mir-27a rs895819 polymorphism positively affects the risk of RSA. In 2016, Wang et al.[18] suggested that SNP rs895819 C>T significantly increased the risk of RSA (which is in agreement with our finding), while studies by Rah et al.[19] and Srivastava et al.[14] showed no relationship between the risk of RSA and the risk of RSA. The variant located in the terminal loop of pre-miR-27a is associated with the risk of non-alcoholic fatty liver disease[20], colorectal cancer[21], type 2 diabetes mellitus[22], and primary ovarian insuffciency[23]. Our bioinformatics data suggest that most miRNA target genes are involved in cancer, bacterial and viral infections, and hepatitis C. Previous studies reported that miR-27a regulates the antimicrobial activities of macrophages by targeting the ***IL-10*** gene (inflammatory response gene)[24], which is consistent with our bioinformatics data. In addition, high levels of miR-27a expression were observed in the villus tissue of patients with RSA, and its upregulation may suppress the cycle progression of trophoblasts and induce apoptosis by targeting the regulation of the expression of cyclin D1 and IGF1[23].

Finally, preliminary data suggested significant differences between miR-146a C>G polymorphism and RSA susceptibility in the northeast Iranian population[25]. However, this study did not identify any significant association with RSA risk, which is consistent with the studies by Jeon et al.[26], Parveen and Agrawal[15], and Babakhanzadeh et al.[27]. Previous studies have shown that miR-146a could promote apoptosis in oocytes during folliculogenesis by binding to the 3'-UTR of the ***FAS*** gene, which may then lead to spontaneous abortion[28].

Study Limitations

First, we analyzed only three miRNAs, whereas there are many miRNAs related to RSA. Second, this study registered 98 patients and 105 healthy individuals from a fraction of the southeast Iranian population. However, a large population-based investigation can be more informative. Third, we did not evaluate the correlation between these variants and relative miRNA expression.

Table 3. Predicted target genes for miR-27a, miR-499a, and miR-146a in miRDB

	HSA-miR-27a 5p		HSA-miR-27a-3p		HSA-miR-499a-5p		HSA-miR-499a-3p		HSA-miR-146a-5p		HSA-miR-146a-3p	
Target rank	Gene symbol	Target score	Gene symbol	Target score	Gene symbol	Target score	Gene symbol	Target score	Gene symbol	Target score	Gene symbol	Target score
1	*RFK*	97	*AFF4*	100	***SOX6***	**100**	*TCF7L2*	99	***TRAF6***	**100**	*CPLX2*	99
2	*LTBP1*	97	*GXYLT1*	100	*VAV3*	99	*FOXN2*	99	*IRAK1*	100	*FOXC1*	99
3	*INO80D*	95	*ARFGEF1*	100	*SLC30A4*	99	*NRIP1*	99	*SEC23IP*	99	*STXBP6*	98
4	*BTF3*	95	*GCC2*	100	*EML4*	99	*MEOX2*	98	*NOVA1*	98	*ZFX*	98
5	*IFI30*	94	*DCUN1D4*	100	*EPM2AIP1*	98	*ZC3H6*	96	*PPP1R11*	97	*RIOK3*	97
6	*HECW2*	94	*PLK2*	100	*REEP1*	98	*ZIC2*	96	*UPP2*	97	*FAM126B*	96
7	***IL2***	**94**	*TNPO1*	100	*TMEM100*	98	***LEPR***	**96**	*WWC2*	97	***TXNIP***	**96**
8	*ADCY1*	92	*TRPV3*	100	*UBE2V2*	98	*ADAMTS9*	96	*BCORL1*	96	*C17orf75*	96
9	*EIF5*	91	*GAB1*	100	*PHLDA2*	97	*RAP2B*	95	*ZNF649*	95	*SNAP23*	95
10	*NPM1*	91	*GRIA4*	99	*IKZF2*	97	*UBE2E3*	95	*SORT1*	95	*SORT1*	95

Conclusion

In conclusion, the present study investigated the association between three miRNA variations and susceptibility to RSA in a fraction of the Iranian population. All polymorphisms except miR-146a C>G significantly increased the risk of RSA in the dominant inheritance model.

Ethics

Ethics Committee Approval: The Zahedan University of Medical Sciences Research Ethics Committee approved this study (decision no: IR.ZAUMS.REC.1396.218, date: 20.01.2024).
Informed Consent: All participants provided informed consent before entering the study.

Authorship Contributions

Concept: G.B., A.G., Design: M.M., Data Collection or Processing: M.M., Analysis or Interpretation: M.M., H.S.G., Literature Search: M.T., Writing: M.M.
Conflict of Interest: No conflict of interest was declared by the authors.
Financial Disclosure: The authors declared that this study received no financial support.

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Clinical Investigation / Araştırma

DOI: 10.4274/tjod.galenos.2024.50980

Turk J Obstet Gynecol 2024;21:166-74

Lymph node evaluation and nodal metastasis prediction in epithelial ovarian cancers: A retrospective study

Epitelyal over kanserlerinde lenf nodu değerlendirmesi ve nodal metastaz tahmini: Retrospektif bir çalışma

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Abstract

Objective: To identify consensus regarding lymph node (LN) evaluation in epithelial ovarian cancer (EOC). The objective of the present study was to evaluate surgico-pathological findings, LN involvement, and the prediction of LN metastasis via preoperative imaging and intraoperative assessment in women with EOC.

Materials and Methods: Women with EOC who underwent cytoreductive surgery (CRS) between Jan 2019 to June 2022 were included. The distribution of histology, stage, and LN metastasis was studied. The predictive value of serum cancer antigen (CA)-125, instead of and radiologically and surgically enlarged LNs with final LN histopathology was studied.

Results: A total of 96 women with EOCs underwent CRS. Fifty women (52%) underwent primary CRS and 46 women (48%) underwent interval CRS. Seventy-five women (78.13%) with EOC underwent pelvic and/or para-aortic lymphadenectomy, out of which 23 (30.67%) were histologically positive. High-grade serous carcinoma was the commonest (n=55, 73.33%) histology. The majority of women, 56 (74.67%) were stage III or IV at presentation. Complete cytoreduction was achieved in 59 (78.66%) patients. The receiver operating characteristics curve showed a cutoff for CA-125 of 1360 U/mL (area under the curve 0.702, p=0.002) for LN metastases. Both radiologically and surgically enlarged LNs significantly predicted LN metastasis on histopathology (p=0.02 and 0.006 respectively). The combined sensitivity, specificity, positive predictive value and negative predictive value of both contrast enhanced computed tomography (CECT) and surgically enlarged LNs were 78.26%, 57.69%, 45%, and 85.71%, respectively.

Conclusion: Serous histology, high-grade tumors, highCA-125 levels, and suspicious LNs on CECT or during surgery were significantly associated with LN metastasis. However, considering the false-negative rate of 21.74%, the combination of radiologically and surgically enlarged LNs cannot be used as the sole surrogate marker for lymphadenectomy.

Keywords: Epithelial ovarian cancer, lymph node metastasis, lymph node evaluation

PRECIS: Higher CA-125 levels (1360 U/mL) and suspicious LNs on CECT or during surgery are significantly associated with LN metastasis.

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Received/Geliş Tarihi: 10.03.2024 **Accepted/Kabul Tarihi:** 06.07.2024

Öz

Amaç: Epitelyal over kanserinde (EOC) lenf nodu (LN) değerlendirmesine ilişkin fikir birliği sağlamak amaçlanmıştır. Bu çalışmanın amacı, EOC'li kadınlarda cerrahi patolojik bulguları, LN tutulumunu ve preoperatif görüntüleme ve intraoperatif değerlendirme yoluyla LN metastazı tahminini değerlendirmektir.

Gereç ve Yöntemler: Ocak 2019 ile Haziran 2022 arasında sitoredüktif cerrahi (CRS) uygulanan EOC'li kadınlar dahil edildi. Histoloji, evre ve LN metastazının dağılımı incelendi. Serum kanser antijeni (CA)-125'in ve radyolojik ve cerrahi olarak gösterilen büyümüş LN'lerin ve son LN histopatolojisinin öngörücü değerleri araştırıldı.

Bulgular: EOC'li toplam 96 kadına CRS uygulandı. Elli kadına (%52) birincil CRS, 46 kadına (%48) aralıklı CRS uygulandı. EOC'li 75 kadına (%78,13) pelvik ve/veya para-aortik lenfadenektomi uygulandı, bunların 23'ü (%30,67) histolojik olarak pozitifti. Yüksek dereceli ayrılan karsinom en sık görülen histolojiydi (n=55, %73,33). Başvuru anında hastaların 56'sında (%74,67) hastalık evresi evre III veya IV idi. Hastaların 59'unda (%78,66) tam sitoredüksiyon sağlandı. Alıcı çalışma özellikleri eğrisi, LN metastazlarını öngörmede CA-125 için 1360 U/mL'lik (eğrinin altındaki alan 0,702, p=0,002) bir kesim değeri gösterdi. Hem radyolojik hem de cerrahi olarak gösterilen büyümüş LN'ler, histopatolojide LN metastazını anlamlı olarak öngördü (sırasıyla p=0,02 ve 0,006). Kontrastlı bilgisayarlı tomografi ile gösterilen LN'lerin birleşik duyarlılığı, özgüllüğü, pozitif tahmin değeri ve negatif tahmin değeri sırasıyla %78,26, %57,69, %45 ve %85,71 idi.

Sonuç: Seröz histoloji, yüksek dereceli tümörler, yüksek CA-125 düzeyleri ve kontrastlı bilgisayarlı tomografide veya ameliyat sırasında şüphe edilen LN'ler LN metastazı ile anlamlı derecede ilişkiliydi. Ancak %21,74'lük yanlış negatiflik oranı dikkate alındığında, radyolojik ve cerrahi olarak gösterilen büyümüş LN'lerin kombinasyonu, lenfadenektomi için tek belirteç olarak kullanılamaz.

Anahtar Kelimeler: Epitelyal over kanseri, lenf nodu metastazı, lenf nodu değerlendirmesi

Introduction

Determination of various histological patterns using comprehensive surgical staging and lymph node (LN) evaluation is crucial for the management and prognosis of epithelial ovarian cancers (EOCs). Thorough research into various factors predicting LN metastasis is required. The present study aimed to evaluate LN involvement in various histotype and predict LN metastasis using preoperative imaging and intraoperative assessment among women with EOC.

The incidence of LN involvement by EOC varies widely. The diagnosis depends mainly on the clinical stage of the disease, histological subtype, and extent of lymphatic dissection[1]. The reported rate of microscopic lymphatic involvement is 13-20% among women with tumors clinically confined to ovary[2]. However, lymphatic involvement is detected in 13-74% of stage III ovarian cancer (OC) and in 33-88% of stage IV OC women[3]. The role of systematic lymphadenectomy remains controversial. For patients with presumed early-stage OC, systematic lymphadenectomy is recommended due to improved overall survival[4]. However, few prospective studies have revealed the survival advantage of routine lymphadenectomy[5]. The lymphadenectomy in ovarian neoplasms (LION) trial showed no advantage of systematic lymphadenectomy in primary debulking surgery in advanced OCs (stage IIB-IV) with clinically negative LNs in terms of overall survival[6]. The National Comprehensive Cancer Network recommended resection of all suspicious or enlarged nodes along with the removal of LNs to have potential metastasis at the time of initial diagnosis, which is selective LN removal[7]. Various imaging methods are frequently employed in the preoperative assessment. Positron emission tomography (PET) has good sensitivity (73.2%) and specificity (96.7%) for detecting metastatic LNs[8]. However, PET is not always feasible as a routine test because of its non-availability and high cost. Computed tomography (CT) scan remains the most widely used preoperative imaging tool in women with ovarian cancer, but its utility is limited considering the varied sensitivity in various studies[8]. Additionally, cancer antigen (CA)-125 was found to be an independent predictor of LN involvement[9,10].

Herein, the primary objective of the study was to evaluate LN metastasis among various histotype of EOC along with the prediction of LN metastasis with enlarged LNs observed on preoperative imaging workup and during surgery on intraoperative clinical examination. The secondary objective of the study was to predict LN metastasis with preoperative CA-125 levels in women with EOC.

Material and Methods

Study Design, Setting, Participants

The present study is an observational retrospective study; conducted in the department of Obstetrics & Gynecology and Surgical Oncology at All India Institute of Medical Sciences (AIIMS), Rishikesh, India, from January 2019 to June 2022. The study protocol was approved by the AIIMS Institutional Ethics Committee (approval number: AIIMS/IEC/21/704, date: December 24, 2021). Women with EOCs aged 18 years or older who underwent cytoreductive surgery (CRS); both primary and interval CRS, with Eastern Cooperative Oncology Group (ECOG) status 0-2 at the time of surgery, were included in the study after providing written informed consent. The exclusion criteria of the study were women who were treated with palliative intent, diagnosed with non-epithelial or borderline tumors, or with recurrent OC or synchronous malignancy.

Data Collection

Demographic characteristics, detailed clinical history, CA-125 level, and preoperative contrast-enhanced computed tomography (CECT) findings were recorded. CRS included sampling of ascitic fluid or peritoneal washing, careful exploration of the pelvic and abdominal cavity, hysterectomy, bilateral salpingo-oophorectomy, omentectomy, peritoneal biopsy from suspected areas, pelvic and/or para-aortic

lymphadenectomy, and removal of all gross disease with the goal of R0 resection. In case of advanced OC, where the tumor is not amenable to R0 resection (patients with diffuse deep infiltration of small bowel mesentery, diffuse and confluent carcinomatosis of stomach and/or small bowel, involvement of superior mesenteric artery, multiple liver/brain/lung metastasis) or patients who are poor surgical candidates (ECOG 3) or more, low serum albumin <3 gm/dL, multiple comorbidities, neoadjuvant chemotherapy (NACT) followed by interval CRS was performed.

Detailed examination of retroperitoneal LNs in CECT scan (performed within 4 weeks prior to surgery) was performed. LNs were considered suspect if they were larger than 1 cm or if their shape deviated from the normal. Intraoperative details included the presence of ascites, largest tumor dimension, laterality, clinical examination of retroperitoneal LNs, including grossly enlarged (more than 1 cm) LN site and size, peritoneal cancer index (PCI) scores, lymphadenectomy details, surgical complexity score (SCS), residual disease, operative time, and blood loss, including any complications. Regarding intra-operative clinical examination of LNs, the opinion of two surgeons was taken; in case of a difference in opinion, evaluation by a third senior gynecology surgeon was sought. Systematic pelvic lymphadenectomy involved the removal of external iliac, internal iliac, and obturator LNs, and para-aortic lymphadenectomy included the removal of common iliac LNs and LNs related to the aorta and vena cava up to the level of the renal vein. Histopathological details, including tumor grade, histological type, number and sites of LN involvement, and final surgicopathological stage, were recorded. The rate of patients with positive LNs according to different histotype was identified, and the possible association between CA-125, radiological and intraoperative clinical examination of LNs with positive LNs (histopathologically proven) was evaluated.

Outcome Measures

Primary outcome measures included histopathological types and LN positivity rate, number (%) of women with LN metastasis among various histotype, positive LNs in women with preoperatively enlarged LNs on imaging, and LN positivity rate among surgically enlarged LNs. Secondary outcome measures included various International Federation of Gynecology and Obstetrics (FIGO) staging of disease and CA-125 levels among all women with EOC and LN metastasis.

Statistical Analysis

Categorical variables are presented as numbers and percentages (%). Quantitative data with normal distribution are presented as mean ± standard deviation and the data with non-normal distribution are presented as median (25^{th} and 75^{th} percentiles). The association of the variables which were quantitative and not normally distributed were analyzed using Mann-Whitney test (two groups) and Kruskal-Wallis test (more than two groups). The optimal cut-off value for CA-125 in patients with LN metastasis was determined using receiver operating characteristics (ROC) curve. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of radiologically enlarged LNs and surgically enlarged LNs were calculated alone and in combination to predict positive LNs. The final analysis was performed using the Statistical Package for Social Sciences (SPSS) software (IBM, Chicago, USA; version 21.0.

Results

A total of 167 women with OC underwent CRS between January 2019 and June 2022. Among them, 71 women were excluded from the study (54 with non-epithelial/borderline tumor, 12 with recurrent OC and 5 with synchronous cancer of endometrium). In total, 96 women underwent CRS. Among the 96 women with EOC, only 75 underwent pelvic and/or para-aortic lymphadenectomy. Out of 75 women with EOC, 35 (47%) underwent primary CRS and 40 (53%) underwent interval CRS. Thus, the final data analysis was done in n=75 women (Supplemental Figure 1).

The mean age of the participants was 48.42±11.6 years. The mean body mass index was 24.06±3.96 kg/m^2. The majority of them, 40 out of 75 (53.33%) were P2-P3 followed by 25 women (33.33%) higher than P3. Eight women (10.67%) were nulliparous. Forty-one women (54.67%) were postmenopausal. The mean duration of menopause was 8.81±5.79 years. Only seven out of 75 (9.33%) had a history of infertility/ovulation induction (Table 1).

The mean initial CA-125 value among women with EOC was 2861.72±11208.85 U/mL with a median (25^{th}-75^{th} percentile) of 894 (312.55-1907.2) U/mL. Most women with EOC had an advanced stage at the time of diagnosis. The most common stage was stage III C (53.3%, 40 out of 75 women) (Supplemental Figure 2). The mean PCI, CC, and SCS of women was 4.71±5.8, 0.36±0.77, and 4.08±1.97, with a range of 0-30, 0-3, and 2-11, respectively. Complete cytoreduction was achieved in 78.66% of women.

As shown in Table 2, the majority of women (55, 73.33%) had high-grade serous carcinoma (HGSC), followed by mucinous (8, 10.67%), endometrioid (4, 5.33%), low-grade serous carcinoma (LGSC) (3, 4.0%), and carcinosarcoma (2, 2.67%). Clear cell carcinoma, mixed carcinoma, and squamous carcinoma comprised one each (1.3%). Among 75 women with EOC who underwent lymphadenectomy along with CRS, 23 (30.67%) had pelvic or para-aortic LN metastasis. Para-aortic LNs were detected in only 16 out of 75 women (21.33%). Pelvic LNs were detected in only 17 out of 75 (22.67%) women. A median of 12 (range 1-60) LNs were removed, with a median of 8 pelvic LNs (range 1-32) and 3 para-aortic LNs (range 1-28) (Supplementary Table 1). Of 23 women with positive retroperitoneal LNs, 20 women (87%) were of HGSC; however, only 1 (infiltrative variety) case out of 8 women with mucinous carcinoma was positive, one woman with LGSC and

Table 1. Baseline characteristics of women with EOC (n=75)

Baseline characteristics	Frequency	Percentage
Age (years)		
20-30	5	6.67%
31-40	16	21.33%
41-50	17	22.67%
51-60	27	36.00%
>60	10	13.33%
Mean ± SD	48.42±11.6	
Median (25^{th}-75^{th} percentile)	50 (40-57.25)	
Range	20-73	
Body mass index (BMI) kg/m²		
18.5-22.99 kg/m² (Normal BMI)	34	45.34%
23-24.99 kg/m² (Overweight)	16	21.33%
>25 kg/m² (Obese)	25	33.33%
Mean ± SD	24.06±3.96	
Median (25^{th}-75^{th} percentile)	23.4 (21.175-25.625)	
Range	18.6-40.3	
Socioeconomic status (Modified Kuppuswamy scale)		
Upper	6	8.00%
Upper middle	23	30.67%
Lower middle	38	50.66%
Lower	8	10.67%
Parity		
P0	8	10.67%
P1	2	2.67%
P2-P3	40	53.33%
>P3	25	33.33%
Mean ± SD	3.01±1.61	
Median (25^{th}-75^{th} percentile)	3 (2-4)	
Range	0-8	
Prior menstrual history		
Irregular	12	16.00%
Regular	63	84.00%
H/0 infertility/ovulation induction	7	9.33%
Menopausal status	41	54.67
Duration of menopause (years)		
Mean ± SD	8.81±5.79	
Median (25^{th}-75^{th} percentile)	9 (4.75-10.25)	
Range	1-20	
Family history of cancer		
No	69	92.00%
Yes	6	8.00%
Contraception		
No	35	46.67%
Barrier	18	24.00%
IUCD	6	8.00%
OCP	3	4.00%
Tubal ligation	13	17.33%

EOC: Epithelial ovarian cancer, SD: Standard deviation

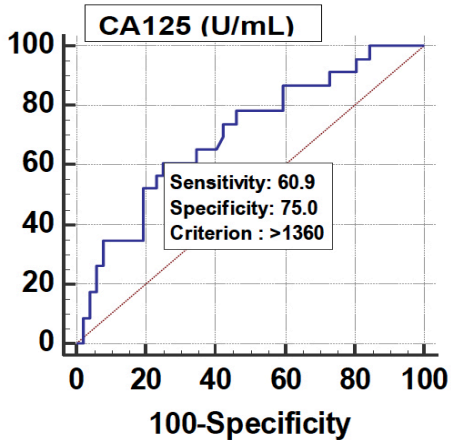

Figure 1. Receiver operating characteristic curve of CA-125 (U/mL) for predicting positive lymph nodes

CA: Cancer antigen

carcinosarcoma was positive each. The numbers of positive LNs among the various histotypes are shown in Table 2.

The median (25^{th}-75^{th} percentile) CA-125 level in positive LNs was 1913.8 (897.1-5048.5) U/mL, which was significantly higher than that in negative LNs (p-value 0.006) (Supplementary Table 2). The discriminatory power of CA125 (U/mL) [area under the curve (AUC) 0.702; 95% confidence interval: 0.585 to 0.802] was acceptable. CA-125 was a significant predictor of positive LNs at a cut-off point of >1360 U/mL with a 70.20% chance of correctly predicting positive LNs and a sensitivity of 60.87%. If CA-125 ≤1360 U/mL, then there was an 81.20% chance of normal LNs (Figure 1, Supplementary Table 3).

Table 3 shows a significant correlation between radiologically enlarged LNs and final histopathological findings (p-value 0.006) and surgically enlarged LNs (p-value 0.020).

Table 4 shows that the sensitivity, specificity, PPV, NPV, and accuracy of detecting LN metastasis with CECT were 56.52%, 78.85%, 54.17%, 80.39%, and 72%, respectively, and those with intraoperative LN assessment alone were 69.57%, 59.62%, 43.24%, 81.58%, and 62.67%, respectively. The combined sensitivity, specificity, PPV, and NPV of both CECT and surgically enlarged LNs were 78.26%, 57.69%, 45%, and 85.71%, respectively.

Combined (radiological + surgical) assessment had a sensitivity of 78.26%, followed by intra-op (69.57%) and radiological (56.52%) assessments in the prediction of positive LNs. Radiological assessment had the lowest sensitivity (56.52%) but higher specificity for predicting positive LN. Women who had positive LNs, 78.26% had enlarged LNs on radiologically or intraoperatively. If an enlarged LN was seen in either radiological or intra-op assessment, there was a 45% probability of a positive LN. If an enlarged LN is not seen on both CECT and intraoperatively, there is an 85.71% chance of no LN metastasis.

Discussion

This study analyzed the pattern of LN metastasis among EOC and evaluated whether CA-125, preoperative CT scan, and intraoperative LN gross examination could reliably predict LN metastasis, in order to decide whether women with EOC could be identified in whom lymphadenectomy could be omitted.

Regarding the spectrum of LN involvement, a significant percentage (23 out of 75, 30.67%) of EOC women were found to have pelvic or para-aortic LN metastasis, whereas in 22.67% of women, pelvic LNs and in 28% of women, para-aortic LN involvement were found. The majority of women (87%) with positive LN metastasis were HGSC. The optimum cut-off

Table 2. Association of positive lymph nodes with histological type

Lymph node status	High grade serous (n=55)	Low grade serous (n=3)	Mucinous (n=8)	Endometrioid (n=4)	Clear cell (n=1)	Squamous cell (n=1)	Mixed (n=1)	Carcinosarcoma (n=2)	Total (n=75)
Total lymph nodes positive									
Negative	35	2	7	4	1	1	1	1	52 (69.33%)
Positive	20	1	1	0	0	0	0	1	23 (30.67%)
Para-aortic lymph nodes positive									
Negative	40	3	8	4	1	1	1	1	59 (78.67%)
Positive	15	0	0	0	0	0	0	1	16 (21.33%)
Pelvic lymph nodes positive									
Negative	41	2	7	4	1	1	1	1	58 (77.33%)
Positive	14	1	1	0	0	0	0	1	17 (22.67%)

Table 3. Association of enlarged LNs with histopathologically positive LNs

Enlarged LNs	Negative (n=52)	Positive (n=23)	Total	p-value
Radiologically enlarged lymph nodes				
No	41 (78.84%)	10 (43.47%)	51 (68%)	0.006†
Yes	11 (21.15%)	13 (56.52%)	24 (32%)	
Surgically enlarged lymph nodes on intra op clinical examination				
No	31 (59.61%)	7 (30.43%)	38 (50.67%)	0.020†
Yes	21 (40.38%)	16 (69.57%)	37 (49.33%)	
Combined radiological + Surgically enlarged LN on intra-op examination				
No	30 (57.69)	5 (21.74%)	35	0.006†
Yes	22 (42.31%)	18 (78.26%)	40	

†: Chi-square test, LN: Lymph node

Table 4. Sensitivity, specificity, positive predictive value and negative predictive value of radiologically enlarged lymph nodes and intra op clinically enlarged lymph nodes for predicting positive lymph nodes

Variables	Radiologically enlarged LNs	Surgically (Intra-op) enlarged LNs	Combined (radiological + surgical assessment)
Sensitivity (95% CI)	56.52% (34.49% to 76.81%)	69.57% (47.08% to 86.79%)	78.26% (56.30% to 92.54%)
Specificity (95% CI)	78.85% (65.30% to 88.94%)	59.62% (45.10% to 72.99%)	57.69% (43.20% to 71.27%)
AUC (95% CI)	0.68 (0.56 to 0.78)	0.65 (0.53 to 0.75)	0.68 (0.56 to 0.78)
Positive predictive value (95% CI)	54.17% (32.82% to 74.45%)	43.24% (27.10% to 60.51%)	45% (29.26% to 61.51%)
Negative predictive value (95% CI)	80.39% (66.88% to 90.18%)	81.58% (65.67% to 92.26%)	85.71% (69.74% to 95.19%)
Diagnostic accuracy	72.00%	62.67%	64.00%

CI: Confidence interval, AUC: Area under the curve, LN: Lymph node

value of serum CA125 in the ROC curve for LN metastasis was 1.360 U/mL with AUC of 0.702 (p-value 0.002) and 60.87% sensitivity and specificity of 75%. Both radiologically and surgically enlarged LNs significantly predicted LN metastasis on histopathology (p=0.02 and 0.006 respectively). Combined (radiological + surgical) assessment had a sensitivity of 78.26%, followed by intra-op (69.57%) and radiological (56.52%) assessments for predicting positive LNs. Radiological assessment had the lowest sensitivity (56.52%) but higher specificity for predicting positive LN. Women who had positive LNs, 78.26% had enlarged LNs on CECT or intraoperatively. If an enlarged LN was seen in either radiological or intra-op assessment, there was a 45% probability of a positive LN. If an enlarged LN is not seen radiologically and intraoperatively, there is an 85.71% chance of no LN metastasis.

Regarding the spectrum of LN metastasis in EOCs, similar to our results, Zhou et al.[11] found LN metastasis in 32.8% women in a retrospective analysis of 256 patients with EOC; majority were HGSC. Similarly, a cross-sectional study of 55 patients with EOC by Andrijono et al.[12] showed an LN positivity rate of 42.9% in serous carcinoma. The discrepancy in the total number of positive LNs between HGSC and LGSC may be attributed to differences in the incidence of different histotypes of EOC in the present study. The LN positivity rate could not be calculated because the present study was underpowered to evaluate subtype-specific associations, particularly for rare histotype. In contrast to the present study, Widschwendter et al.[13] found that 51.8% of women had LN metastasis (pelvic and/or para aortic) in a retrospective analysis of 114 women with EOC. The reason could be higher LN retrieval and the non-inclusion of NACT cases.

Various other studies also showed a correlation between CA-125 and LN metastasis. Sudolmus et al.[14] concluded that the cut-off value for LN metastases was 7192 U/mL in the ROC curve, which was significant in logistic regression analysis (p=0.005) but associated with a high rate of false positivity in the Turkish population. Zhang et al.[15] concluded that serum CA-125 combined with D-dimer had good predictive value for LN metastasis in EOC.

Regarding the prediction of LN metastasis by CT scan, Uysal et al.[16] found the sensitivity, specificity, PPV, NPV, and accuracy of 62%, 52%, 57%, 57%, and 57%, respectively, in a retrospective analysis of 89 women with EOC. Concordance with our results, another retrospective analysis done by Widschwendter et al.[11] concluded that CT scan has a sensitivity of 40.7%, specificity of 89.1%, PPV of 80%, and NPV of 58.3% in the prediction of LN metastasis in EOC patients.

Regarding the prediction of LN metastasis by LN palpation during surgery, Arango et al.[17] found a sensitivity, specificity, PPV, and NPV of 72%, 81%, 56%, and 89%, respectively, in a prospective study of 126 women with EOC. Khunnarong et al.[18] analyzed 124 women with EOC and concluded that 81% had sensitivity, 91% specificity, 65% PPV, 96% NPV, and 90% accuracy. Harter et al.[19] performed a retrospective analysis of 195 women with EOC and inferred similar results. A meta-analysis of 89 women with EOC performed by Mimoun et al.[20] concluded that intra-op clinical examination for the prediction of LN metastasis has a sensitivity of 79%, specificity of 85%, and accuracy of 86%. Hailing Xiang et al. constructed a nomogram integrating CT-reported LN status, child-bearing status, tumor laterality, and stage, which showed good calibration and discrimination with an AUC of 0.775, significantly improving performance over the CT results (0.699, p=0.0002)[21]. Recently, a prospective multicentric study conducted by the FRANCOGYN group evaluated the utility of a new diagnostic tool, consisting of pelvic and/or para-aortic LN metastasis on CT and/or PET/CT scan, initial PCI ≥10 and/or diaphragmatic carcinosis, and initial CA-125 ≥500 U/mL, in predicting LN metastasis in women with advanced EOC. The group at a high risk of LN metastasis had 83.5% sensitivity, 2.73% LR+, and 79.3% observed probability of LN metastasis[10].

However, comparisons across studies should be made with utmost caution because of differences in study design and study populations. The sensitivity, specificity, PPV, and NPV of the combination of radiological + surgically enlarged LN for detecting LN metastasis were 78.26%, 57.69%, 45%, and 85.71%, respectively, in the present analysis. The assessment of retroperitoneal LNs by a combination of CECT+ intraoperative clinical evaluation is not sufficient to preclude lymphadenectomy in the case of normal-appearing LNs; the probable reason could be the presence of microscopic tumor metastasis without a visible bulky LN in the present study.

Our study showed that positive radiological findings combined with surgical assessment triage women into a group at high risk of LN metastases, with a clear indication for pelvic and para-aortic lymphadenectomy, as described in the literature and particularly in the LION trial[6].

Negative radiological and intraoperative assessment triage women into groups at low risk of LN metastases. However, it does not appear sufficient to conclusively rule them out in view of the considerable number of false-negative (21.74%) in the combined assessment in the present study. Nonetheless, it must be noted that in the LION trial, despite the 55.7% false-negative rate, no survival difference was observed between the "lymphadenectomy" and "no lymphadenectomy" groups in advanced ovarian cancer.

Strengths and Limitations of the Study

Our study evaluated the prediction of LN metastasis with the combined assessment of preoperative CECT scan and intraoperative gross examination of LNs, along with CA-125 levels. To date, no study has evaluated LN involvement in consideration of the above-mentioned factors in combination. To reduce observational bias, the opinions of at least two surgeons were obtained during the intraoperative clinical evaluation of retroperitoneal LNs.

In this study, patients were enrolled retrospectively, which has

the inherent potential to introduce selection bias. Our institution is a tertiary referral oncology center; therefore, most patients were primarily referred with low-performance status and advanced stage. The present study identified a high percentage of interval debulking surgery (IDS) cases due to poor ECOG status at presentation and the COVID-19 pandemic. Depending on these factors, the number of removed pelvic and para-aortic LNs was less than the minimum number described in the literature. The present study was underpowered to evaluate subtype-specific associations, particularly for rare histotype.

Conclusion

Serous histology, higher CA-125 levels (1360 U/mL, AUC 0.702, p=0.002), and suspicious LNs on CECT and during surgery were significantly associated with LN metastasis. However, normal LNs both in size and morphology, as assessed by the combination of CECT and intraoperative findings, were positive in 21.74% of women in the present study, suggesting that a combination of radiologically and surgically enlarged LNs cannot be used as sole surrogate markers for lymphadenectomy. A pragmatic approach to complete surgical staging with systematic lymphadenectomy for ovarian cancer is needed. However, the effect of this is yet to be known; since the present study did not evaluate overall survival and progression-free survival in women. Further prospective randomized trials in a larger population are needed to confirm these findings.

Ethics

Ethics Committee Approval: The study protocol was approved by the AIIMS Institutional Ethics Committee (approval number: AIIMS/IEC/21/704, date: December 24, 2021).
Informed Consent: All participants provided informed consent before entering the study.

Authorship Contributions

Surgical and Medical Practices: A.B., S.R., R.K.S., J.C., R.M., S.R., Concept: P.V., A.B., S.R., R.K.S., Design: P.V., A.B., S.R., R.K.S., Data Collection or Processing: P.V., I.S., A.H., Analysis or Interpretation: R.K.S., A.G., S.R., Literature Search: P.V., J.C., R.M., Writing: P.V., A.B.
Conflict of Interest: No conflict of interest was declared by the authors.
Financial Disclosure: The authors declared that this study received no financial support.

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Supplementary Table 1. Lymph node yield of all EOC women with LND on histopathology

Lymph node yield	Mean ± SD	Median (25^{th}-75^{th} percentile)	Range
Total LN yield	13.61±12.5	12 (1-22)	0-60
LN yield (para-aortic)	4.33±5.89	2.5 (0-7)	0-28
LN yield (pelvic)	9.28±8.21	8 (1-15.25)	0-32

SD: Standard deviation, EOC: Epithelial ovarian cancer, LN: Lymph node, LND: Lymph node dissection

Supplementary Table 2. Association of CA-125 (U/mL) with histopathologically positive LNs

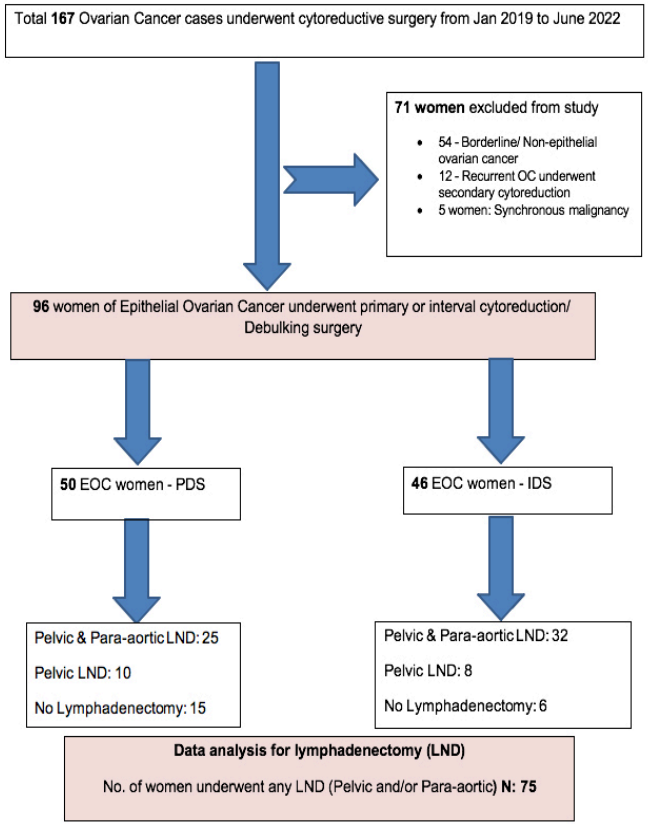
CA125 (U/mL)	Negative (n=52)	Positive (n=23)	Total	p-value
0-35 IU/L	4 (7.69%)	0 (0%)	4 (5.33%)	0.306*
>35 IU/L	48 (92.31%)	23 (100%)	71 (94.67%)	
Mean ± SD	3407.23±14966.95	3519.01±4076.48	3441.51±12622.52	0.006‡
Median (25^{th}-75^{th} percentile)	665.35 (344.85-1395)	1913.8 (897.1-5048.5)	946 (363.05-2268.5)	
Range	4.2-108.290	117.4-14.507	4.2-108.290	

‡: Mann-Whitney test, *: Fisher's exact test, SD: Standard deviation, LN: Lymph node, CA: Cancer antigen

Supplementary Table 3. Receiver operating characteristic curve of CA-125 (U/mL) for predicting positive lymph nodes

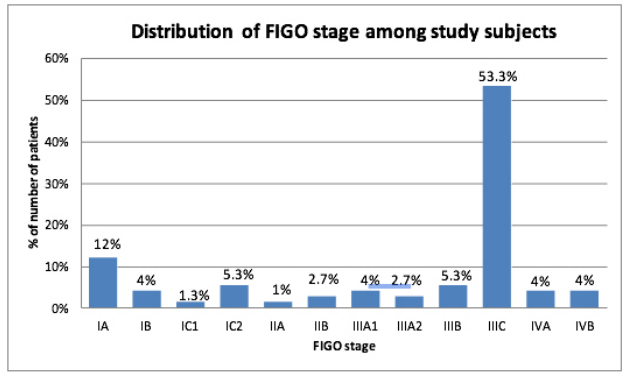
Variables	Value
Area under the ROC curve (AUC)	0.702
Standard error	0.066
95% confidence interval (CI)	0.585 to 0.802
P-value	0.0022
Cut-off	>1360 U/mL
Sensitivity (95% CI)	60.87% (38.5-80.3%)
Specificity (95% CI)	75% (61.1-86.0%)
PPV (95% CI)	51.9% (31.9-71.3%)
NPV (95% CI)	81.2% (67.4-91.1%)
Diagnostic accuracy	70.67%

ROC: Receiver operating characteristics, CA: Cancer antigen, PPV: Positive predictive value, NPV: Negative predictive value



Supplemental Figure 1. Flow-chart for analysis of all women with EOC under study

EOC: Epithelial ovarian cancer, LND: Lymph node dissection



Supplemental Figure 2. Distribution of FIGO stage among women with EOC

EOC: *Epithelial ovarian cancer, FIGO: International Federation of Gynecology and Obstetrics*

Clinical Investigation / Araştırma

DOI: 10.4274/tjod.galenos.2024.89439

Turk J Obstet Gynecol 2024;21:175-9

Negative effects of ethanol on ovarian reserve and endometrium thickness: An animal study

Etanolün yumurtalık rezervi ve endometrium kalınlığı üzerine olumsuz etkileri: Bir hayvan çalışması

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Abstract

Objective: This study aimed to assess the effect of ethanol on the ovarian reserve and endometrium of rats by evaluating anti-Müllerian hormone (AMH) levels and follicle counts.

Materials and Methods: We performed histological follicle counting and AMH measurements to evaluate ovarian reserve. The study included 16 Wistar albino rats evenly distributed into two groups of eight rats each. The rats in the intervention group (group 1) were administered ethanol at a daily dose of 2.5 g/kg via oral gastric lavage for 30 days, whereas the control group (group 2) received water as a placebo via oral gastric lavage for the same period. At the end of 30 days, the animals were sacrificed, and 2 mL blood samples were collected for AMH measurements. Laparotomy was performed to remove the ovaries and uterus.

Results: Despite the lack of a meaningful distinction in the quantity of primordial and primary follicles between the two groups, a substantial disparity was observed in the overall follicle count and AMH levels. Specifically, the intervention group exhibited significantly lower total follicle counts and AMH levels than the control group ($p \leq 0.001$). The researchers also found that the endometrium of ethanol-treated rats was significantly thinner than that of control rats ($p \leq 0.001$).

Conclusion: This study concluded that ethanol consumption can negatively affect reproductive ability and the success of in vitro fertilization treatment by reducing ovarian reserve and thinning the endometrium.

Keywords: Ethanol, endometrium, ovarian reserve, rat

Öz

Amaç: Bu çalışmada, anti-Müllerian hormon (AMH) düzeyleri ve folikül sayıları değerlendirilerek etanolün sıçanların ovaryan rezervi ve endometriyumu üzerindeki etkisinin değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntemler: Araştırmacılar ovaryum rezervini değerlendirmek için histolojik folikül sayımı ve AMH ölçümleri gerçekleştirmiştir. Çalışmaya her biri sekiz sıçandan oluşan iki gruba eşit olarak dağıtılmış 16 Wistar albino sıçan dahil edilmiştir. Müdahale grubundaki sıçanlara (grup 1) 30 gün boyunca oral gastrik lavaj yoluyla günlük 2,5 g/kg dozunda etanol uygulanırken, kontrol grubuna (grup 2) aynı süre boyunca oral gastrik lavaj yoluyla plasebo olarak su verilmiştir. Otuz günün sonunda hayvanlar sakrifiye edilmiş ve AMH ölçümü için 2 mL kan örneği alınmıştır. Yumurtalıkları ve uterusu çıkarmak için laparotomi yapıldı.

Bulgular: İki grup arasında primordial ve primer folikül miktarında anlamlı bir fark olmamasına rağmen, genel folikül sayısı ve AMH seviyelerinde önemli farklılıklar vardı. Spesifik olarak, müdahale grubunda toplam folikül sayısı ve AMH seviyeleri kontrol grubuna kıyasla önemli ölçüde daha düşüktü ($p \leq 0,001$). Araştırmacılar ayrıca etanol ile tedavi edilen sıçanların endometriyumunun kontrol sıçanlarına göre önemli ölçüde daha ince olduğunu tespit etmiştir ($p \leq 0,001$).

PRECIS: Ethanol reduces ovarian reserve and endometrial thickness in rats, lowering AMH levels and total follicle counts, thereby negatively affecting reproductive health.

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Received/Geliş Tarihi: 31.07.2024 **Accepted/Kabul Tarihi:** 20.08.2024

Sonuç: Çalışma, etanol tüketiminin yumurtalık rezervini azaltarak ve endometriyumu incelterek üreme yeteneğini ve in vitro fertilizasyon tedavisinin başarısını olumsuz etkileyebileceği sonucuna varmıştır.

Anahtar Kelimeler: Etanol, endometriyum, yumurtalık rezervi, sıçan

Introduction

Alcoholism has been shown to have a negative impact on the female reproductive system, leading to amenorrhea, ovulation disorders, premature menopause, spontaneous abortion, and infertility[1,2]. Studies have also shown that ovarian reserve is reduced by alcohol consumption[1]. Although the consequences of ethanol exposure on the reproductive system of females have been meticulously documented, the specific mechanisms of alcohol's mechanism of action on the ovarian reserve and endometrium are not well understood.

Recent research indicates that ethanol suppresses the luteinizing hormone through its action on the hypothalamus, and chronic ethanol exposure has been associated with increased levels of serum estradiol[3]; however, the evidence regarding the effects of ethanol on peripheral reproductive tissues and their physiology is conflicting.

The ovarian reserve has been suggested to clearly indicate the fertility status of a woman as it has been demonstrated that reproductive capability is directly proportional to ovarian reserve. Due to these findings, many methods for determining ovarian reserve have been identified in the literature. Among these, the total ovarian follicle count and the measurement of anti-Müllerian hormone (AMH) levels can be used to predict ovarian reserve; consequently, these tests have become the most widely used methods for assessing ovarian reserve[4].

The primary aim of this study was to evaluate the effect of ethanol on AMH levels and ovarian reserve, as determined by histological analysis in a rat model. We also aimed to demonstrate the negative effects of alcohol consumption on reproductive ability.

Materials and Methods

Sixteen female Wistar albino rats weighing 180-210 grams and five to six months old were used in this study. All stages and procedures of this research were conducted in the Guinea Pig Experimental Animal Laboratory. During the experiments, the researchers adhered to strict animal care and use guidelines approved by the Institutional Review Board. All rats were maintained at 22±2 °C and subjected to a 12-h/12-h light/dark cycle without restriction of food or water.

Sixteen Wistar albino rats were randomly divided into two groups of eight rats each. Rats in the experimental group (group 1) were administered 2.5 g/kg ethanol daily via oral gastric lavage for 30 days. Rats in the control group (group 2) were given water as a placebo for the same period and using the same method. The rats eliminated ethanol at approximately 7.9 mmol/kg h[5]. To prevent possible effects of blood ethanol levels on AMH measurements in the study group, laparotomy was performed in both groups on the day after the last ethanol administration. A total of 2 mL of intracardiac blood was taken to evaluate AMH, and the ovaries and uteri of all animals were obtained with immediate laparotomy. Ovarian follicle counts were taken according to types (primordial, primary, secondary, tertiary, and total) in all rats and endometrial thickness was histologically evaluated.

After 30 days, 2 mL of intracardiac blood was taken to evaluate AMH, and the ovaries and uteri of all animals were obtained with immediate laparotomy. Ovarian follicle counts were taken according to types (primordial, primary, secondary, tertiary, and total) in all rats, and endometrial thickness was histologically evaluated.

Before laparotomy, a 2 mL blood sample was obtained via intracardiac aspiration after appropriate anesthesia. Blood serum was separated by centrifugation at 1000 x g for 15 min at 4 °C. Serum samples were stored at 20 °C until assayed. AMH levels (ng/mL) were determined using a BioTek Synergy HT Microplate Reader (USA) capable of measuring absorbance at 450 nm, which was used according to the manufacturer's instructions. In this procedure, a Cusabio Rat AMH kit was used, and the correction wavelength was set to 600 nm-630 nm by a blinded researcher in a private medical laboratory. All samples were tested on the same plate in triplicate measurements. The minimum detection limit of the AMH enzyme-linked immunosorbent assay kit was 0.051 nanograms per milliliter (ng/mL), and the inter-assay and intra-assay variations were 15%.

5 µm thick slices of ovarian sections and five randomly selected samples from each ovary were used to assess follicular activity. Hematoxylin-eosin staining was performed, and a pathologist evaluated the samples using a light microscope and a blinded method. All follicles were counted and categorized into primordial, primary, secondary, and tertiary stages, according to standard microscopic anatomy textbooks. The ovarian reserve was determined by summing these four categories.

The endometrial thickness (nm) was also measured, and 100 cells were evaluated in each section. In addition, each tenth section of 100 slices was examined at 400X magnification to count the number of glands in the area.

Before the start of the study, written approval was obtained from the Experimental Animals Local Ethics Committee of the Faculty of Medicine of Niğde Ömer Halisdemir University (approval number: 2024/10; date: 31.05.2024).

Statistical Analysis

For statistical analyses, the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) software version 15.0 was used. Normality tests, including the Shapiro-Wilk test,

were conducted to assess whether the variables followed a normal distribution. If a variable failed to show a normal distribution, the Mann-Whitney U test was employed. Variables with a normal distribution were analyzed using the t-test. The significance level was set at $p<0.05$. The results are expressed as the mean ± standard deviation, and power analysis of the values was performed using Sigma-Aldrich 3.5 software, as indicated below the tables.

Results

Both groups showed comparable numbers of primordial and primary follicles; however, the overall follicle count in the study group was notably lower than that in the control group (12.10±4.94 vs. 28.60±6.80, p≤0.001). Moreover, AMH levels were considerably lower in the ethanol group than in the control group (24.55±28.03. 91.38±26.54, p≤0.001). Furthermore, endometrial thickness was significantly lower in the ethanol group than in the control group (487±53 vs. 312±58, p≤0.001). All measured parameters are summarized in Table 1, Figure 1, and Figure 2, and endometrial thickness comparisons between the groups are illustrated in Figure 3.

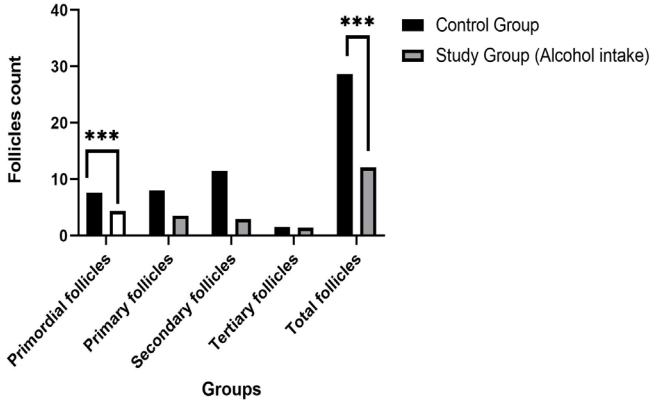

Figure 1. Bar graphs of the follicle count
***: p<0.01

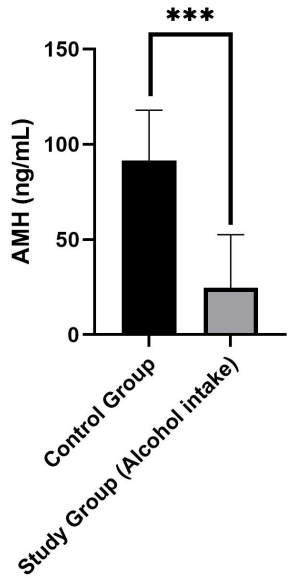

Figure 2. Bar graph of AMH levels
***: p<0.01
AMH: Anti-Müllerian hormone

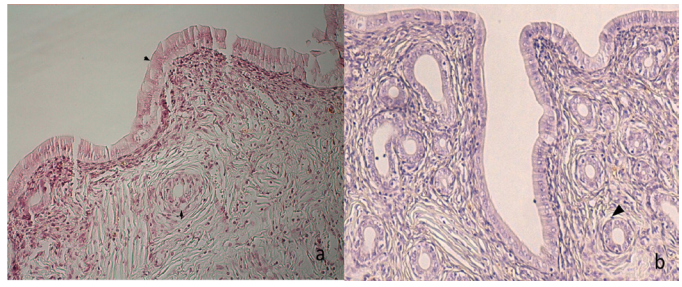

Figure 3. Comparison of endometrial histology
a. Histological examination of the control group (H&E)(x400)
b. Histological examination of the alcohol intake (H&E)(x400)
H&E: Hematoxylin-eosin

Table 1. Results of ovarian follicule tests and endometrium thickness of the groups

Parameters	Control group	Study group (alcohol intake)	p
Primordial follicles	7.63±4.86	4.30±2.65	0.108
Primary follicles	8.02±3.54	3.55±1.60	0.039
Secondary follicles	11.47±3.11	2.90±2.41	<0.001
Tertiary follicles	1.55±0.75	1.41±0.74	0.74
Total follicles	28.60±6.80	12.10±4.94	<0.001
AMH (ng/mL)	91.38±26.54	24.55±28.03	<0.001
Endometrium (nm)	487±53	312±58	<0.001

AMH: Anti-Müllerian hormone

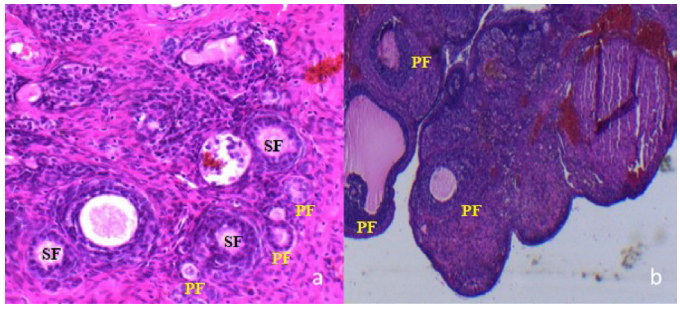

Figure 4. Comparison of ovarian histology (SF; Secondary follicle, PF; Primordial follicle)

a. Histological examination of the control group (H&E)(x400)

b. Histological examination of the alcohol intake (H&E)(x400)

H&E: Hematoxylin-eosin

Figure 4 displays a histological image that exhibits evident changes in the ovaries of ethanol-treated rats compared with the control group.

Discussion

Alcohol has been proven to have detrimental effects on the neuroendocrine, cardiovascular, and immune systems, and this is a widely accepted fact[6,7]. Despite the known adverse effects of ethanol on the female reproductive system, few studies have focused on the toxicity of alcohol to specific tissues[8]. Therefore, identifying the influence of ethanol on various organs of the female reproductive system is an important research focus, especially since available studies have shown varying degrees of alcohol-induced damage.

As mentioned previously, ovarian reserve determination has become a central part of reproductive assessment in females. The measurement of AMH levels is a good proxy for ovarian reserve[9,10]. This has been demonstrated through evidence obtained from various studies[11]. Thus, we utilized AMH measurements to determine and compare the ovarian reserve of rats in this study. We also directly counted the number of follicles via histological evaluation of tissues.

Normally, the highest levels of AMH expression are observed in the granulosa cells of secondary follicles and preantral and small antral follicles[12]. When this finding is evaluated considering a study by Chuffa et al.[13] which reported advanced atresia of secondary, antral, and preovulatory follicles in the granulosa layer of rats exposed to ethanol, it seems that the mechanism of AMH reduction is associated with the damage to the granulosa layer by ethanol. However, this may not be the only explanation, as other mechanisms may contribute to the overall reduction in AMH levels associated with alcohol consumption. In this study, using a prospective randomized rat model, we demonstrated that ethanol ingestion leads to decreased AMH levels and adverse effects on ovarian reserve. Although our results agree with those of especially controlled studies and some clinical studies[14,15], most clinical studies report opposing findings. For instance, a large population-based study by Dólleman et al.[16] failed to find any relationship between alcohol consumption and AMH levels according to age. This was also true in various studies of the same type conducted in several countries[17-20]. However, it is important to note that the measures used for female fertility varied from study to study, and not every study focused on alterations in AMH levels or other methods of laboratory analyses.

Chuffa et al.[13] investigated the effects of ethanol on the types and numbers of follicles. Their findings revealed that rats treated with ethanol experienced a decrease in the number of primordial follicles, with no noticeable effect on the number of primary or tertiary follicles.

Somewhat in contrast with these results, our findings demonstrated that the secondary and total follicle counts were significantly decreased in the rat group receiving ethanol. We also found that follicle counts were correlated with AMH levels in our study group.

Thin endometrium, which is often a result of a hypoestrogenic state, is significantly associated with implantation failure and pregnancy loss[21]. Lack of estrogen may be due to the lower number of follicles, which in turn leads to a vicious cycle. Studies suggesting the adverse effects of alcohol on reproductive health have shown poor pregnancy outcomes in those exposed to alcohol during pregnancy[22]. Despite the presence of convincing arguments for both sides of the debate, the adverse effects of alcohol use on the success of in vitro fertilization and the quality of embryos are widely accepted[23]. Considering that alcohol consumption may cause a thin endometrium, it is evident that the implantation of embryos may also suffer from alcohol use, both in the long and short term. Our results add to these adversities by suggesting that ovarian reserve is negatively affected by ethanol consumption in a rat model of daily alcohol consumption.

Study Limitations

Although we believe our results are convincing, it is important to consider that the metabolic differences between humans and rats, especially in terms of liver function, reproductive capabilities, and evolutionary differences, could explain the lack of agreement between our results and most clinical studies. These data are important to consider when evaluating the effects of ethanol on ovarian reserve.

Conclusion

To the best of our knowledge, this study is the first to evaluate ovarian reserves in rats exposed to ethanol for 30 days by analyzing AMH levels and histological features of ovarian follicles. In addition, this study clearly revealed that alcohol use was associated with decreased ovarian reserve and a thin endometrium based on histological findings.

Acknowledgments

The author would like to thank histologist Esra Balcıoğlu for her help.

Ethics

Ethics Committee Approval: Before the start of the study, written approval was obtained from the Experimental Animals Local Ethics Committee of the Faculty of Medicine of Niğde Ömer Halisdemir University (approval number: 2024/10; date: 31.05.2024).
Informed Consent: Not necessary.

Authorship Contributions

Surgical and Medical Practices: E.K., Concept: E.K., Design: E.K., M.E.A., Data Collection or Processing: M.E.A., Analysis or Interpretation: E.K., M.E.A., Literature Search: E.K., M.E.A., Writing: E.K.
Conflict of Interest: No conflict of interest was declared by the authors.
Financial Disclosure: The authors declared that this study received no financial support.

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Clinical Investigation / Araştırma

DOI: 10.4274/tjod.galenos.2024.99132

Turk J Obstet Gynecol 2024;21:180-9

Evaluation of placental bed uterine in L-NAME-induced early-onset preeclampsia (EO-PE) like the rat model

L-NAME ile indüklenen erken başlangıçlı preeklampsi (EO-PE) sıçan modelinde uterin plasental yatağın değerlendirilmesi

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Abstract

Objective: Preeclampsia (PE) is the leading cause of maternal death worldwide and is associated with long-term morbidity in both mothers and newborns. Animal modeling is considered a functional source for understanding PE pathogenesis, diagnostic standards, and therapeutic approaches.

Materials and Methods: This study aimed to demonstrate and evaluate the use of N-nitro-L-arginine methyl ester (L-NAME) in a Wistar rat model under conditions similar to PE. A total of 12 rats were divided into 4 groups, each consisting of 3 members, including the pregnant control group and treatment groups administered low-dose (PE 25 mg/kg L-NAME/day), medium-dose (PE 50 mg/kg L-NAME/day), and high-dose L-NAME (PE 75 mg/kg L-NAME/day) L-NAME from gestational day 4 to 19. Measurements included blood pressure, creatinine, and proteinuria levels, placental histological changes, and placental tissue hypoxia-inducible factor 1-alpha, and plasma endothelial nitric oxide synthase levels.

Results: The results showed that intervention with L-NAME at 75 mg/kg body weight/day (PE3) induced PE earlier than that with 50 mg/kg body weight/day L-NAME.

Conclusion: The model conditions also support further research into PE pathogenesis.

Keywords: eNOS, HIF1α, L-NAME, MAP, preeclampsia, proteinuria, spiral arteries

Öz

Amaç: Preeklampsi (PE) dünya çapında anne ölümünün önde gelen nedenidir ve hem annelerde hem de yenidoğanlarda uzun süreli morbidite ile ilişkilidir. Hayvan modelleri, PE patogenezini, tanı standartlarını ve tedavi yaklaşımlarını anlamak için işlevsel bir kaynak olarak kabul edilir.

Gereç ve Yöntemler: Bu çalışmada, N-nitro-L-arginin metil esterin (L-NAME) Wistar sıçan modelinde PE'ye benzer koşullar altında kullanımının gösterilmesi ve değerlendirilmesi amaçlandı. Gebeliğin 4-14 günlerinde olan toplam 12 sıçan, gebe kontrol grubu ve düşük doz L-NAME (PE 25 mg/kg L-NAME/gün), orta doz L-NAME (PE 50 mg/kg L-NAME/gün) ve yüksek doz L-NAME (PE 75 mg/kg L-NAME/gün) uygulanan tedavi grupları dahil olmak üzere her biri 3 üyeden oluşan 4 gruba ayrıldı. Ölçümler kan basıncını, kreatinin ve proteinüri düzeylerini, plasental histolojik verileri ve plasental doku hipoksisi ile indüklenen faktör 1-alfa ve plazma endotelyal nitrik oksit sentaz seviyelerini içeriyordu.

Bulgular: Sonuçlar, 75 mg/kg vücut ağırlığı/gün (PE3) L-NAME müdahalesinin, 50 mg/kg vücut ağırlığı/gün L-NAME müdahalesinden daha erken PE'yi tetiklediğini gösterdi.

Sonuç: Model koşulları aynı zamanda PE patogenezine yönelik daha ileri araştırmaları da desteklemektedir.

Anahtar Kelimeler: eNOS, HIF1α, L-NAME, MAP, preeklampsi, proteinüri, spiral arterler

PRECIS: Treatment of rats with L-NAME at a dose of 75 mg/kg/d results in inadequate spiral artery remodeling, HBP, proteinuria, and IUGR, thus clearly mimicking the syndrome of EO-PE.

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Received/Geliş Tarihi: 19.03.2024 **Accepted/Kabul Tarihi:** 03.06.2024

Introduction

Preeclampsia (PE) is a leading cause of maternal mortality worldwide and is associated with long-term morbidity in both mothers and newborns[1]. It is a hypertensive condition characterized by a diastolic[2] blood pressure (DBP) of 90 mmHg and a systolic blood pressure (SBP) of 140 mmHg, accompanied by proteinuria (0.3 g/24 hours) appearing after the 20th week of pregnancy[3]. The current classification is based on the onset and severity of symptoms, but it does not accurately reflect the underlying pathophysiological processes. The different PE classes include early onset (EO) <34 weeks, late onset (LO) ≥34 weeks, or preterm (<37 weeks), and term (≥37 weeks) PE[4]. EO or premature PE is more often complicated by fetal growth restriction and more severe symptoms than LO or term PE[5]. The main pathological feature of EO-PE is incomplete transformation of spiral arteries[6]. This condition manifest as a multisystem syndrome with maternal and neonatal morbidities exceeding those of normal pregnancies[7]. The pathogenesis of EO-PE is diverse and is associated with factors such as excessive inflammation, oxidative stress, metabolic disturbances, and apoptosis[7,8]. According to current knowledge, the disease develops through preclinical and clinical stages[10]. Important pathological modifications, including inadequate trophoblast invasion and remodeling of spiral arteries at the placental base, are recognized as critical factors in the preclinical phase[11]. The term placental bed describes the maternal-fetal interface or the area where the placenta attaches to the uterus, which requires adequate vascularization for fetal development[12].

Experimental animal models are valuable for examining PE pathogenesis, diagnosis, and treatment options[13]. In reproductive research, rats have significant anatomical and behavioral advantages over mice[14]. Many models have been projected to meet or at least resemble the above criteria, including reductions in uterine perfusion pressure, nitric oxide synthase (NOS) knockout rats [parallel to the N-nitro-L-arginine methyl ester (L-NAME) model], transgenic, sFlt-1 infusion, and alpha tumor necrosis factor infusion models[15]. Physiologically, these models represent hypoxia, nitric oxide (NO) dysregulation, renin-angiotensin deviation, angiogenesis disturbances, and disproportionate maternal immune responses. The importance of NO in endothelial cells lining the arteries in controlling vascular tone has been previously reported[16]. Heart rate, blood volume, and cardiac output all increase during pregnancy, although blood pressure usually remains at or slightly below pre-pregnancy levels[17]. NOS inhibition and L-NAME have been shown to reduce hypertension in pregnant rats while maintaining normal blood pressure levels until delivery. Additionally, during pregnancy, NO is crucial for controlling the cardiovascular system[18]. Considering that PE is associated with vascular endothelial dysfunction and significant inflammation, it is crucial to create animal models that replicate the pathology of the circulatory system to gain a better understanding of the onset and progression of PE[19].

Previous research has shown a potential relationship between PE onset and placental development, particularly dysfunction during early pregnancy[5]. PE syndrome in mothers is believed to be caused by vascular dysfunction, oxidative stress, and metabolic abnormalities, although the exact mechanisms remain unclear[20]. However, there has been no evaluation of uteroplacental ischemia as the onset of the problem, which is the onset of EO-PE. Hypoxia due to reduced blood flow can trigger the production of several vasoactive chemicals, potentially disrupting the location of the placenta in the uterus[21]. Many PE models show high blood pressure during pregnancy, but specific characteristics and features are still debated. Each disease has advantages and limitations, with insufficient symptoms or an inability to indicate further symptom development, but none of these characteristics reflect human conditions at the placental level. Therefore, this study aimed to evaluate vascular defects, kidney injury, and uterine damage at the placental base in an L-NAME-induced PE model.

Materials and Methods

Materials

The reagent used in this study, L-NAME, was purchased from Sigma-Aldrich St. Louis, Missouri, USA (Cas. No: 51298-62-5). Proteinuria (Cat. No. FY-RA 4983) and creatinine (Cat. No. FY-EU14140) were obtained from Eiyue Biological Company, China, while hypoxia-inducible factor 1α (HIF1α) (Cat. No. RK 03528) was acquired from ABclonal, Company Inc., UK. Female Wistar rats (Rattus norvegicus) were provided by the Laboratory of Integrated Research and Testing (LPPT) at Gadjah Mada University (UGM), Jogjakarta. On November 1, 2022, the Research Ethics Committee of the Faculty of Medicine, Universitas Sebelas Maret (UNS), with number 301/UNS/27.06.9.1/TU.00/2022, approved the research protocol.

Animal Experiments

This research used an *in vivo* experimental method with a pre-posttest control group design, except for assessing HIF1α and endothelial nitric oxide synthase (eNOS) levels. The samples were 30 female Wistar rats weighing 180-200 g and 6 males weighing 250-350 g, all obtained from the LPPT 4 Animal Experiment Center, with the females being 8 and 12 weeks old. In a 12-hour light-dark cycle, the rats were kept in a pathogen-free atmosphere with a humidity of 55±15% and a temperature of 22±2 °C. Access to food and water was provided ad libitum, and testing was performed with a 5:1 ratio between females and males. There were 12 confirmed pregnant rats identified using vaginal swabs, which were then randomly divided into 4 groups. The groups received the following treatments: (1) Pregnant control (PC) (n=3); (2) PE1 (n=3) administered with L-NAME 25 mg/kg/day; (3) PE2 (n=3) administered with 50 mg/kg/day; and (4) PE3 (n=3) administered with 75 mg/kg/day. Pregnant rats were given continuous doses of L-NAME mixed with drinking water on the 4th or 19th day of pregnancy through gavage and were administered until GD19.

Blood Pressure Measurement

Blood pressure was measured on GD0, GD5, GD10, GD14, and GD18 using a CODA non-invasive blood pressure monitoring technique (BP-2010A, Softron, Beijing, China). For 5 min, rats were placed in a heated jacket at 40 °C to increase blood flow to tails. Once the blood pressure had stabilized, the detector was placed near the base of the tail, and measurements were performed. The 5 repetitions were performed, and the average value was calculated.

Tissue Preparation for Measurement of Spiral Artery Diameter, Neutrophil Count, Percentage of Placental Uterine Layer Necrosis, and Placental HIF1α Levels

The uterine tissue was fixed as part of the endometrium where the placenta is located. Tissue processing stages included grossing and fixation (8-48 hours), dehydration, and clearing embedding (24 hours at 58 °C), whereas paraffin blocks were prepared by blocking, cutting, identification, and incubation processes. Hematoxylin-eosin (H&E) staining included deparaffinization, hematoxylin staining, mounting, and histopathological observation of uterine tissue under a light microscope (Olympus CX32; Olympus, Tokyo, Japan). Histopathological preparations were performed at the UGM PA Lab to assess HIF1α levels in placental tissue. To collect the supernatant, 0.1 g was combined with 0.9 mL of PBS solution and centrifuged for 5 min at 5000 rpm and 4 °C.

Measurement of eNOS Levels in Blood

A 1-cc blood sample was centrifuged for 10 min at a speed of 1500 rpm to extract plasma eNOS levels for serum formation. This was followed by the preparation of the Abclonal Kit reagent (Cat. No. RK 03528).

Urine Analysis Measurements

The estimated urine output consisted of GD4, GD10, and GD17, and rats were kept in individual metabolic cages (Techniplast, Italy). Subsequently, 24-h urine protein and creatinine levels were measured in each group using kits from Eiyue Biological Company, China.

Statistical Analysis

Results are reported as mean ± standard deviation. In addition, One-Way ANOVA and Tukey's post-hoc test with a 95% confidence interval were applied to statistical analysis. The analysis was performed using GraphPad Prism version 9.1.1 software.

Results

Changes in Systolic, Diastolic, and Mean Arterial Pressure in Pregnant Rats

Figures 1a, b, and c show the variations in SBP, DBP, and mean arterial pressure (MAP) at each time point during pregnancy. The 4 groups of rats did not differ significantly in SBP, DBP, or MAP before L-NAME administration. After L-NAME treatment, these parameters indicated progressive increases, with SBP, DBP, and MAP in the PE2 and PE3 groups slightly increasing toward the end of pregnancy (Figure 1). Specifically, SBP, DBP, and MAP improved significantly in the PE3 group compared with the control ($p<0.05$) and the L-NAME-treated group ($p<0.05$).

Renal Function Modifications in Rats Receiving L-NAME Injections

Urine parameters were measured to characterize kidney filtration and excretion, which may be affected by injury and

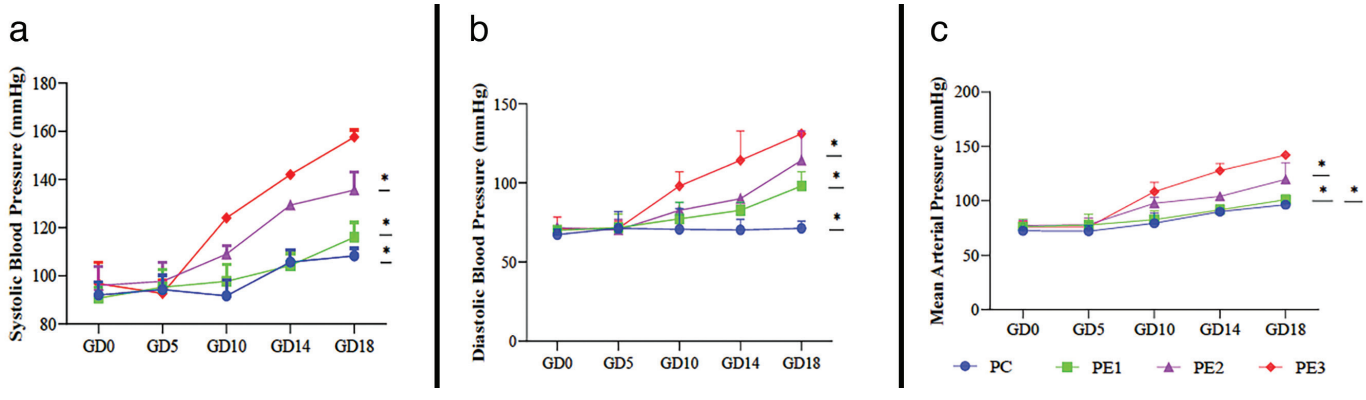

Figure 1. The CODA non-invasive blood pressure method (BP-2010A, Softron, Beijing, China) was used to determine the blood pressure of pregnant rats non-invasively. Trends of deviation in (a) SBP, (b) DBP, and (c) MAP of each group during pregnancy. *: Using repeated measures analysis of variance with $p<0.05$, significance was assessed in relation to the control group. The values are shown using a mean ±95% confidence interval. PC rats comprise the PC group. Three doses of L-NAME were administered to the rats: 25 mg/kg for PE1, 50 mg/kg for PE2, and 75 mg/kg for PE3

PC: Pregnant control, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, MAP: Mean arterial pressure, L-NAME: N-nitro-L-arginine methyl ester, PE: Preeclampsia

affect reabsorption. The results showed that before treatment, the urine protein levels of the 4 groups were similar for GD4 and GD10, and the creatinine levels were similar only for GD4, and there were no differences in the levels of proteinuria and creatinine in the GD4. Additionally, the urine protein and creatinine levels of pregnant rats treated with L-NAME were significantly higher than those of the PC group ($p<0.05$; Figure 2a and b).

Changes in the Structure and Function of the Placental Layer in Pregnant Rats Injected with L-NAME

Increased neutrophil activation, necrosis percentage, and dilation of spiral artery diameter were observed in the placental base and structure of uterine spiral arteries in pregnant rats (GD19). Histopathological images of placental myometrium and uterine spiral arteries in pregnant rat groups PC, PE1, PE2, and PE3 (H&E; magnification, x400; scale bar, 25 µm for neutrophil count and uterine spiral artery diameter; magnification, x100 for neutrophil count). Values are presented as mean ± standard deviation.

Figure 3 shows that the number of neutrophils in the L-NAME-treated groups was higher than that in the PC group (3.26±0.69), and significant differences were found in the PE2 (17.29±1.67), and PE3 group (22.26±0.13) ($p<0.05$).

Figures 4a and b show that the percentage of necrosis was lower in the L-NAME-treated groups than in the PC group (5.16±2.69). There were significant differences between PE1 (10.08±0.98 %), PE2 (10.29±1.67), and PE3 groups (15.26±2.13) ($p<0.05$).

Figures 5a and b show that the diameter of the uterine spiral artery in the L-NAME-treated group was smaller than that in the PC group (162.54±0.025). There were significant differences between PE2 (162.42±0.021) and PE3 (162.34±0.023) ($p<0.05$) (Figure 6).

Vasoconstriction Reactions in Pregnant Rats Treated with L-NAME

Generally, the pathogenesis of PE is associated with vasoconstriction factors. In this study, tissue EL tests and plasma assays were used to detect the levels of HIF1α and eNOS in the placental plasma of the PC, PE1, PE2, and PE3 groups (Figures 7a and b). Significant differences in HIF1α levels were recorded in the PE3 group (2.50±0.51 pg/mg) $p<0.05$ compared to PC (1.75±0.11 pg/mg), as shown in Figure 7a. The effects of different L-NAME treatment regimens on eNOS levels in the circulation of pregnant rats in each group were determined by plasma analysis toward the end of pregnancy (GD19). The eNOS levels in each L-NAME-treated group were significantly decreased compared with PC (84.12±0.53 ng/dL; $p<0.05$).

Adverse Pregnancy Outcomes in the L-NAME-treatment Groups

The study groups experienced adverse pregnancy outcomes, primarily due to inadequate remodeling of spiral arteries and inflammation in the uterus and placenta. Rats administered high-dose L-NAME (75 mg/kg) had significantly smaller placentas than controls. There was also a significant decrease in fetal weight ($p<0.05$) and shorter crown-rump length. Furthermore, the PE2 and PE3 groups showed significant differences in fetal weight, crown-rump length, placental diameter, and weight compared with the PC group (Figures 7a-d).

Discussion

The administration of high-dose L-NAME (75 mg/kg) evaluated on gestational days 10, 14, and 18 increased blood pressure (SBP, DBP, and MAP), elevated proteinuria on GD17, and creatinine levels from GD10 to 17. The treatment also increased the number of neutrophils, increased the occurrence of necrosis

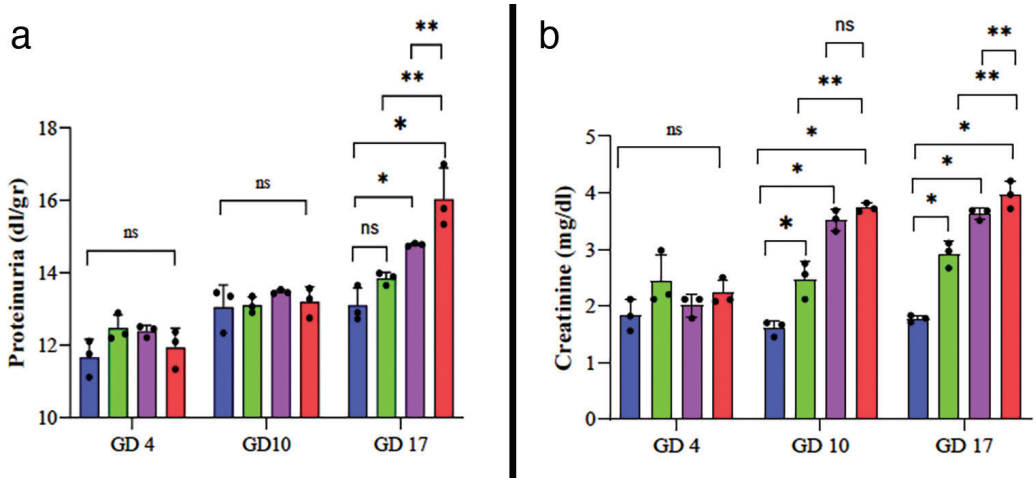

Figure 2. Urine Parameters during PE. The urine proteinuria (a) and creatinine (b) levels are displayed per day. All parameters increased on day 10 of PE pregnancy compared with the control. Urine creatinine levels increased on day 10 of PE. Statistical analysis was performed using repeated-measures ANOVA. Mean ± standard deviation is shown (n=3-4). P-values are displayed *: $p<0.05$ and ns >0.05. **: Bonferroni test $p<0.05$ vs. PE3

PE: Preeclampsia

at the placental base, reduced the diameter of uterine spiral arteries, elevated placental HIF1α levels, decreased plasma eNOS levels, and heightened the incidence of intrauterine fetal death and intrauterine growth restriction.

The specific identification parameters for EO-PE are SBP 140 mmHg and DBP 90 mmHg[1]. In this investigation, GD4 or GD18 were selected as the optimal starting points for optimizing L-NAME administration. A comprehensive literature review validated the use of L-NAME at doses of 25, 50, and 75 mg/kg per day to induce PE in pregnant rats. The results showed increased SBP, DBP, and MAP in hypertensive rats after L-NAME administration, with pressure differences of up to 15 mmHg observed in the PE2 and PE3 groups from GD10. These blood pressure differences persisted until birth on GD19, with the PE3 group showing the greatest variation. All rat groups, except for PE3, demonstrated a typical pregnancy trend, including decreases in MAP, DBP, and SBP toward late pregnancy. Meanwhile, rats in the PE3 group showed a continuous increase in blood pressure throughout pregnancy rather than a decrease. These results were inconsistent with[22], where 40 SD rats (Sprague-Dawley rats) treated with PE 50 mg/mL had significantly higher SBP than the control[23]. Differences in the results may be due to variations in the types of rats used. According to research, the normal blood pressure of SD rats is higher than that of Wistar[24]. Low to medium doses or late administration failed to produce the average blood pressure characteristic of PE pregnancy and did not significantly affect SBP, DBP, or MAP.

Proteinuria parameters were measured to characterize kidney filtration and excretion. Research has proven that proteinuria and creatinine levels significantly decrease during and after PE compared with the control[25]. Both parameters were measured for GD4, 10, and 17 in all groups. There were no significant changes in proteinuria across all groups on GD4 and 10. On day 17, proteinuria significantly increased in the early PE2 and PE3 groups compared with the control. These results were consistent with[26], where 4 groups given 50 mg/kg PE had significantly higher proteinuria than the relatively PC group. Differences in the results may be due to variations in the types of rats used. There were no significant changes in

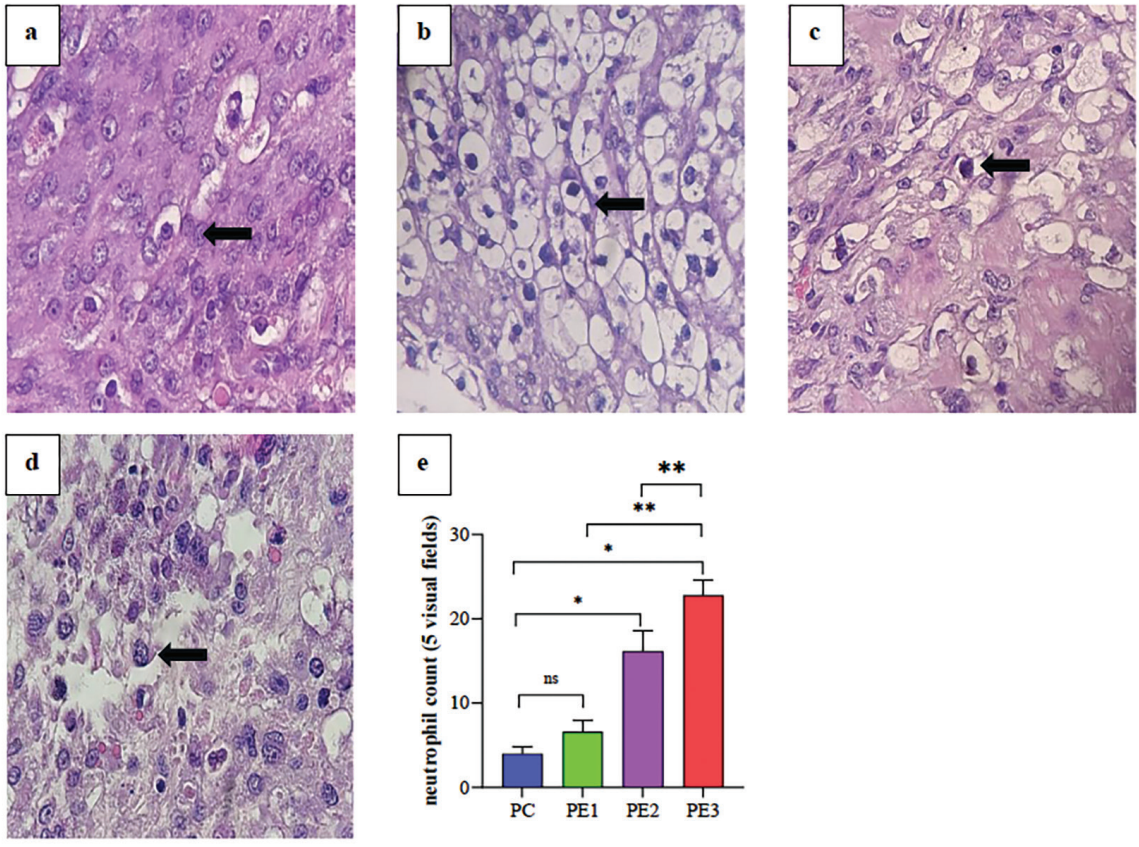

Figure 3. Changes in the design and process of placental bed oxidative stress in pregnant rats (GD19). (a) Histopathological images of the myometrial layer in pregnant rats (PC) control group, (PE1) group treated with 25 mg/kg body weight per day, (PE2) group treated with 50 mg/kg body weight per day, and (PE3) group treated with 75 mg/kg body weight per day (H&E; magnification, x400; scale bar, 25 µm). Additionally, black indicators indicate neutrophils in 5 fields of view. (b) The number of neutrophils in pregnant rats on GD19. *: Tukey post-hoc test significant p≤0.05 vs. PC; ns p>0.05 vs. PC. **: p<0.05 vs. PE3

PE: Preeclampsia, H&E: Hematoxylin-eosin

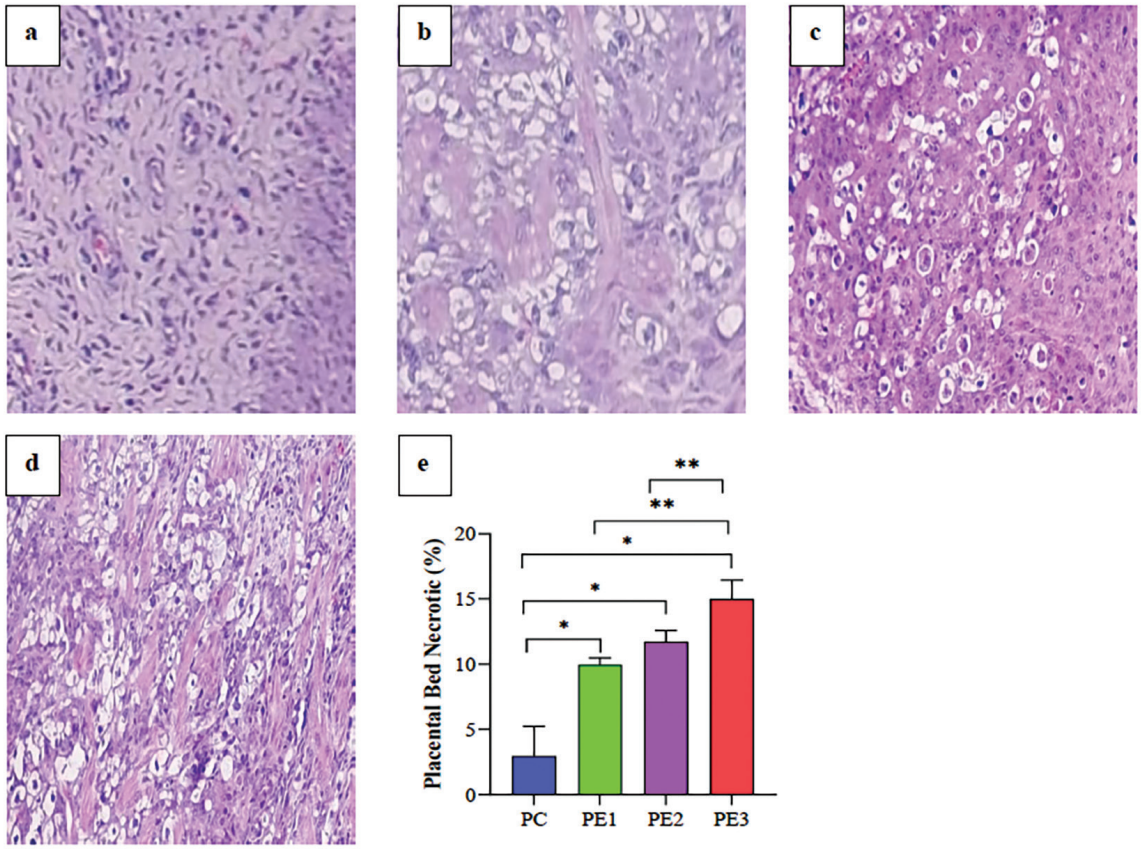

Figure 4. Variation in the shape and function of the placental base in pregnant rats, along with the percentage of inflammatory necrosis (GD19). (a) Histopathological images of the myometrial layer in (PC), control, (PE1), group treated with 25 mg/kg body weight per day, (PE2), and (PE3) groups treated with 75 mg/kg body weight per day in pregnant rat groups (H&E; magnification, x100; scale bar, 25 µm). The percentage of necrosis in 5 fields of view is indicated by black boxes. (b) Proportion of pregnant rats on GD19 experiencing necrosis. *: Tukey post-hoc test significant p≤0.05 vs. PC; ns p>0.05 vs. PC. **: p<0.05 vs. PE3

PE: Preeclampsia, PC: Pregnant control, H&E: Hematoxylin-eosin

creatinine levels across all groups on GD4, but a significant increase was observed on days 10 and 17. These results may be attributed to L-NAME, which induces glomerular damage by increasing glomerular pressure. The increase in pressure leads to thickening of the blood vessel walls in the kidneys[27] and also creates stress, which results in the loss of protein in the urine, resulting in proteinuria[28].

The term "placental bed" describes the interface between the mother and fetus, namely, the area inside the uterus where the placenta attaches to the uterine wall. Administration of 75 mg/kg body weight per day L-NAME successfully induced the highest increase in neutrophil counts compared with the PE, PE1, and PE2 groups. This result was consistent with the research conducted by[29] using 35 Sprague-Dawley rats induced with RUPP. The results showed that there was a higher neutrophil count in rats with PE[29]. Generally, PE is characterized by reduced placental perfusion accompanied by ischemia and hypertension during pregnancy. Women with this condition also show increased inflammation and a higher number of neutrophils in their blood vessels compared with healthy women. The main pathological feature of EO-PE is the imperfect transformation of spiral arteries, resulting in placental hypoperfusion and decreased fetal nutrient supply. Based on the results, the administration of L-NAME at 75 mg/kg body weight per day reduced the diameter of the uterine spiral arteries compared with the PE, PE1, and PE2 groups. This was supported by[11], where 16 placental beds from Cesarean hysterectomy specimen collections showed a difference in diameter between the Normotensive (500 micrometers) and PE (200 micrometers) groups. The administration of L-NAME at a dose of 75 mg/kg body weight per day as an NO inhibitor can trigger placental hypoxia and ischemia, as evidenced by the narrowing of the average diameter of spiral arteries, which is a significant characteristic of EO-PE.

The vasoconstriction reaction in pregnant rats treated with L-NAME was also evidenced by placental HIF1α levels and eNOS levels in the blood. HIF1α levels decreased with the use of 75 mg per kilogram body weight per day and showed a significant difference compared with the PC group. This

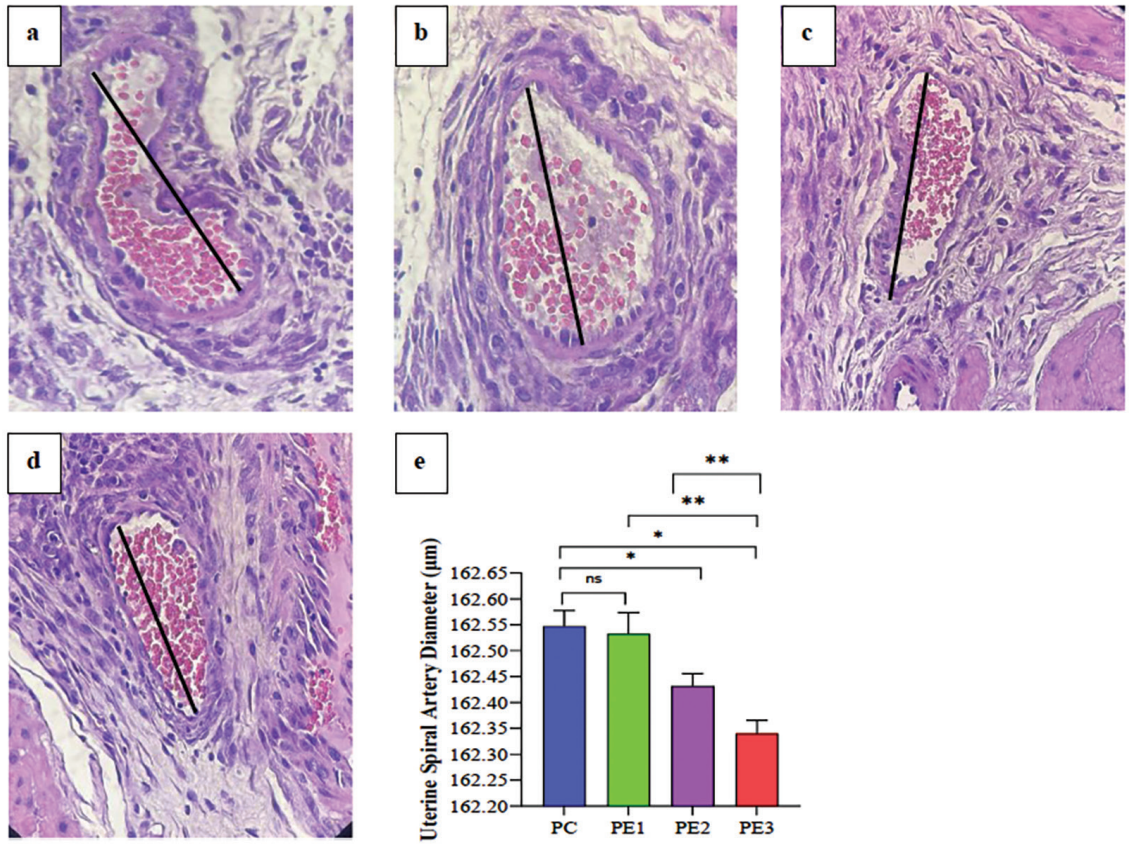

Figure 5. Modifications in the anatomy and function of uterine spiral artery remodeling in pregnant rats (GD19). (a) Histopathological images of the diameter of the uterine spiral artery in pregnant rats (PC), control group, (PE1) group treated with 25 mg/kg body weight per day, (PE2) 50 mg per kg body weight per day, and (PE3) 75 mg/kg body weight per day (H&E; magnification, x400; scale bar, 25 µm). The diameter of the uterine spiral artery is indicated by black lines spanning from outer to inner. (b) Pregnant rats on GD19 with the uterine spiral artery diameter. *: Significant p≤0.05 vs. PC in Tukey's post-hoc test; ns) p>0.05 vs. PC; **: p<0.05 vs. PE3

PE: Preeclampsia, PC: Pregnant control, H&E: Hematoxylin-eosin

result was consistent with a previous study in which 15 rats were administered L-NAME, with 2 (13.4%) and 13 (86.6%) showing light and medium immunohistochemical staining for HIF1α expression in placental tissue, respectively[30]. Abnormalities in early placental development, starting from inadequate trophoblast invasion into spiral arteries, lead to decreased uteroplacental perfusion and hypoxia[4]. The decreased eNOS levels in the PE3 group were consistent with the findings in 84 pregnant women at the Department of Obstetrics and Gynecology, University Hospital at FMRP-USP. A significant decrease in plasma eNOS concentration was observed in PE compared with HP[31]. The reduction in eNOS levels is associated with hemodynamic decline caused by systemic vascular dilatation, which affects the level of organ hypoperfusion and contributes to the development of PE[32].

The administration of L-NAME also affected pregnancy outcomes, with significant differences observed between the PE3 and PC groups regarding placental diameter and weight, as well as fetal weight and length. Similarly, the authors found that 9 SD rats administered L-NAME had lower body weights compared to those treated with aspirin or quercetin alone[22]. Endothelial dysfunction caused by L-NAME leads to narrowing of the uteroplacental spiral arteries, which hinders the flow of nutrients from the mother to the fetus. The model examined in this research is easy to create and has potential utility in evaluating the pathophysiological indicators of PE, pregnancy outcomes, and complexity and identifying therapeutic targets for prevention. However, placental histopathological lesions in PE were not analyzed in this study. Further studies are needed to confirm the effect of histopathological features on the placenta of L-NAME-induced PE rats.

Conclusion

In conclusion, the administration of L-NAME on 75 mg/kg BW/day (PE3) induced EO-PE, consistent with the rat model. This method is reliable for investigating the pathogenesis of PE. The model facilitates the investigation of changes in blood pressure,

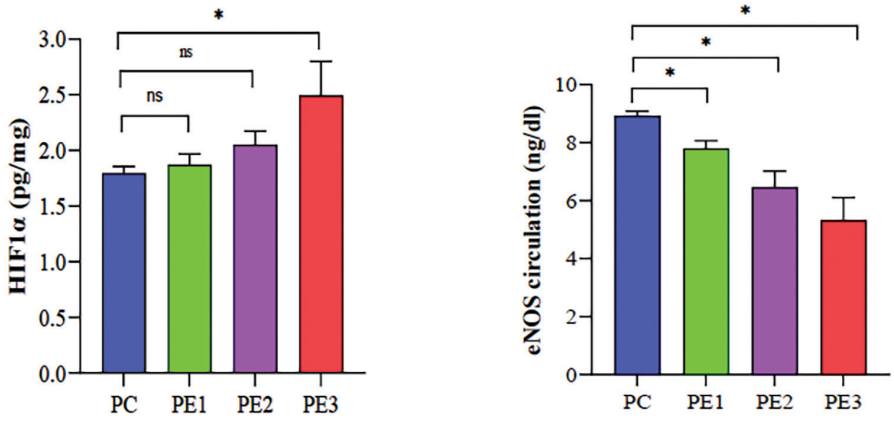

Figure 6. Using Elisa assays, placental hypoxia levels in each group of pregnant rats at the end of pregnancy (GD19) were measured and presented as means ± standard deviation. Legend: From GD4, PC, PE1 rats were given low-dose L-NAME (25 mg/kg body weight/day); PE2 rats were given medium-dose L-NAME (50 mg/kg); and PE3 rats were given high-dose L-NAME (75 mg/kg body weight/day) on GD4. *: Significance by Tukey post-hoc test compared to PC group ($p<0.05$), Tukey Post-hoc test not different compared to PC group (ns)

PE: Preeclampsia, PC: Pregnant control, L-NAME: N-nitro-L-arginine methyl ester

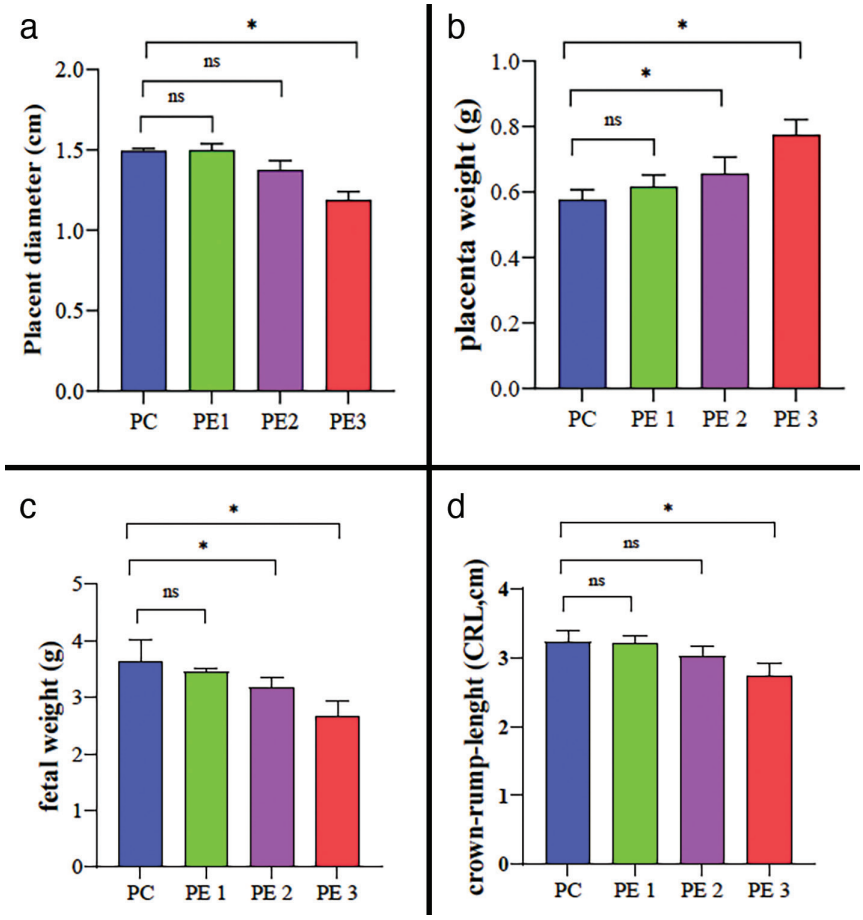

Figure 7. Impact of Pregnancy and Trophoblast Placental Structure Modifications in Rats During Pregnancy (GD19). (a) Placental diameter for each group separately. (b) Placental weight according to status. (c) Fetal significance in each group. (d) Height in each group from crown to rump. (D). Values are presented as mean ± standard deviation. The PC group underwent normal pregnancy; PE1, PE2, and PE3 rats underwent treatment with low-dose L-NAME (25 mg/kg body weight/day), medium-dose (50 mg/kg), and high-dose (75 mg/kg) starting from GD4. GD (Gestation Day); L-NAME *: Significant $p\leq0.05$ vs. PC; ns $p>0.05$ vs. PC (Tukey post-hoc test)

PE: Preeclampsia, PC: Pregnant control, L-NAME: N-nitro-L-arginine methyl ester

kidneys, blood vessels, and the uterine placental layer over time, as well as the discovery of new biomarkers in the early stages of pregnancy.

Ethics

Ethics Committee Approval: On November 1, 2022, the Research Ethics Committee of the Faculty of Medicine, Universitas Sebelas Maret (UNS), with number 301/UNS/27.06.9.1/TU.00/2022, approved the research protocol.
Informed Consent: Written informed consent was obtained from the patients.

Authorship Contributions

Surgical and Medical Practices: F.F., D.I., Concept: F.F., D.I., S.S., S.So., Design: F.F., D.I., S.S., S.So., Data Collection or Processing: F.F., D.I., Analysis or Interpretation: F.F., D.I., Literature Search: F.F., D.I., S.S., S.So., Writing: F.F.
Conflict of Interest: No conflict of interest was declared by the authors.
Financial Disclosure: This research was supported by the Aisyah Lampung Foundation.

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Review / Derleme

DOI: 10.4274/tjod.galenos.2024.55058

Turk J Obstet Gynecol 2024;21:190-207

How safe is high-intensity focused ultrasound? An intriguing solution for obstetric and gynecological diseases: A systematic review

Yüksek yoğunluklu odaklanmış ultrason ne kadar güvenlidir? Obstetrik ve jinekolojik hastalıklara ilgi çekici bir çözüm: Sistematik bir inceleme

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Abstract

High-intensity focused ultrasound (HIFU) is a non-surgical and noninvasive treatment modality that depends on external ultrasound energy sources that induce focused mass ablation and protein degeneration in the treatment area via thermal energy penetration under the intact skin. We aim in our study to collectively evaluate the safety of HIFU for the treatment of different obstetric and gynecological diseases in the literature. We searched PubMed, Scopus, and Science Direct databases, without restriction on date or language, from the inception of these databases until January 20, 2024. We also examined the references of the included studies in the Mendeley archive for eligible articles. We found a total of 706 studies. After the screening and selection process, 56 participants were included. Our dichotomous outcomes were pooled in our single-arm meta-analysis as risk ratio (RR) and with 95% confidence interval (CI) while our continuous outcomes were pooled as mean change and 95% CIs. Fixed- or random-effects models were applied depending on the heterogeneity detected. Our systematic review and meta-analysis included 56 studies including 11.740 patients. Depending on the Society of Interventional Radiology (SIR) classification for adverse effects. The results of this meta-analysis for the type A category that did not require clinical intervention found that pain in the treatment site estimated RR with 95% CI: 0.61 (0.33, 0.89), abnormal vaginal discharge 0.16 (0.073, 0.24), low-grade fever (<38 °C) 0.005 (0.002, 0.009). Sensory abnormalities of the lower limbs were examined in 3390 individuals and observed in only 19 patients who experienced gradual relief of symptoms within one month after treatment. Regarding SIR type B, 99 of a total of 6.437 patients had small vesicles and superficial burns with pooled RR and 95% CI: 0.012 (0.007, 0.018). In terms of groin or perianal and lower abdominal pain, our RRs with 95% CIs were 0.1 (0.067, 0.13) and 0.38 (0.25, 0.51). However, vaginal bleeding was detected in only 32 out of a total of 3.017. Major adverse events like lumber disc herniation, thrombocytopenia, and renal failure, were unmentionable. Additionally, our included studies did not record any deaths. HIFU, either alone or in combination with oxytocin or any other enhancing agent, is safe for patients with different gynecological and obstetric diseases. In terms of efficacy, it showed promising results compared with traditional treatment lines. To our knowledge, we are the first and most comprehensive meta-analysis in the literature that has studied the different safety outcomes related to HIFU as a treatment modality for different obstetric and gynecological diseases with a very large sample size, making our evidence strong and less attributed to errors.

Keywords: HIFU, ectopic pregnancy, endometriosis, gestational trophoblastic diseases, adenomyosis

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Received/Geliş Tarihi: 29.03.2024 **Accepted/Kabul Tarihi:** 10.07.2024

Öz

Yüksek yoğunluklu odaklanmış ultrason (HIFU), sağlam deri altına termal enerji nüfuzu yoluyla tedavi alanında hedeflenmiş kitle ablasyonunu ve protein dejenerasyonunu tetikleyen harici ultrason enerji kaynaklarına dayanan, cerrahi olmayan ve invazif olmayan bir tedavi yöntemidir. Çalışmamızda literatürdeki farklı obstetrik ve jinekolojik hastalıkların tedavisinde HIFU güvenliğini toplu olarak değerlendirmeyi amaçladık. PubMed, Scopus ve Science Direct veritabanlarını, bu veri tabanlarının başlangıcından 20 Ocak 2024'e kadar tarih ve dil sınırlaması olmaksızın araştırdık. Ayrıca uygun makaleler için Mendeley arşivine dahil edilen çalışmaların referanslarını da inceledik. Toplam 706 çalışma bulduk. Eleme ve seçme sürecinin ardından 56 katılımcı dahil edildi. İkili sonuçlarımız tek kollu meta-analizimizde risk oranı (RR) şeklinde ve %95 güven aralığı (GA) ile bir araya getirilirken, sürekli sonuçlarımız ortalama değişiklik şeklinde ve %95 GA ile bir araya getirildi. Tespit edilen heterojenliğe bağlı olarak sabit veya rastgele etki modelleri uygulandı. Sistematik incelememiz ve meta-analizimiz 11.740 hastanın dahil olduğu 56 çalışmayı içeriyordu. Olumsuz etkiler Girişimsel Radyoloji Derneği'nin (SIR) sınıflandırmasına göre sınıflandırıldı. Klinik müdahale gerektirmeyen tip A kategorisi için bu meta-analizin sonuçlarına göre tedavi bölgesindeki ağrının %95 GA ile tahmin edilen RR değeri 0,61 (0,33, 0,89), anormal vajinal akıntının RR değeri 0,16 (0,073, 0,24) ve düşük dereceli ateşin (<38 °C) RR değeri 0,005 (0,002, 0,009) idi. Alt ekstremitelerdeki duyusal anormallikler 3.390 kişide incelendi ve tedaviden sonraki bir ay içinde semptomların kademeli olarak azaldığı yalnızca 19 hastada gözlemlendi. SIR tip B kategorisi ile ilgili olarak, toplam 6.437 hastanın 99'unda küçük veziküller ve yüzeysel yanıkların %95 GA ile havuzlanmış RR değeri 0,012 (0,007, 0,018) olarak saptandı. Kasık ağrısı veya perianal ağrı ve alt karın ağrısı açısından %95 GA ile RR değerleri 0,1 (0,067, 0,13) ve 0,38 (0,25, 0,51) idi. Ancak toplam 3.017 olgunun sadece 32'sinde vajinal kanama tespit edildi. Lomber disk hernisi, trombositopeni ve böbrek yetmezliği gibi önemli yan etkilere rastlanmadı. Ayrıca derlediğimiz çalışmalarda herhangi bir ölüm kaydedilmedi. HIFU, tek başına veya oksitosin veya başka herhangi bir güçlendirici ajanla kombinasyon halinde, farklı jinekolojik ve obstetrik hastalıkları olan hastalar için güvenlidir. Etkinlik açısından geleneksel tedavi yöntemleriyle karşılaştırıldığında HIFU ümit verici sonuçlar göstermiştir. Bildiğimiz kadarıyla, bu meta-analiz farklı obstetrik ve jinekolojik hastalıklar için bir tedavi yöntemi olarak HIFU ile ilgili farklı güvenlik sonuçlarını, kanıtlarımızı güçlendiren ve hata payımızı düşüren çok büyük bir örneklem büyüklüğüyle inceleyen literatürdeki ilk ve en kapsamlı meta-analizdir.

Anahtar Kelimeler: HIFU, ektopik gebelik, endometriozis, gestasyonel trofoblastik hastalıklar, adenomiyoz

Introduction

High-intensity focused ultrasound (HIFU) is a newly discovered non-invasive modality used to treat various obstetric and gynecological diseases. Based on its high ability to focus thermal energy and ultrasonic waves at a targeted location, it is used for local ablation of tumor masses like uterine myomas and fibroid masses. Recently, it gained confidence in the treatment of adenomyosis, gestational trophoblastic disease, endometriosis, and ectopic pregnancy (EP)[1,2].

Adenomyosis is defined as the growth of ectopic endometrial tissues in the myometrium caused by various factors. It commonly occurs during the childbearing period. It manifests as menorrhagia, dysmenorrhea, and uterine enlargement. Surgery or medication is a treatment option for this disease. The only curative treatment is hysterectomy[3,4].

Endometriosis is defined as the presence of endometrial glands outside the uterus, and it mainly affects females during the reproductive period. However, endometriosis is a benign disorder that tends to propagate, invade, and proliferate under the effect of female hormones, and it is treated either by surgical excision or medication[5,6].

Gestational trophoblastic neoplasia (GTN) is a condition affecting human placental trophoblastic cells that usually occurs secondary to hydatidiform mole and is characterized by abnormal proliferation of these cells with an increase in serum beta-human chorionic gonadotropin (β-hCG) levels. Chemotherapy is the first-line treatment for GTN. However, surgery may be an additional option for high-risk, chemoresistant, or unsuitable cases[7,8].

EP refers to the implantation of the embryo in any site rather than the endometrial cavity[9]. Tubal pregnancy is the most common type of EP and is associated with the highest mortality rate[10]. HIFU causes lesion ablation through thermal and cavitation effects. It is a non-invasive, safe, and effective treatment in oncology[11].

With HIFU's broad application in the treatment of uterine fibroids, osteosarcoma, liver cancer, and other solid tumors, it gained interest from patients and physicians for the treatment of the following adenomyosis, EP, endometriosis, and gestational trophoblastic diseases[12,13]. Therefore, in our study, we aimed to thoroughly compile the existing literature to investigate the safety of HIFU as an intriguing solution for different obstetric and gynecological diseases.

Methods

Our study design closely adheres to the latest guidelines reported in the Cochrane Handbook for Systematic Reviews of Interventions. Moreover, we followed the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement guidelines for systematic review and meta-analysis[14,15].

Literature Search

A comprehensive search was conducted across the following databases PubMed/MEDLINE, Scopus, and ScienceDirect. This meticulous search included articles published from inception until January 20, 2024. Additionally, we examined the reference lists of eligible articles and previous meta-analyses to identify any citations related to our research topic. Our search strategy was a combination of the following search terms: (HIFU and EP) Or (HIFU and endometriosis) Or (HIFU and gestational trophoblastic diseases) Or (HIFU and adenomyosis).

Eligibility Criteria

Two reviewers independently examined all retrieved references and eligible articles. Our Inclusion criteria were based on the following characteristics: Patients who experienced HIFU and

had adenomyosis or EP or gestational trophoblastic diseases or endometriosis, studies where individuals were subjected to HIFU application, and studies revealing the safety outcomes of HIFU by counting the number of patients who experienced side effects due to HIFU and categorizing the side effects according to the committee.

Numerous studies were excluded from our analysis for the following reasons: 1) studies on animals; 2) articles not written in English; 3) only abstracts available; and 4) studies lacking outcome data.

Data Gathering

Data for this systematic review and meta-analysis were extracted in specific electronic offline sheets, capturing specific information from each included study. Extracted data included study ID, study design and publication year, total sample size, geographic distribution of the study, mean participant age, gender distribution, duration of follow-up, conclusions, and primary outcomes.

Assessment of Risk of Bias

Our case reports and cohort quality were evaluated using Murad et al.[16] tool. This tool comprises the following parameters: selection, ascertainment, causality, and reporting. From a total score of eight, we assigned the quality of assessed studies as good (>6.5, fair, or poor if matched >5-6.5, or less than 5, respectively. National Institutes of Health[17] used for assessment of some of our cohort studies. The evaluation process was based on scores to categorize the quality of our included studies as "good", "fair", or "poor". Moreover, any discrepancies were resolved by discussion with a third assessor.

Data Synthesis

Our dichotomous data were pooled in this single-arm meta-analysis as risk ratio (RR) and with 95% confidence interval (CI) while our continuous outcomes were pooled as mean change and 95% CIs. Fixed- or random-effects models were applied depending on the heterogeneity detected. We conducted a single-arm meta-analysis. We first apply a random effect model and then, according to the degree of heterogeneity, we choose between random and fixed models. We express statistical heterogeneity using the I^2 statistics chi-squared test. We also used Open-Meta-Analyst software for all statistical analyses.

Results

Literature Search Results

Our search across distinct databases yielded 706 studies. Subsequently, after eliminating duplicate studies, 584 studies were included for screening. A meticulous review of the titles and abstracts led to the identification of 87 articles suitable for full-text evaluation. Finally, a total of 56 articles were included in our systematic review and meta-analysis. A visual representation of the study selection process is presented in the PRISMA flow diagram in Figure 1.

Characteristics of the Included Studies

Our systematic review and meta-analysis included 56 studies[13,18-61], encompassing 11.740 patients. Regarding geographic distribution, the majority of our studies were conducted in China from 2011 to 2023. The mean age was 39 years old. While the mean body mass index of our population ranged from 21 to 23 kg/m^2. The studies in this systematic review evaluate HIFU for the treatment of the following disorder: Adenomyosis, endometriosis, gestational trophoblastic diseases, and EP. The baseline characteristics, summary, and citations of our included studies are comprehensively discussed in Table 1.

Risk of Bias Assessment

Our 14 case report studies were judged from good to fair according to the Murad et al.[16], tool, while the other 42 included cohort studies were designed as either retrospective or prospective cohorts and all showed fair quality. Risk of bias assessment tables are presented in Tables 2-6.

Outcomes

We classified adverse events according to the Society of Interventional Radiology (SIR) guidelines as follows:

Type A category that did not require clinical intervention includes pain at the treatment site, which estimated pooled RR with a 95% CI of 0.61 (0.33, 0.89). however, abnormal vaginal discharge estimated 0.16 (0.073, 0.24), In terms of low-grade fever (<38 °C) RR and 95% CI; 0.005 (0.002, 0.009) Figures 2-4. depicts the forest plots for pain at the treatment site, abnormal vaginal discharge, and fever.

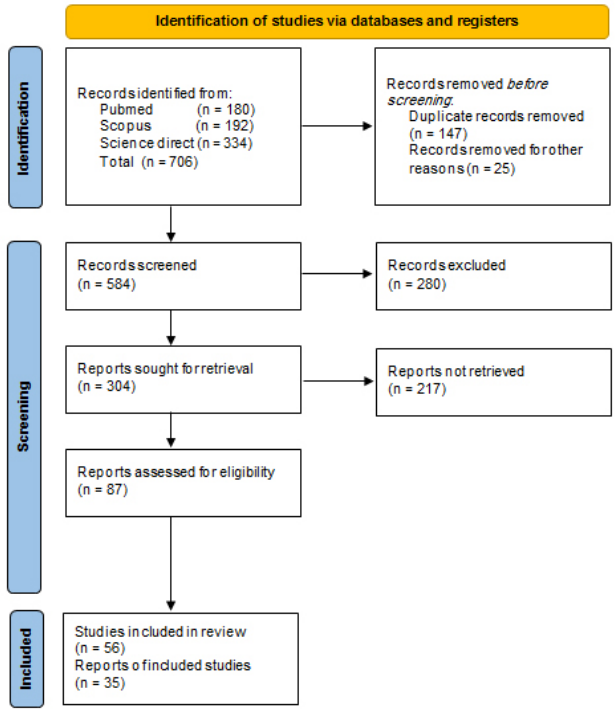


Figure 1. A visual representation of the study selection process is showed in the PRISMA flow diagram

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-analyses

Table 1. Baseline characteristics, summary, and citations of our included studies

First author's name	Year of publication	Title	Type of study	Location of study
Jeng et al.[27]	2020	500 Cases of high-intensity focused ultrasound (HIFU) Ablated Uterine Fibroids and Adenomyosis	Retrospective cross-sectional analysis	Taiwan
Shui et al.[31]	2015	High-intensity focused ultrasound (HIFU) for adenomyosis: Two-year follow-up results	This retrospective two-year follow-up study	Chongqing, China
Yao et al.[28]	2021	Microbubble contrast agent SonoVue combined with oxytocin improves theefficiency of high-intensity focused ultrasound ablation for adenomyosis	Prospective randomized controlled trial	Kunming, China
Xu et al.[29]	2023	Comparison of high-intensity focused ultrasound for the treatment of internaland external adenomyosis based on magnetic resonance imaging classification	Retrospective study	Chongqing, China
Li et al.[26]	2021	High-intensity focused ultrasound in the management of adenomyosis:long-term results from a single center	Retrospective analysis	Changsha, China
Gong et al.[20]	2017	High intensity focused ultrasound treatment of adenomyosis: The relationship between the features of magnetic resonance imaging on T2 weighted images and the therapeutic efficacy	Retrospective study	China
Zhao et al.[73]	2023	High intensity focused ultrasound treatment for adenomyosis: comparison ofefficacy based on MRI features	Retrospective study	Suining, China
Wei et al.[38]	2023	Comparison of pregnancy outcomes in infertile patients with different types ofadenomyosis treated with high-intensity focused ultrasound	Retropectve review	Guangxi, China
Feng et al.[36]	2017	Safety of ultrasound-guided high-intensity focused ultrasound ablation for diffuse adenomyosis: A retrospective cohort study	Retrospective cohort study	Chongqing, China
Zhou et al.[35]	2011	Ultrasound-guided high-intensity focused ultrasound ablation for adenomyosis: the clinical experience of a single center	Prospective clinical trial	Chongqing, China
Gong et al.[25]	2022	Evaluation of high intensity focused ultrasound treatment for different types ofadenomyosis based on magnetic resonance imaging classification	Retrospective study	Chongqing, China
Lee et al.[23]	2018	Comparison of effectiveness of epidural analgesia and monitored anesthesiacare for high-intensity focused ultrasound treatment of adenomyosis	Retrospective case-control study	Seoul, Republic of Korea
Yu et al.[24]	2017	Treatment of cornual pregnancy in a patient with adenomyosis by high-intensity focused ultrasound (HIFU) ablation	Case report	Shandong, China
Yu et al.[22]	2023	Factors influencing USgHIFU ablation for adenomyosis with NPVR 50%	Retrospective study	Chongqing, China
Lee et al.[21]	2015	Ultrasound-guided high-intensity focused ultrasound treatment for uterine fibroid & adenomyosis: A single center experience from the Republic of Korea	Retrospective analysis	Republic of Korea
Hong et al.[33]	2019	Complication Following Ultrasound-Guided High-Intensity Focused Ultrasound for the Treatment of Uterine Adenomyosis: Case Report of CT Imaging Features	Case report of CT imaging features	Korea
Xiong et al.[34]	2015	Ultrasound-guided high-intensity focused ultrasound (USgHIFU) ablation for the treatment of patients with adenomyosis andprior abdominal surgical scars: A retrospective study	Retrospective study	Chongqing, China
Jingqi et al.[18]	2018	Clinical Usefulness of the Microbubble Contrast Agent SonoVue in Enhancing the Effects of High-Intensity Focused Ultrasound for the Treatment of Adenomyosis	Prospective study	Chongqing, China
Cheng et al.[19]	2015	Contrast-Enhanced Ultrasound for Evaluation of High-Intensity Focused Ultrasound Treatment of Benign Uterine Diseases	Retrospective study	China

Table 1. continued

First author's name	Year of publication	Title	Type of study	Location of study
Fan et al.[32]	2012	Feasibility of MRI-guided high intensity focused ultrasound treatment for adenomyosis	Prospective and clinical trail	Chongqing, China
Liu et al.[30]	2023	High Intensity Focused Ultrasound Ablation for Juvenile Cystic Adenomyosis: Two Case Reports and Literature Review	Case studies and literature review	Suining, china
Qu et al.[39]	2022	Long-term outcome of ultrasound-guided focused ultrasound ablation for gestational trophoblastic neoplasia in the cesarean scar: a case report	Case report	Chongqing, China
Liu et al.[7]	2023	High-intensity focused ultrasound as a pretreatment combined with hysteroscopic resection for gestational trophoblastic neoplasia with chemotherapy intolerance: a case report	Case report	China
She et al.[40]	2021	High-intensity focused ultrasound ablation as an adjuvant surgical salvage procedure in gestational trophoblastic neoplasia chemotherapy with chemoresistance or recurrence: two case reports	Case report	Zunyi City, China
Hu et al.[41]	2022	Exploring the Diagnostic Performance of Magnetic Resonance Imaging in Ultrasound-Guided High-Intensity Focused Ultrasound Ablation for Abdominal Wall Endometriosis	Prospective	China
Liu et al.[74]	2023	Safety and efficacy of microwave ablation for abdominal wall endometriosis: A retrospective study	Retrospective	China
Philip et al.[42]	2020	Transrectal high-intensity focused ultrasound (HIFU) for management of rectosigmoid deep infiltrating endometriosis: results of Phase-I clinical trial	Prospective	France
Shi et al.[43]	2020	High-Intensity Focused Ultrasound in the Treatment of Abdominal Wall Endometriosis	Retrospective	China
Yang and Zhang[75]	2023	Efficacy and safety of high-intensity focused ultrasound ablation for rectus abdominis endometriosis: a 7-year follow-up clinical study	Retrospective	China
Luo et al.[77]	2017	Ultrasound-guided high-intensity focused ultrasound treatment for abdominal wall endometriosis: a retrospective study	Retrospective	China
Zhu et al.[47]	2017	A comparison between high-intensity focused ultrasound and surgical treatment for the management of abdominal wall endometriosis	Retrospective	China
Xiao-Ying et al.[76]	2019	Clinical analysis of high-intensity focused ultrasound ablation for abdominal wall endometriosis: a 4-year experience at a specialty gynecological institution	Retrospective	China
Zhao et al.[78]	2018	Comparison of ultrasound-guided high-intensity focused ultrasound ablation and surgery for abdominal wall endometriosis	Retrospective	China
Lee et al.[46]	2019	Abdominal wall endometriosis treatment by ultrasound-guided high-intensity focused ultrasound ablation: a case report	Case report	Korea
Nguyen[79]	2020	Magnetic Resonance Imagingguided Highintensity Focused Ultrasound Ablation for Endometriosis of the Abdominal Wall	Case report	Vietnam
Wang et al.[44]	2021	The safety of echo contrast-enhanced ultrasound in high-intensity focused ultrasound ablation for abdominal wall endometriosis: a retrospective study	Retrospective	China
Wang et al.[45]	2011	Ultrasound-guided high-intensity focused ultrasound treatment for abdominal wall endometriosis: Preliminary result	Prospective	China

Table 1. continued

First author's name	Year of publication	Title	Type of study	Location of study
Stehouwer et al.[80]	2018	Magnetic Resonance Imaging Guided High Intensity Focused Ultrasound is a Non-Invasive Treatment Modality for Patients with Abdominal Wall Endometriosis	Case report	Netherlands
Wang et al.[53]	2022	High-intensity focused ultrasound compared with uterine artery chemoembolization with methotrexate for the management of cesarean scar pregnancy	Retrospective cohort	China
Hong et al.[48]	2017	Outcome of high-intensity focused ultrasound and uterine artery embolization in the treatment and management of cesarean scar pregnancy	Retrospective cohort	China
Huang et al.[49]	2022	Clinical analysis of high-intensity focused ultrasound (HIFU) combined with hysteroscopy-guided suction curettage (HGSC) in patients with cervical pregnancy	Retrospective cohort	China
Liu et al.[51]	2020	Clinical outcome of high-intensity focused ultrasound as the preoperative management of cesarean scar pregnancy	Prospective cohort	China
Liu et al.[50]	2022	Clinical efficacy and safety of high-intensity focused ultrasound (HIFU) ablation in treatment of cesarean scar pregnancy (CSP) I and II	Retrospective cohort	China
Peng et al.[52]	2022	Analysis of the type of cesarean scar pregnancy impacted on the effectiveness and safety of high intensity focused ultrasound combined with ultrasound-guided suction curettage treatment	Retrospective cohort	China
Zhu et al.[13]	2015	High-Intensity Focused Ultrasound Combined with Suction Curettage for the Treatment of Cesarean Scar Pregnancy	Prospective cohort	China
Yuan et al.[54]	2021	Focused Ultrasound Ablation Surgery combined with ultrasound-guided suction curettage in the treatment and management of Cesarean Scar Pregnancy	Retrospective cohort	China
Xiao et al.[55]	2017	Cesarean Scar Pregnancy: Comparing the Efficacy And Tolerability Of Treatment With High-Intensity Focused Ultrasound And Uterine Artery Embolization	Retrospective cohort	China
Xiao et al.[81]	2014	Cesarean scar pregnancy: noninvasive and effective treatment with high intensity focused ultrasound	Prospective cohort	China
Peng et al.[56]	2022	High-intensity focused ultrasound ablation combined with systemic methotrexate treatment of intramural ectopic pregnancy: A case report	Case report	China
Li et al.[57]	2022	Comparison of high-intensity focused ultrasound ablation and uterine artery embolization in the management of cervical pregnancy	Prospective cohort	China
He et al.[58]	2011	A preliminary clinical study on high-intensity focused ultrasound therapy for tubal pregnancy	Prospective cohort	China
Huang et al.[59]	2014	High-intensity focused ultrasound combined with dilatation and curettage for Cesarean scar pregnancy	Case report	China
Jiang et al.[60]	2019	The treatment of cervical pregnancy with high-intensity focused ultrasound followed by suction curettage: report of three cases	Case report	China
Yu et al.[24]	2017	Treatment of cornual pregnancy in a patient with adenomyosis by high-intensity focused ultrasound (HIFU) ablation	Case report	China
Mu et al.[61]	2022	Evaluation of the treatment of high intensity focused ultrasound combined with suction curettage for exogenous cesarean scar pregnancy	Retrospective cohort	China
Liu et al.[7]	2023	High-intensity focused ultrasound as a pretreatment combined with hysteroscopic resection for gestational trophoblastic neoplasia with chemotherapy intolerance: a case report	Case report	China

Table 2. Showed some studies that report major adverse events and their number

Study ID	Foot drop	Thrombocytopenia	Abdominal hernia	Lumbar disc herniation	Acute renal failure	Bowel perforation
Jeng et al.[27]	NR	1/546	0/546	NR	4/546	1/546
Hong et al.[33]	NR	1/1	1/1	NR	1/1	1/1
Lee et al.[46]	1/618	NR	NR	1/618	NR	NR

Table 3. NIH quality assessment tool for observational cohort and cross-sectional studies

N	ID	Quality rating: good (11-14 points) or fair (7.5-10.5 points) or poor (0-7 points), Yes = 1/No = 0.5/NR & NA & CD = 0
1	Hu et al.[41]	**Fair**
2	Hong et al.[48]	**Fair**
3	Huang et al.[49]	**Fair**
4	Liu et al.[51]	**Fair**
5	Liu et al.[50]	**Fair**
6	Peng et al.[52]	**Fair**
7	Zhu et al.[13]	**Fair**
8	Yuan et al.[54]	**Fair**
9	Xiao et al.[55]	**Fair**
10	Xiao et al.[81]	**Fair**
12	Li et al.[57]	**Fair**
13	He et al.[58]	**Fair**
17	Mu et al.[61]	**Fair**

Table 4. Included studies for adenomyosis

ID	Quality rating: good (11-14 points) or fair (7.5-10.5 points) or poor (0-7 points), Yes = 1/No = 0.5/NR & NA & CD = 0
Jeng et al.[27]	**Good**
Shui et al.[31]	**Fair**
Yao et al.[28]	**Fair**
Xu et al.[29]	**Fair**
Li et al.[26]	**Fair**
Gong et al.[20]	**Good**
Zhao et al.[73]	**Fair**
Wei et al.[38]	**Fair**
Feng et al.[36]	**Fair**
Zhou et al.[35]	**Fair**
Gong et al.[25]	**Fair**
Lee et al.[23]	**Fair**
Yu et al.[22]	**Fair**
Lee et al.[21]	**Fair**
Xiong et al.[34]	**Fair**
Jingqi et al.[18]	**Fair**
Cheng et al.[19]	**Fair**

Table 5. ROB of endometriosis

ID	Quality rating: good (11-14 points) or fair (7.5-10.5 points) or poor (0-7 points), Yes = 1/No = 0.5/ NR & NA & CD = 0
Hu et al.[41]	**Fair**
Liu et al.[50]	**Fair**
Philip et al.[42]	**Fair**
Shi et al.[43]	**Fair**
Yang and Zhang[75]	**Fair**
Luo et al.[77]	**Fair**
Zhu et al.[47]	**Fair**
Xiao-Ying et al.[76]	**Good**
Zhao et al.[78]	**Fair**
Wang et al.[44]	**Good**
Wang et al.[45]	**Fair**

We also found that patients treated with HIFU may experience sensory abnormalities in the lower limbs (Lower limb paraesthesia), but it is very rare that only occurred in 19 patients out of 3390 individuals additionally, we noticed gradual relief of symptoms within one month after treatment (Figure 5). Forest plots for lower limb paraesthesia.

Regarding SIR type (B), 99 of a total of 6.437 patients had small vesicles and superficial burns, with pooled RR and 95% CI: 0.012 (0.007, 0.018). In terms of groin or perianal pain, our RR with 95% CI was 0.1 (0.067, 0.13). lower abdominal pain RR and 95% CI; 0.38 (0.25, 0.51). However, vaginal bleeding was detected in only 32 out of a total of 3.017 (Figures 6-9). Forest plots for superficial burns, groin pain, lower abdominal pain, vaginal bleeding.

Major adverse events SIR type (C&D) like lumber disc herniation, thrombocytopenia, and renal failure, were unmentionable. Our included studies did not record any deaths at all (Table 2). Showed some studies reporting major adverse events and their number (Figure 10). Forest plots for death.

Table 2 showed some studies reporting major adverse events and their number.

Back or Sacral Pain

Sacral pain was evaluated in 11 studies with 4183 patients in our pooled RR with 95% CI=0.3 (0.1, 0.5). the pooled studies represent major heterogeneity, so we used a random effect model the I^2=100%, and chi-p=0.0001 (Figure 11). Represents the forest plots for sacral pain.

Table 6. Murad et al.[16] assessment tool for case report study

ID	Quality rating: good (6.5-8 points) or fair (5-6.5 points) or poor (4.5-0 points)
Lee et al.[23]	**Good**
Nguyen[79]	**Good**
Stehouwer et al.[80]	**Fair**
Yu et al.[24]	**poor**
Hong et al.[33]	**Fair**
Fan et al.[32]	**Good**
Liu et al.[30]	**Poor**
Qu et al.[39]	**Poor**
Liu et al.[50]	**Poor**
She et al.[40]	**Poor**
Peng et al.[52]	**Fair**
Huang et al.[59]	**Fair**
Jiang and Xue[60]	**Fair**
Yu et al.[24]	**Fair**

Leg or Buttock Pain

Leg or buttock pain was evaluated in 18 studies involving 8143 patients in our pooled RR analysis with 95% CI=0.25 (0.15, 0.35). The pooled studies represent major heterogeneity with I^2=100%, and chi-p=0.0001 (Figure 12) depicts the forest plots for leg or buttock pain.

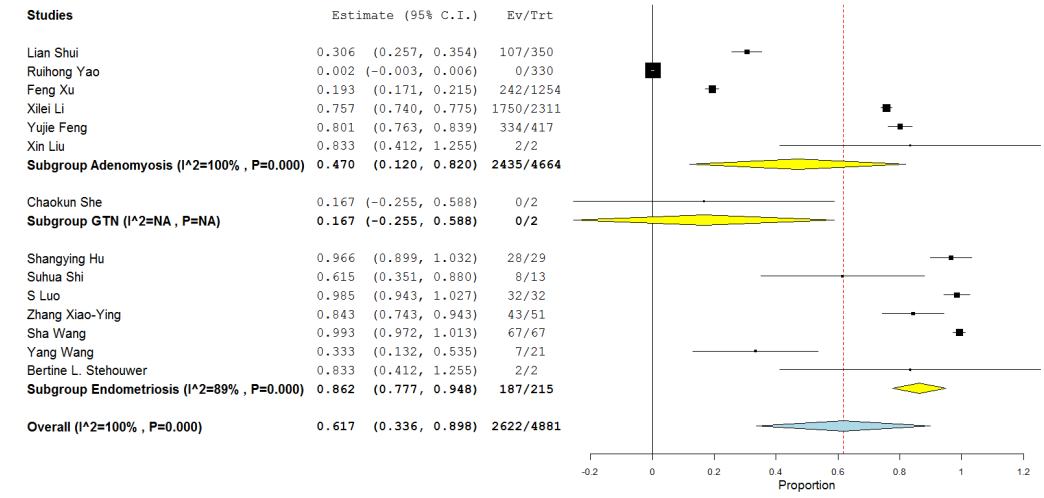
Nausea & Vomiting

Nausea and vomiting were examined in 11 studies totaling 4.183 patients, with only 112 experiencing nausea and 111 experiencing vomiting. Our pooled RRs and 95% CIs for nausea and vomiting were; 0.024 (0.01, 0.03) and 0.023 (0.009, 0.037), respectively. The pooled studies on this outcome were heterogeneous with I^2 82% and chi-p=0.001 for nausea and 84% and 0.001 for vomiting. Figures 13, 14 show the forest plots for nausea and vomiting.

Hematuria

Hematuria was evaluated in 11 studies totaling 4.573 patients; only 91 cases were found to have hematuria. Additionally, our pooled RR with 95% CI=0.25 (0.15, 0.35). The pooled studies represent major heterogeneity with I^2=87%, and chi-p=0.0001 (Figure 15). Represents the forest plot of hematuria.

2



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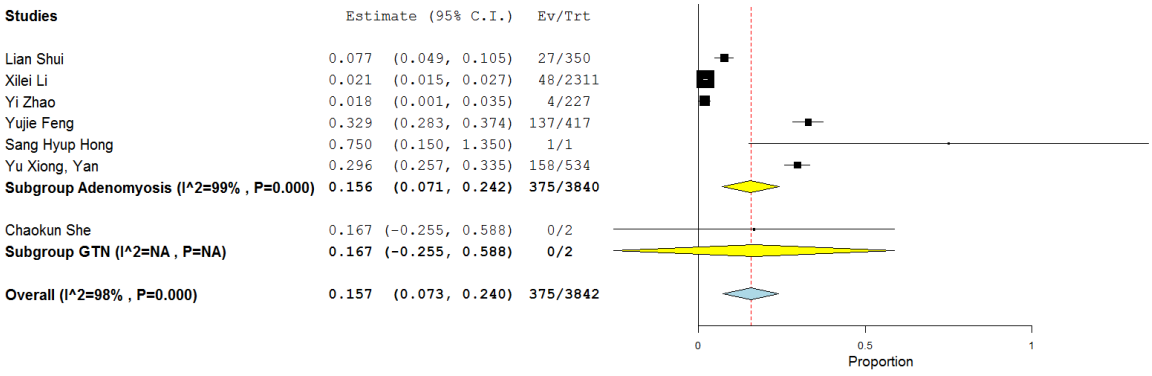

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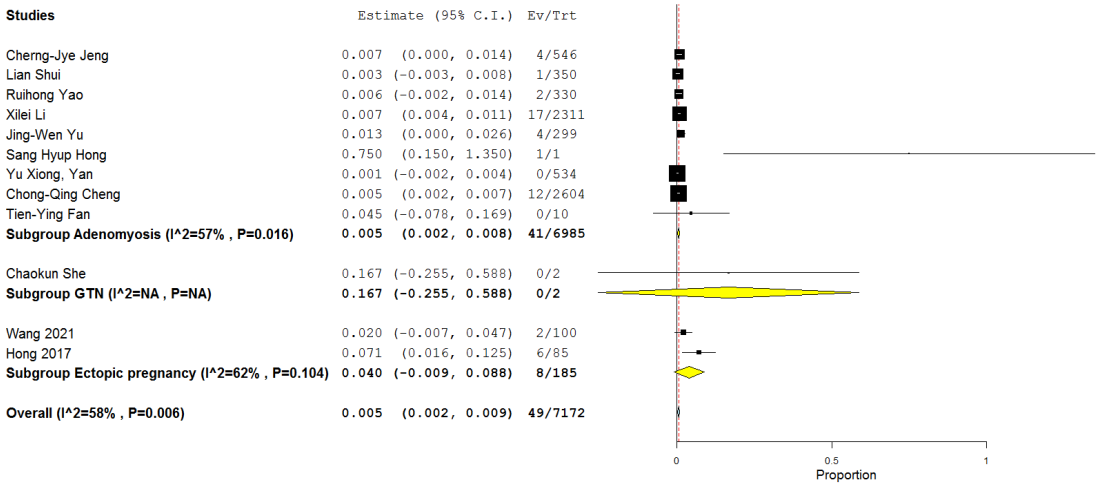

Figure 2-4. Depicts the forest plots for pain in the treatment site, abnormal vaginal discharge, and fever

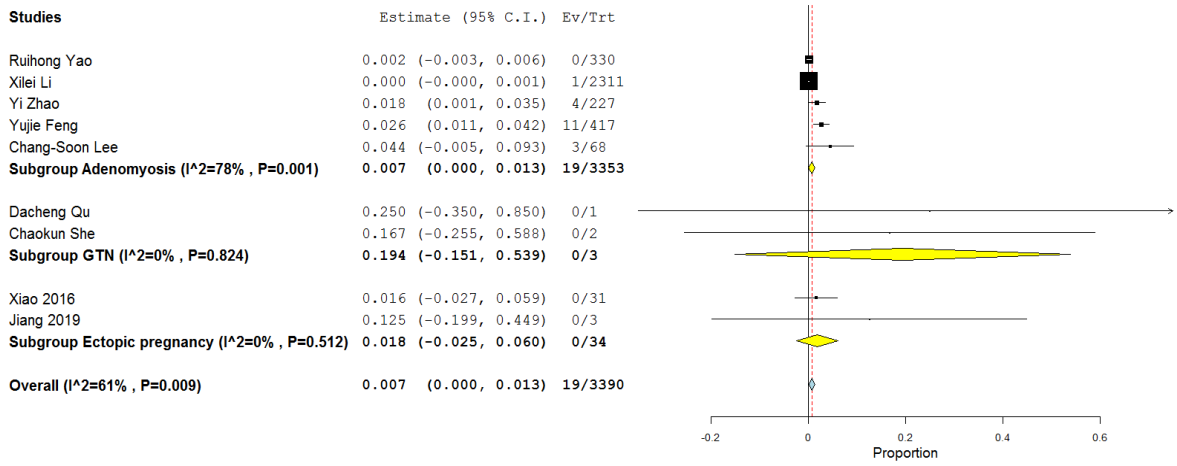

Figure 5. Shows the forest plots for lower limb paraesthesia

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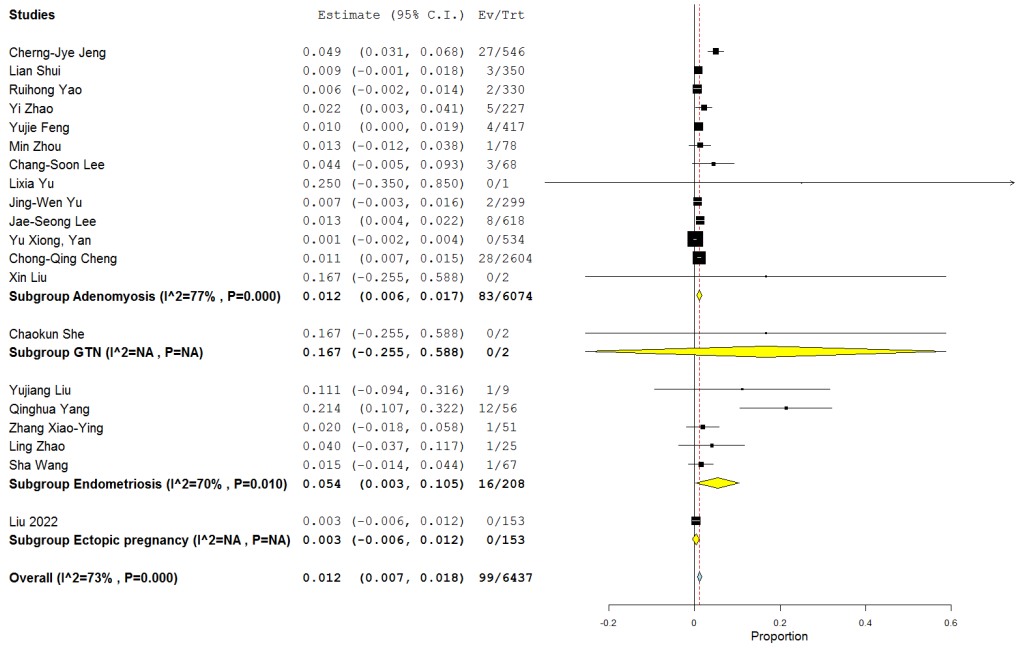

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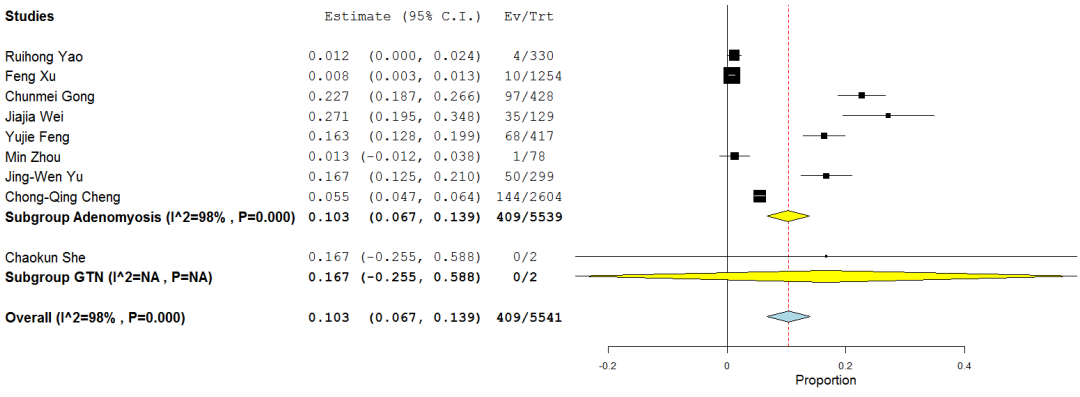

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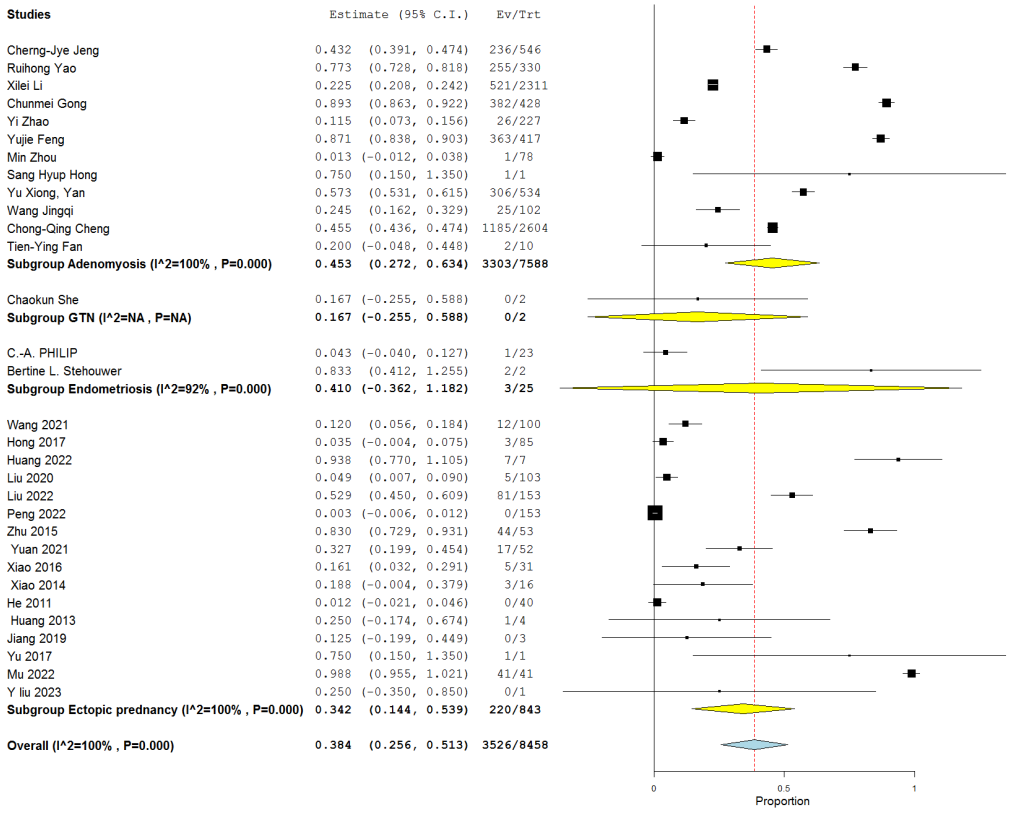

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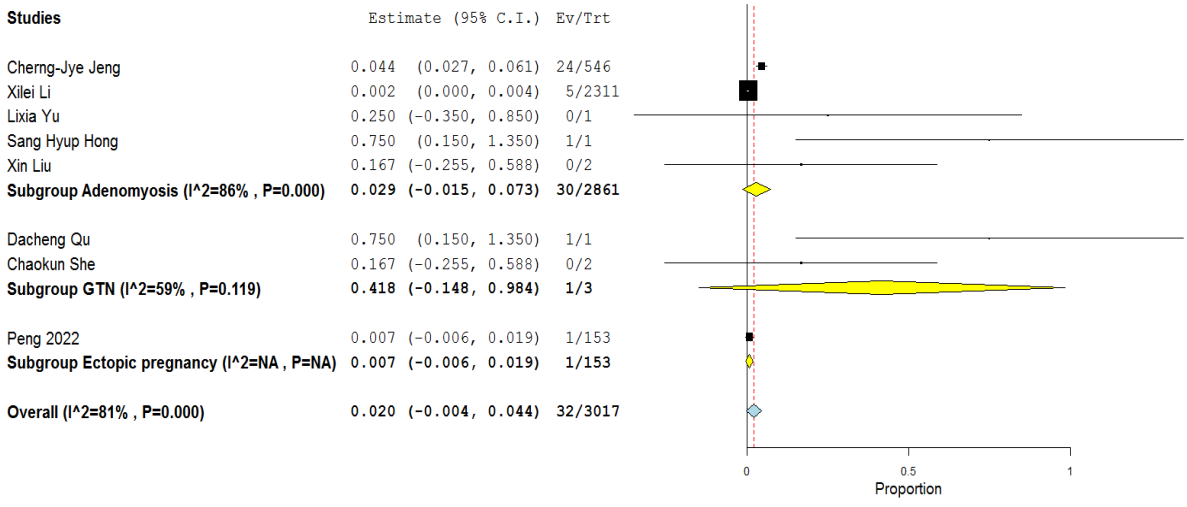


Figures 6-9. Represents the forest plots for superficial burns, groin pain, and lower abdominal pain, vaginal bleeding

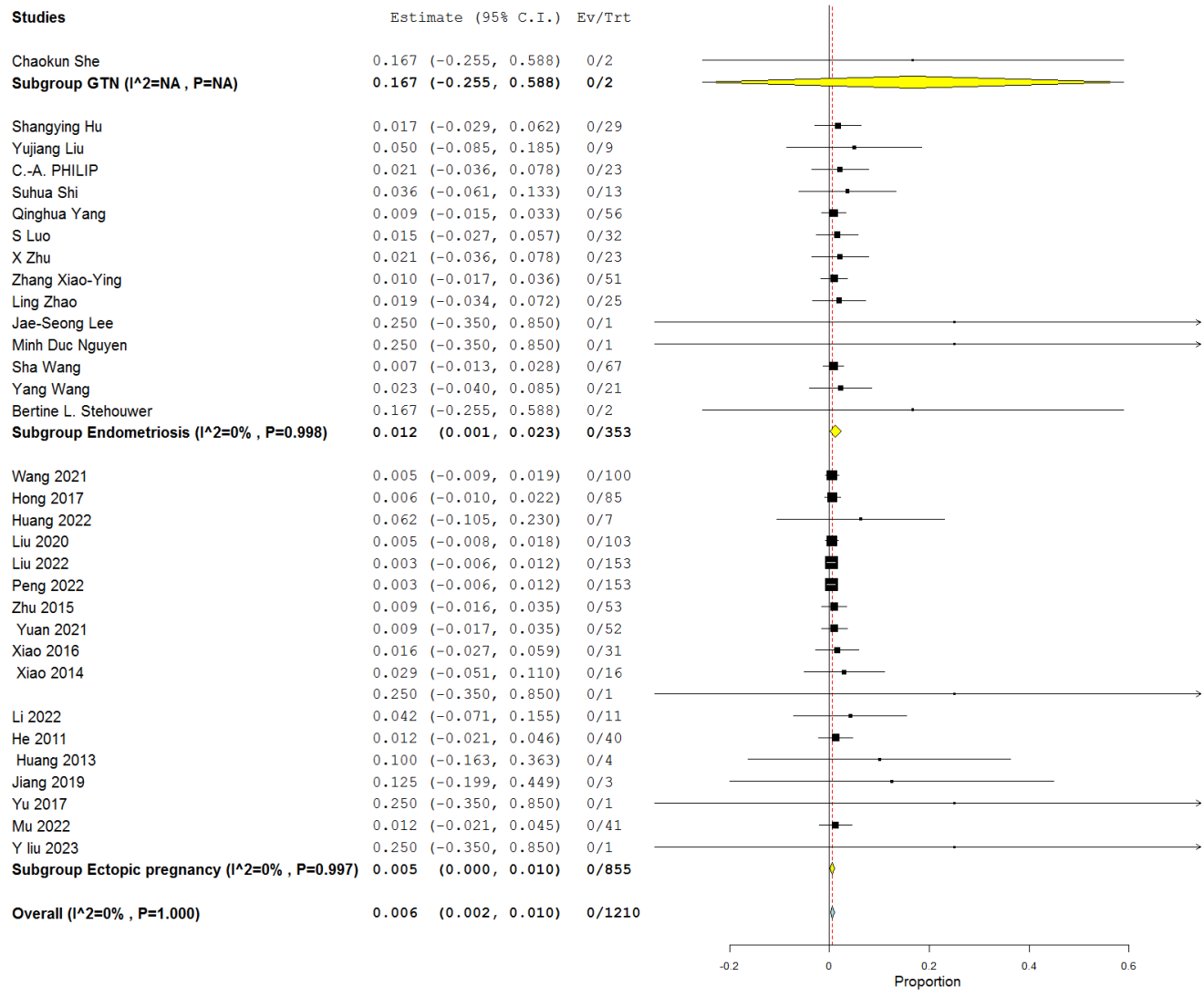


Figure 10. Represents the forest plots for death

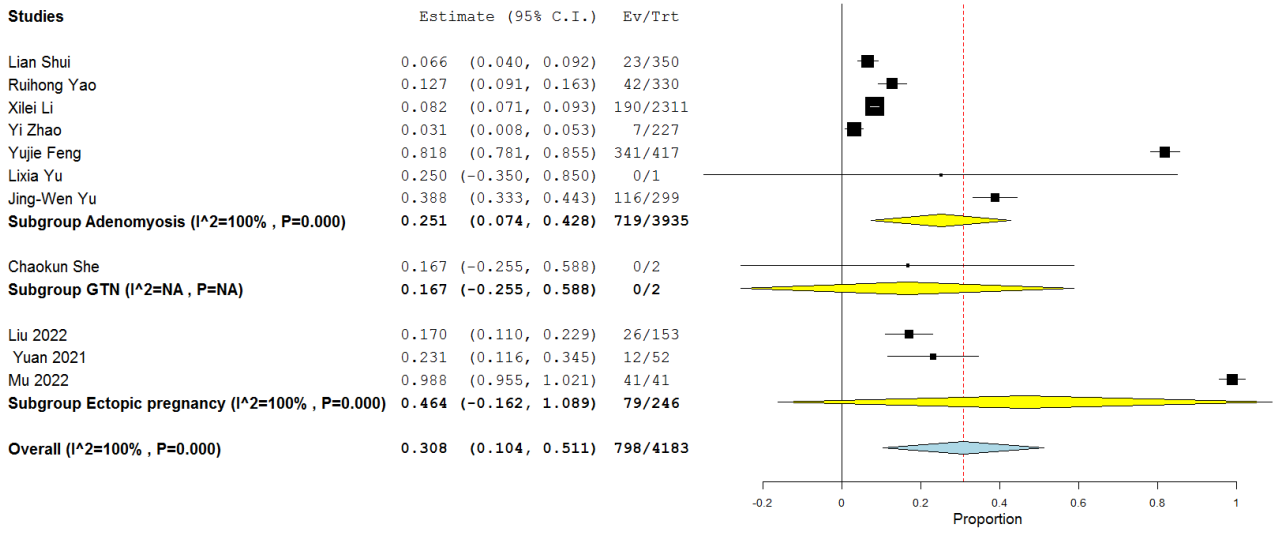


Figure 11. Represents the forest plots for sacral pain

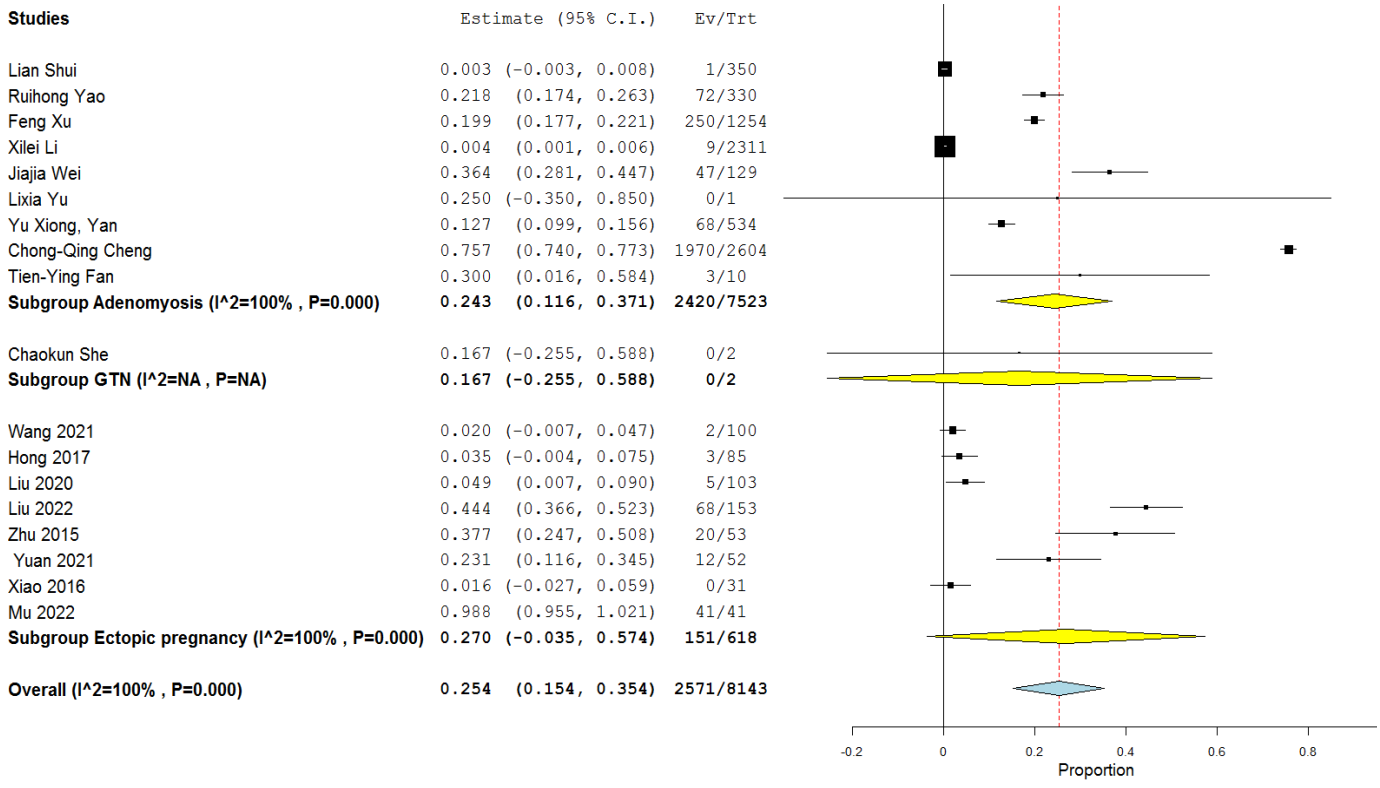


Figure 12. Depicts the forest plots for leg or buttock pain

Discussion

This systematic review and meta-analysis provides direct evidence of HIFU in terms of safety. Our systematic review and meta-analysis investigated the different adverse events of HIFU in individuals with adenomyosis, EP, endometriosis, or gestational trophoblastic disease. across 56 studies, including approximately 11.740 patients. In terms of mild to moderate adverse events that did not require clinical intervention, we found that pain at the treatment site estimated RR with 95% CI: 0.61 (0.33, 0.89), abnormal vaginal discharge 0.16 (0.073, 0.24), low-grade fever (<38 °C) 0.005 (0.002, 0.009). Sensory abnormalities of the lower limbs were examined in 3.390 individuals and observed in only 19 patients who experienced gradual relief of symptoms within one month after treatment. However, regarding adverse events that required treatment, 99 of a total of 6.437 patients had small vesicles and superficial burns, with pooled RR and 95% CI: 0.012 (0.007, 0.018). In terms of groin or perianal and lower abdominal pain, our RRs with 95% CIs were 0.1 (0.067, 0.13) and 0.38 (0.25, 0.51). However, vaginal bleeding was detected in only 32 out of a total of 3.017.

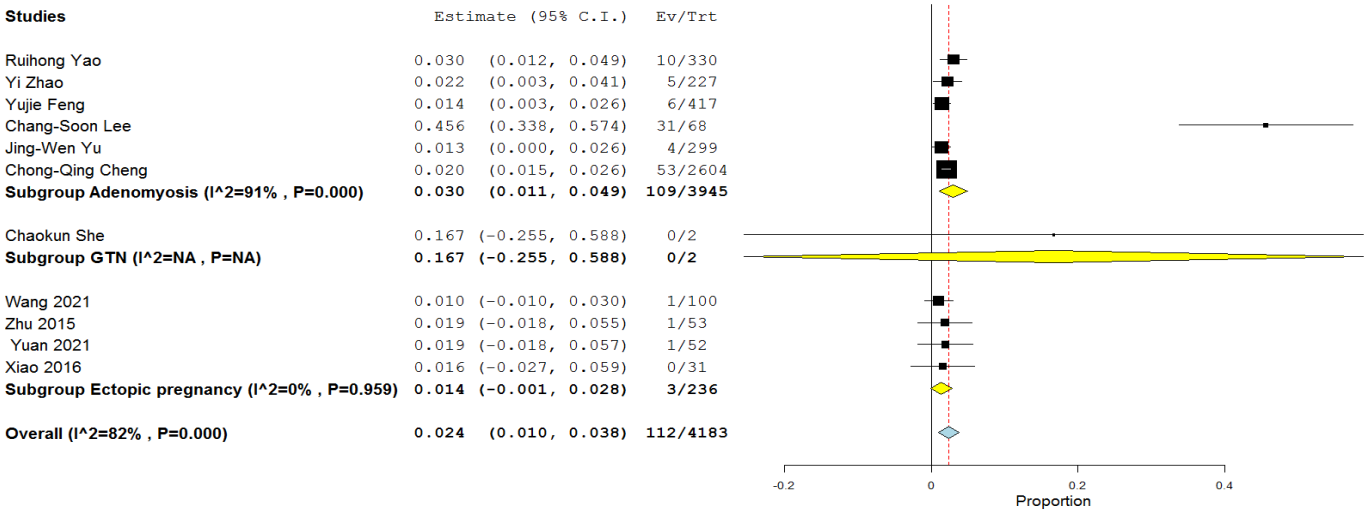
Major adverse events like lumber disc herniation, thrombocytopenia, and renal failure, were unmentionable. Additionally, our included studies did not record any deaths. The geographical distribution of the cohort is mainly China, Korea, and Hong Kong.

In women, benign breast lumps are the most common complaint and are more likely to be attacked than malignant breast lumps. One of them is fibro-adenomas. The only noninvasive transcutaneous ablative therapy that has been shown to treat a variety of solid mass types is HIFU or HIF-U. It is possible to rapidly build up enough energy in the region to cause coagulative necrosis and ablate the target lesion.

According to Liang et al.[62], following HIFU, approximately 25% of the patients in their research had subcutaneous edema and mild skin redness. None of the patients presented with any evidence of significant epidermis burns.

According to Wang et al.[63], five out of the 88 patients experienced skin blistering after receiving HIFU therapy; these patients healed with conservative measures. Furthermore, in their study, there were no serious side effects, such as multiple organ failure or malfunction, severe heat damage, bleeding, or intestinal perforation.

13



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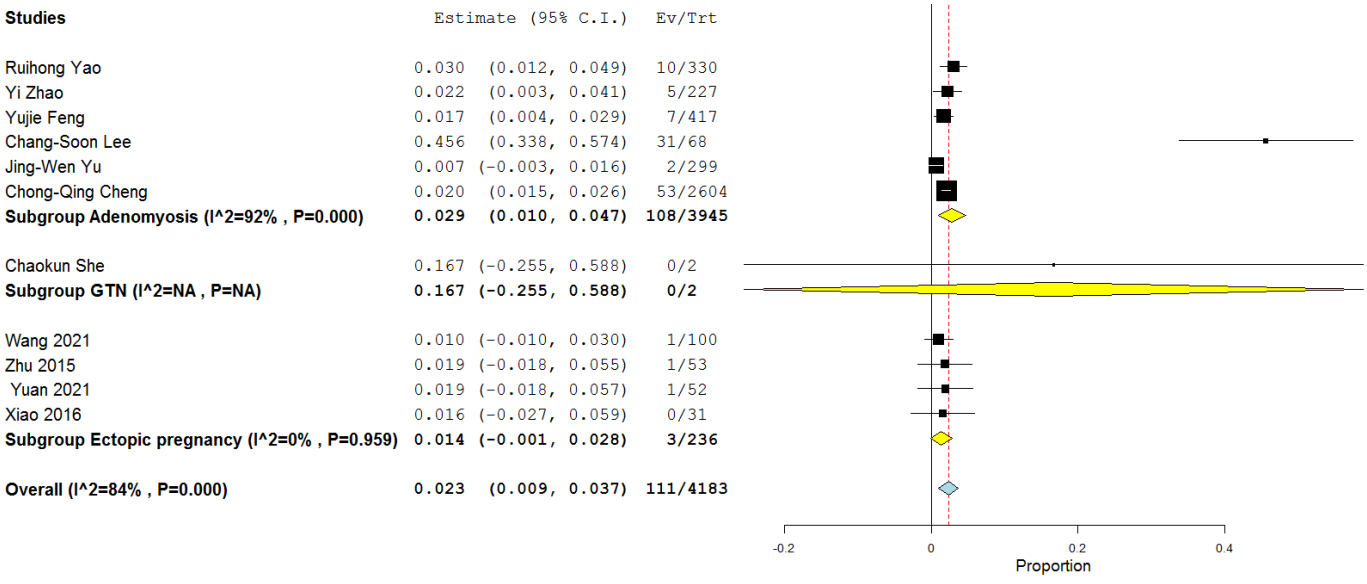

Figure 13, 14. Show the forest plots for nausea and vomiting

The incidence frequency of hepatic ectopic pregnancy (HEP), a relatively uncommon form of EP, has been estimated at 1 in 15.000 instances[64].

Wang et al.[65] discovered 31 cases of HEP in the literature within 60 years. Due to the liver's high vascularity, 26 cases required laparotomy, which is associated with a significant risk of severe surgical hemorrhage.

Although hepatocellular carcinoma (HCC) has not been treated with HIFU regularly, it has been reported to be treated with excellent success, particularly when the tumor mass is less than 3 cm. For cases in which the HCC mass was less than 3 cm, Ng et al.[66] found efficacy exceeding 90%.

Furthermore, Xu's study[67] included all patients with HCC who received HIFU treatment. After 2 years, the survival rate of stage 1 HCC was approximately 80%, whereas that of stage 2 HCC was approximately 51.4%. Furthermore, except for different levels of skin burns, no known adverse effects were associated with decline in liver function.

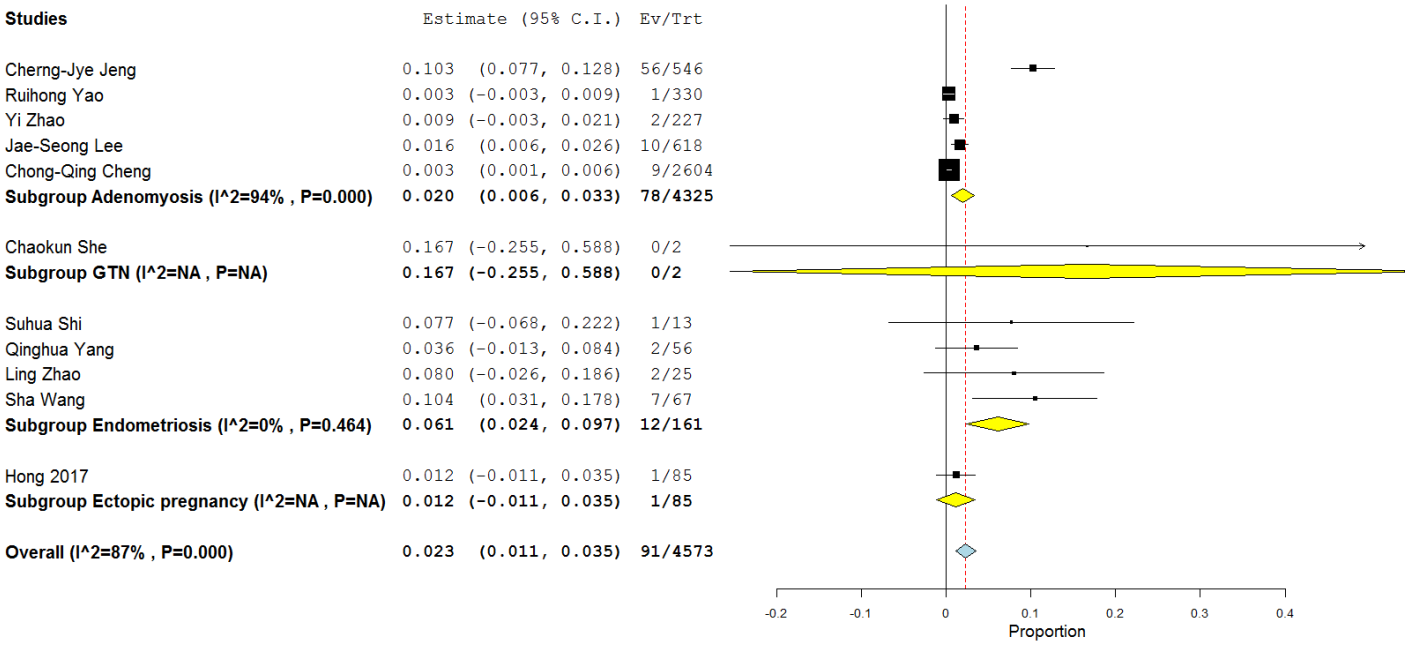

Figure 15. Represents the forest plot for hematuria

Therefore, we consider HIFU is a potential therapeutic option for HEP given its non-invasiveness compared with laparotomy and its safety for the function of hepatic tissue.

One prevalent gynecological issue that frequently requires surgical treatment is uterine fibroids. Due to their ability to prevent surgical morbidity and preserve the uterus, minimally invasive procedures like ultrasound-guided high-intensity focused ultrasound (USG HIFU), are becoming increasingly common. To manage symptoms and fibroid development, medical interventions, such as selective estrogen receptor modulators and gonadotropin-releasing hormone analogs, may be used. Unfortunately, the adverse effects or frequent failure of these therapies make them unsuitable for long-term use[68].

However, although surgical therapies for fibroids, including hysterectomy and myomectomy, are effective, up to 10% of individuals experience postoperative problems[69-71].

One patient presented with L4 nerve radiculopathy after presenting with foot drop and left lower limb paralysis following USG HIFU. Pregabalin was administered, and the patient was treated conservatively. In three months, she recovered completely and experienced no long-term effects. During the course of HIFU, no further significant adverse effects were observed. A few minor problems, such as mild lower abdominal pain or discomfort, resolved on their own. After treatment, all patients returned to their regular activities, with the exception of one who had L4 nerve radiculopathy. None of the HIFU cases required blood transfusion, and no skin burn incidents were documented[72].

Our study has several strengths. No previous systematic reviews have been published on this topic, so we are first. Additionally, we have a very large sample size of 11,74,000 among 56 studies that make our evidence less liable for negative results and more robust. However, our study was not free from limitations. First is lack of a comparator because of the single-arm design, also our included studies exhibited different designs, so we used different methodologies in assessment. HIFU was compared on diverse gynecological and obstetric diseases, and this gives potential biases in our analysis. These limitations require meticulous analysis and cautious interpretation of the results.

Conclusion

HIFU, either alone or in combination with oxytocin or any other enhancing agent, is safe for patients with different gynecological and obstetric diseases. In terms of efficacy, it showed promising results compared with traditional treatment lines. To our knowledge, we are the first and most comprehensive meta-analysis in the literature that has studied the different safety outcomes related to HIFU as a treatment modality for different obstetric and gynecological diseases with a very large sample size, making our evidence strong and less attributed to errors.

Ethics

Authorship Contributions

Design: M.M.A., E.A.H.F., C.R.M., Data Collection or Processing: M.M.A., C.R.M., E.O., P.K.R., E.A.H.F., Analysis or Interpretation: M.M.A., E.A.H.F., C.R.M., E.O., P.K.R.,

Literature Search: M.M.A., E.A.H.F., C.R.M., E.O., P.K.R., Writing: E.A.H.F.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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Review / Derleme

DOI: 10.4274/tjod.galenos.2024.59944

Turk J Obstet Gynecol 2024;21:208-18

The effects of growth hormone supplementation in poor ovarian responders undergoing In vitro fertilization or Intracytoplasmic sperm injection: A systematic review and meta-analysis of randomized controlled trials

İn vitro fertilizasyona veya intrasitoplazmik sperm enjeksiyonuna zayıf yumurtalık yanıtı veren kadınlarda büyüme hormonu takviyesinin etkileri: Randomize kontrollü çalışmaların sistematik bir derlemesi ve meta-analizi

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Abstract

To evaluate the effect of growth hormone (GH) supplementation on outcomes of in vitro fertilization (IVF) or Intracytoplasmic sperm injection (ICSI) for women with poor ovarian response. Relevant randomized controlled trials (RCTs) were obtained through search in several databases including PubMed, Scopus, Clinicaltrials.gov, Google Scholar, and Cochrane Library. Outcome measures included live birth rate, clinical pregnancy rate, cycle cancelation rate, number of retrieved oocytes, number of transferred embryos, total dose of gonadotropin, duration of gonadotropin treatment, and peak estradiol level. Additionally, a meta-regression analysis was performed to acknowledge any potential linear relationships between these outcomes and IVF success. After analyzing 18 RCTs comprising of 1870 patients, the study found that GH supplementation improved the number of retrieved oocytes [standardized mean difference (SMD), 0.65; 95% confidence interval (CI), 0.29-1.00] and transferred embryos group (SMD, 0.80, 95% CI, 0.39, 1.21) as well as peak E2 level (SMD, 1.20; 95% CI, 0.59, 1.81). While reduced the total dose and duration of gonadotropin treatment (SMD, -0.82, 95% CI, -1.25, -0.39, and SMD, -0.63, 95% CI, -1.04, -0.22, respectively). The meta-regression analysis found no linear relationship between clinical pregnancy, live birth rate, or cycle cancelation rate and the outcomes measured ($p>0.1$). Based on the available evidence, GH supplementation appears to improve the outcomes of IVF or ICSI in women with poor response. However, there is a need for further RCTs with larger sample sizes to determine the cost-effectiveness of adding GH to conventional protocols of IVF/ICSI for treating infertility in women with poor ovarian response.

Keywords: Growth hormone, gonadotropin, IVF, meta-analysis, systematic review

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Received/Geliş Tarihi: 28.06.2024 **Accepted/Kabul Tarihi:** 27.08.2024

Öz

Bu çalışmanın amacı zayıf yumurtalık yanıtı olan kadınlarda büyüme hormonu (GH) takviyesinin in vitro fertilizasyon (IVF) veya intrasitoplazmik sperm enjeksiyonu (ICSI) sonuçları üzerindeki etkisini değerlendirmektir. İlgili randomize kontrollü çalışmalar (RKÇ) PubMed, Scopus, Clinicaltrials.gov, Google Scholar ve Cochrane Kütüphanesi dahil olmak üzere çeşitli veri tabanlarında arama yapılarak elde edildi. Sonlanım ölçümleri arasında canlı doğum oranı, klinik gebelik oranı, siklus iptal oranı, alınan oosit sayısı, transfer edilen embriyo sayısı, toplam gonadotropin dozu, gonadotropin tedavisinin süresi ve en yüksek östradiol seviyesi yer aldı. Ek olarak, bu sonuçlar ile IVF başarısı arasındaki olası doğrusal ilişkileri belirlemek için bir meta-regresyon analizi gerçekleştirildi. Toplam 1870 hastayı kapsayan 18 RKÇ'nin analizinden sonra, GH desteğinin alınan oosit sayısını [standartlaştırılmış ortalama fark (SOF), 0,65; %95 güven aralığı (GA), 0,29-1,00] ve transfer edilen embriyo grubunu (SOF, 0,80, %95 GA, 0,39, 1,21) ve pik E2 seviyesini (SOF, 1,20; %95 GA, 0,59, 1,81) iyileştirdiği; Gonadotropin tedavisinin toplam dozunu ve süresini azalttığı (sırasıyla SOF, -0,82, %95 GA, - 1,25, -0,39 ve SOF, -0,63, %95 GA, - 1,04, -0,22) bulundu. Meta-regresyon analizi klinik gebelik, canlı doğum oranı veya siklus iptal oranı ile ölçülen sonlanımlar arasında doğrusal bir ilişki bulamadı (p>0,1). Mevcut kanıtlara göre, GH takviyesi zayıf yanıt veren kadınlarda IVF veya ICSI sonuçlarını iyileştiriyor gibi görünmektedir. Ancak, zayıf yumurtalık yanıtı olan kadınlarda kısırlığı tedavi etmek için IVF/ICSI'nın geleneksel protokollerine GH eklemenin maliyet etkinliğini belirlemek için daha büyük örneklem boyutlarına sahip daha fazla RKÇ'ye ihtiyaç vardır.

Anahtar Kelimeler: Büyüme hormonu, gonadotropin, IVF, meta-analiz, sistematik inceleme

Introduction

Diminished ovarian reserve (DOR) affects a significant percentage of women, between 8-15%, and its incidence increases in women over 40, affecting more than half of them[1]. Poor responders (POR) are women with DOR who experience difficulties producing enough mature oocytes, leading to lower embryo quality and higher cycle cancellation rates[2]. The European Society for Human Reproduction and Embryology introduced a standardized set of criteria named Bologna in 2011 to diagnose women with poor ovarian response[3]. To diagnose a patient with POR, at least two of the following three criteria must be met: (i) advanced maternal age (40 years or older) or another risk factor for POR; (ii) a history of poor ovarian response (three or fewer oocytes retrieved or a previous cycle that was canceled); and (iii) abnormal results from ovarian reserve tests (antral follicle count [AFC] of less than 5-7 follicles or Anti-Mullerian hormone [AMH] levels below 0.5-1.1 ng/mL). If a patient experiences two instances of POR despite receiving maximal stimulation, she can be diagnosed with the condition, even if the other criteria are not met. When considering treatment strategies for stimulating ovarian function in POR, one potential option is to administer gonadotropin-releasing hormone (GnRH) agonists[4]. While this approach has shown promise, it can also have its limitations. For example, it may inhibit ovarian function and response and require an increased dose of gonadotropin, thus leading to early luteinizing hormone (LH) secretion and potentially contributing to in vitro fertilization (IVF) failure rates[5]. In an effort to improve outcomes, various ovarian hyper-stimulation protocols have been explored, including the use of growth hormone as an additional treatment in stimulation protocols. However, an ideal protocol for POR has yet to be established, and more research is needed to optimize treatment strategies.

GH is a protein originating from the ovary and pituitary gland that targets the uterine and ovarian tissues in the female reproductive system. In general, GH promotes the overall ovarian health through its antioxidant effect[6]. However, the specific effects of GH are mediated by its receptors in myometrium[7], uterine decidua[8], granulosa cells and stroma of human ovary[9]. Ovarian GH, by creating a GH-GH receptor complex, increases the phosphorylation of janus kinase-2 and subsequently activates STAT molecules, thereby changing gene expression and cell performance. It also intercedes the development of primordial follicles to pre-antral in a paracrine manner. With gonadotropin receptors upregulation, GH increases the sensitivity of granulosa cells to follicle-stimulating hormone (FSH) and LH. Moreover, some studies have suggested its steroidogenesis effect, too. Additionally, GH enhances the oocytes quality by increasing the nucleal and cytoplasm maturation, which is done by the inhibitory effect of GH on connexin 43 and the increase of cumulus cells. Finally, GH improves implantation through its proliferative effect on uterine decidua cells[6]. Growth hormone is primarily released by the pituitary gland and plays a key role in cell growth, development, and metabolism.

GH is secreted mostly by pituitary gland, and affects cell growth, development as well as metabolism[10]. The use of GH as a co-treatment for various ovarian stimulation protocols in reproductive medicine has been the subject of extensive research. GH stimulates insulin-like growth factor 1 (IGF-1) production in both the liver and ovarian follicles. IGF-1 helps regulate steroidogenesis, enhances gonadotropin effect on granulosa and theca cells, and increases ovarian sensitivity to gonadotrophins, thereby advancing early follicular development, preventing of antral follicles involution, and promoting oocyte maturation[11-13]. Furthermore, recent studies indicate that GH takes part in enhancing follicular survival and cell proliferation as well as promoting high-quality embryos and increasing implantation rate[14,15]. However, the effect of GH administration on IVF/intracytoplasmic sperm injection (ICSI), outcomes is still not clearly understood, with studies showing contradictory results. While some research has shown that growth hormone (GH) positively affects oocytes, the endometrium, and improves embryo development outcomes, others have not replicated these results[16-19]. In conclusion, t the majority of evidence suggests GH is crucial for follicular development, estrogen production, and the maturation of

oocytes via IGF-1, while its effect on the efficacy of IVF or ICSI techniques remains a subject of ongoing research.

The addition of GH to IVF/ICSI protocols has been the subject of several recent studies, including a 2020 meta-analysis which reported improved outcomes for poor ovarian responders[20]. Since then, several new randomized controlled trials (RCTs) have been published investigating the influence of GH on IVF/ICSI outcomes. To provide a thorough understanding of the subject, our aim is to conduct a rigorous meta-analysis of the available evidence from relevant RCTs to date.

Materials and Methods

In this meta-analysis, we aim to investigate the impact of GH supplementation on the outcomes of IVF or ICSI in poor ovarian responders. We have taken measures to ensure the rigor and transparency of our study, including registering our research protocol on the Open Science Framework and utilizing a checklist for search strategy, screening, and data selection. Our methodology conforms to the Preferred Reporting Items for Systematic Reviews and Meta-analyses guidelines to maintain a high standard of reporting[21]. The systematic review protocol was registered in Open Science Framework (https://osf.io/bv9zm).

Literature Search

A comprehensive literature search was conducted up to May 25, 2023 to identify relevant RCTs and systematic reviews. The following databases were searched: Cochrane Library, PubMed, SCOPUS, Google Scholar, and Clinical Trials. To generate subsets of relevant citations, we used a combination of Medical Subject Headings (MESH) and text words. Two subsets were created for studies of poor ovarian response, using keywords related to "reserve" and "poor". Additional two subsets were also created for studies on IVF/ICS and GH supplementation, using relevant keywords. the subsets were combined using the "AND" operator to generate a refined set of citations, and a publication type filter was applied to obtain only RCTs and systematic reviews. To adhere to the search engine specifications for each database, the search strategy was adjusted accordingly. There were no restrictions on language or date. Additionally, we screened the reference lists of relevant systematic reviews and included studies to identify any additional articles that met our inclusion criteria. Two independent reviewers conducted the search and screened all records for eligibility. Any differences were addressed through discussion and agreement among the reviewers.

Criteria for Selecting Studies

Prior to conducting the literature search, we established the inclusion and exclusion criteria for this meta-analysis. To be considered for inclusion, studies had to be RCTs that met the following criteria: inclusion of women characterized as POR, women subjected to IVF or ICSI with any ovarian stimulation protocol, and reporting of clinical pregnancy outcomes. Studies that used adjuvant treatments alongside GH or lacked a comparison group not using GH were excluded from the analysis.

Extraction of Relevant Data

Two reviewers (AA, FA) undertook a meticulous evaluation of each study's title and abstract to determine its eligibility for inclusion in this meta-analysis. Studies that did not meet the inclusion criteria were excluded. The complete texts of the remaining studies were reviewed to confirm they met the eligibility criteria for the data extraction process. Next, the following information was selected for extraction in three sets: 1. patient-specific factors [i.e. what POR criteria are met, age, body mass index (BMI), and the duration of infertility], 2. study design (i.e. number of participants, details of IVF or ICSI protocol, doses and details of GH administration), 3. outcomes (i.e. pregnancy rate, live birth rate, number of retrieved oocytes, number of transferred embryos, rate of cycle cancellation, and total dose of gonadotropins administered, and peak estradiol level).

Study Quality Assessment

To ensure the reliability and accuracy of the meta-analysis, two independent authors (FZ, QB) evaluated the quality of the included literature using the Cochrane risk of bias assessment tools for RCTs. Any inconsistencies in the evaluations were resolved by a third author, (FA).

Statistical Analysis

This meta-analysis was conducted on the effects of co administration of gonadotropins and GH during the ovarian stimulation on IVF outcome for POR's patients compared to a control group using Stata version 15 (Stata Corp, College Station, TX, USA). After the authors have extracted data, meta-analysis has been on the adequate data. We have gathered all information in Tables 1-3 to be systematically studied and explained in the result section. Standardized mean difference (SMD) between the control and the patient's group was selected as the main unit of analysis for each variable. The cut-off values have been set by Cohen for the interpretation of medium, small, and large effect sizes (0.5, 0.2, and 0.8, respectively). Analyses have been performed employing the random effects model. The authors have assessed heterogeneity by I2 statistics and values larger than 50% were announced as moderate to high heterogeneity. We also have done meta-regression when we found enough studies to examine the relationship between pregnancy rate, and live birth rate as potential effect modifiers. Publication bias was assessed visually using funnel plots and quantitatively through Begg's and Egger's regression tests.

Results

Study Selection and Included Studies

After an extensive search using MESH terms, a total of 234 studies were identified. Of these, 37 duplicates were removed.

Table 1. Characteristics of the studies included

Publication, country of origin	Population (GH + control)	Inclusion criteria	Intervention	Gonadotropins treatment	Stimulation protocol	Selected outcomes
Safdarian et al.[24] 2019, Iran	70 (35+35)	Bologna criteria	2.5 mg/day GH CD8-trigger	300 IU/day CD3-trigger	GH: GnRH antagonist protocol C: GnRH antagonist protocol	Duration of gonadotropin treatment Total doses of gonadotropin No. of collected oocytes No. of transferred embryos Clinical pregnancy rate, live birth rate, cycle cancelation rate
Bassiouny et al.[16] 2016, Egypt	141 (68+73)	Bologna criteria	2.5 mg/day GH CD6-trigger	300-450 IU/day HMG IM CD2-trigger	GH: GnRH antagonist protocol C: GnRH antagonist protocol	Total dosage of HMG Duration stimulation No. of collected oocytes No. of embryos transferred Clinical pregnancy rate Live birth rate Cycle cancelation rate
Bayoumi et al.[17] 2015, Egypt	172 (84 + 88)	Bologna criteria	2.5 mg/day GH CD6-trigger	300-450 IU/ day HMG three days after GnRh-a until trigger	GH: Microflare stimulation protocol C: Microflare stimulation protocol	Total dosage of HMG Duration of stimulation No. of embryos transferred No. of collected oocytes Clinical pregnancy rate, cycle cancelation rate
Choe et al.[28] 2018, South Korea	127 (62+65)	Bologna criteria	20 mg GH three times at mid-luteal, late luteal, and menstrual cycle day 2	225-375 IU/day FSH from CD3-trigger	GH: GnRH antagonist protocol C: GnRH antagonist protocol	Total doses of gonadotropin No. of collected oocytes No. of embryos transferred Clinical pregnancy rate
Dakhly et al.[22] 2018, Egypt	240 (120+120)	Bologna criteria	2.5 mg GH from previous cycle day 21 until trigger	300 IU/day FSH from CD2/3-trigger	GH: Long GnRH agonist protocol C: Long GnRH agonist protocol	Duration of stimulation, Dosage of gonadotropins E2 levels No. of collected oocytes No. of transferred embryos Canceled cycles rate, Clinical pregnancy rate, Live birth rate
Dor et al.[29] 1995, Israel	14 (7+7)	Oestradiol <500 pg/ mL, less than three oocytes retrieved in two previous IVF cycles	18 IU GH on days 2, 4, 6 and 8 of the cycle	300 IU/day FSH on CD3-CD7 and 300 IU/day HMG On CD8-trigger	GH: GnRH agonist short protocol C: GnRH agonist short protocol	Total dosage of HMG No. of collected oocytes No. of transferred embryos
Eftekhar et al.[18] 2012, Iran	82 (40+42)	Failed IVF cycles ≥1; oocytes ≤3; E2 levels <500 pg/mL on HCG day	4 IU/day GH from previous cycle day 21 until trigger	IU/day of HMG on CD2-trigger	GH: GnRH antagonist protocol C: GnRH antagonist protocol	Duration of stimulation Total dosage of HMG E2 levels Cancelation rate No. of collected oocytes No. of embryos transferred Clinical pregnancy rate
Lee et al.[30] 2019, Taiwan	184 (94+90)	Bologna criteria	4, 4, 2 IU GH for three days in a row	NA	GH: Ultra-long GnRH agonist protocol C: Ultra-long GnRH agonist protocol	Total dosage of HMG E2 levels No. of collected oocytes No. of embryos transferred Clinical pregnancy rate

Table 1. Continued

Publication, country of origin	Population (GH + control)	Inclusion criteria	Intervention	Gonadotropins treatment	Stimulation protocol	Selected outcomes
Kucuk et al.[5] 2008, Turkey	61 (31+30)	Responded poorly to high dose gonadotropin in first cycle	4 mg/day GH from previous cycle day 21	450 IU/day rFSH until trigger (starting day: NA)	GH: GnRH agonist long protocol C: GnRH agonist long protocol	Duration of stimulation, Total dosage of FSH E2 levels No. of embryos transferred, Pregnancy rate
Zafardoust et al.[25] 2022, Iran	194 (97+97)	Bologna criteria	5 mg/day GH from previous cycle day 21 until trigger	75-300 IU/ day of FSH on CD2/3-trigger	GH: GnRH antagonist protocol C: GnRH antagonist protocol	Duration of stimulation Total dosage of FSH E2 levels Cancelation rate Clinical pregnancy rate Live birth rate
Mohammad et al.[23] 2021, Egypt	156 (78+78)	Bologna Criteria W/O advanced maternal age	4 IU/day GH from CD2 - 1 day before oocyte retrieval	450 IU/day HMG from CD2-trigger	GH: Ultra-short GnRH antagonist C: Ultra-short GnRH antagonist	Duration of stimulation E2 levels Cancelation rate No. of collected oocytes No. of embryos transferred Clinical pregnancy rate
Norman et al.[31] 2019, Australia	130 (65+65)	At least one IVF cycle with oocytes ≤5; age ≤ 41; FSH ≤15 IU/l	12 IU/day GH from CD1- trigeer	NA	GH: GnRH antagonist protocol C: GnRH antagonist protocol	Total dosage of rFSH duration of stimulation No. of collected oocytes No. of embryos transferred live birth rate Clinical pregnancy rate, cancelation rate
Owen et al.[32] 1991, UK	25 (13+12)	At least one previous IVF cycle with poor response (e.g. oocytes ≤6, embryos ≤3)	24 IU GH on alternate days during stimulation	225 IU/day HMG from CD1 until trigger	GH: Microflare protocol C: Microflare protocol	Duration of HMG Total dosage of HMG No. of embryos No. of collected oocytes Pregnancy rate, live birth rate
Suikkari et al.[33] 1996, Finland	22 (16+6)	Oocytes ≤2 or ≥48 amples of HMG	4 IU/day or 12 IU/day GH from menstrual cycle day 3 d until trigger	300 IU/day FSH from from menstrual cycle day 3 until trigger	GH: GnRH-a flare up protocol C: GnRH-a flare up protocol	Cancelation rate Total dosage of FSH E2 levels No. of collected oocytes Pregnancy rate Live birth rate
Gong et al.[26] 2020, China	105 (52+53)	Bologna criteria	4 IU/day GH on day 2 of the previous menstrual cycle until trigger	FSH from day 2 of the menstrual cycle	GH: GnRH antagonist protocol C: GnRH antagonist protocol	Total dosage of FSH Duration of stimulation E2 levels No. of collected oocytes No. of embryos Cancelation rate Clinical pregnancy rates
Bergh et al.[34] 1994, Sweden	20 (10+10)	Failed IVF attempts ≥2, oocytes <5, age 25-38 years	0.1 IU/kg GH during stimulation for a maximum of 25 days	75-300 IU/day HMG or rFSH for 10 to 25 days	GH: GnRH agonist long protocol C: GnRH agonist long protocol	Total dosage of gonadotropins Duration of stimulation E2 levels No. of collected oocytes Cancelation rate Clinical pregnancy rates

Table 1. Continued

Publication, country of origin	Population (GH + control)	Inclusion criteria	Intervention	Gonadotropins treatment	Stimulation protocol	Selected outcomes
Tesarik et al.[35] 2005, Spain	100 (50+50)	Age 41-44 years	8 IU/day GH from CD7	450 IU/day of rFSH and 150 IU/day of HMG for 5 days (adjusted until trigger)	GH: GnRH agonist long protocol C: GnRH agonist long protocol	Total dosage of gonadotropins Duration of stimulation E2 levels No. of collected oocytes No. of embryos Live birth rate Clinical pregnancy rates Cancelation rate
Zhuang et al.[27]1994, China	27 (12+15)	Previous poor response	12 IU GH on alternate days	2 IU HMG given on alternate days for 12 days (at same time as GH)	GH: GnRH agonist long protocol C: GnRH agonist long protocol	Total dosage of gonadotropins Duration of stimulation E2 levels No. of collected oocytes No. of embryos Live birth rate Clinical pregnancy rates Cancelation rate

GH: Growth hormone, CD: Cluster of differentiation, C: Control, HMG: Human menopausal gonadotropin, GnRH: Gonadotropin-releasing hormone, IVF: In vitro fertilization, HCG: Human chorionic gonadotropin, FSH: Follicle-stimulating hormone, rFSH: Recombinant follicle stimulating hormone

Table 2. The results of subgroup analysis

	Number of study	Standard mean difference	95% CI	I2
Age	14	-0.02	-0.14, 0.11	41.9%
BMI	12	-0.05	-0.16, 0.06	15.5%
Duration of infertility	8	-0.00	-0.12, 0.12	0.0%
Oocytes retrieved	13	0.65	0.29, 1.00	90.6%
Total gonadotropin	11	-0.82	-1.25, -0.39	92.8%
Duration of gonadotropin stimulation	11	-0.63	-1.04, -0.22	91.7%
Transferred embryo	11	0.80	0.39, 1.21	91.4%
Peak E2 level	11	1.20	0.59, 1.81	96.1%

CI: Confidence interval, BMI: Body mass index

Then, the abstracts of the remaining articles went through further assessment, which resulted in exclusion of 163 studies for not meeting the inclusion criteria. The full text of the remaining 34 studies were retrieved, and 16 of them were excluded following the reasons outlined in Figure 1. At last, only 18 studies matched the selection criteria and were included for meta-analysis. Eligible studies were published from 1991-2022, and included a total of 1870 women identified as POR. Among them, 934 women received GH co-treatment during ovarian stimulation and were assigned to the intervention group,

Table 3. The results of meta-regression analysis

	Primary outcomes	Coef (95% confidence interval)	p-value
Oocyte retrieved			
	Cycle cancellation rate	0.019 (-1.86, 1.80)	0.982
	Clinical pregnancy rate	-0.740 (-5.73, 4.25)	0.750
	Live birth rate	-3.154 (-10.83, 4.52)	0.318
Total gonadotropin			
	Cycle cancellation rate	3.166 (-1.44, 7.77)	0.152
	Clinical pregnancy rate	0.753 (-7.74, 9.25)	0.845
	Live birth rate	8.183 (-8.23,24.60)	0.239
Duration of gonadotropin			
	Cycle cancellation rate	1.337 (-0.61,3.29)	0.156
	Clinical pregnancy rate	-19.455 (-53.96, 15.03)	0.170
	Live birth rate	4.551 (-7.22, 16.32)	0.343
Transferred embryo			
	Cycle cancellation rate	-1.743 (-4.95, 1.47)	0.241
	Clinical pregnancy rate	0.495 (-8.65, 9.64)	0.905
	Live birth rate	-9.305 (-48.57, 29.96)	0.415
Peak E2 level			
	Cycle cancellation rate	-1.743 (-4.95, 1.47)	0.247
	Clinical pregnancy rate	0.495 (-8.65, 9.64)	0.905
	Live birth rate	-9.305 (-48.57, 29.96)	0.415

while the remaining 936 women, who received conventional COS, were assigned to the comparison group. The definition of poor ovarian response was not consistent across the studies due to some being published before the establishment of the Bologna criteria. Out of articles included, four were carried out in Egypt[16,17,22,23], three were from Iran[18,24,25], two from China[26,27], and one from South Korea[28], Israel[29], Taiwan[30], Turkey[5], Australia[31], UK[32], Finland[33], Sweden[34], and Spain[35]. All articles aimed to determine whether GH co-treatment could enhance IVF or ICSI outcomes for patients with poor ovarian response. GnRH agonists were used in 10 studies[5,17,22,23,29,30,32-35], and total of five RCTs used GnRH antagonists[16,18,24,25,28]. Detailed information on these studies can be found in Table 1.

Demographics

A total of 14, 12, and 8 studies reported the age[5,16-18,22-26,28,30,31,34,35], and duration of infertility[18,22-24,26,28] of the study participants, respectively. The findings of the analysis indicated that there was no significant difference with regard to these demographic factors among studies, exhibiting low to moderate heterogeneity (age: I2=41.9%, BMI: I2=15.5%, duration of infertility: I2=0.0%). Nevertheless, a random-effects model was used as illustrated in Figure 2.

Number of Retrieved Oocytes

A total of 15 studies reported number of retrieved oocytes. two of which[29,32] did not provide standard deviation of data, finally, 13 studies[16-18,22-24,26-28,30,31,34,35] including 1554 patients (770 in the GH group and 784 in the control group) were included in the meta-analysis. A significant increase in the number of retrieved oocytes was found in the GH group compared to the control group (SMD, 0.65; 95% CI, 0.29-1.00), as shown in

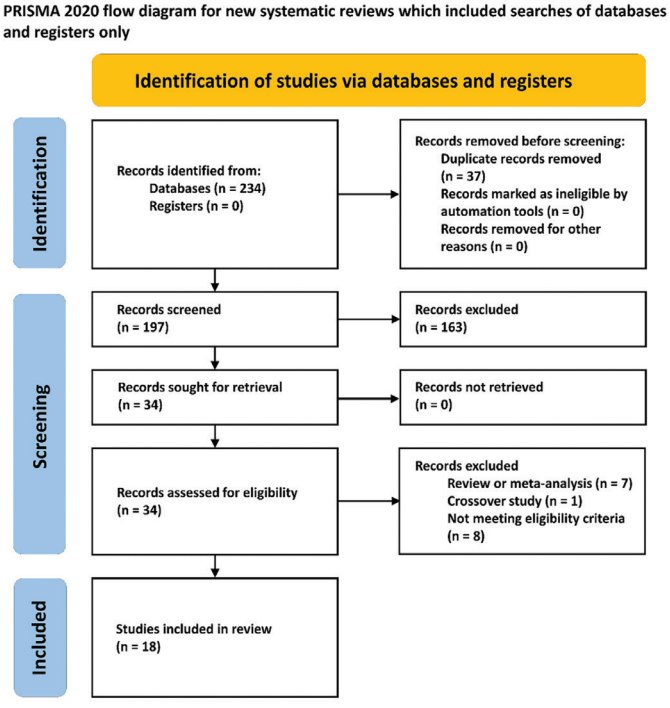

Figure 1. PRISMA flow diagram for current systematic review

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-analyses for Protocols

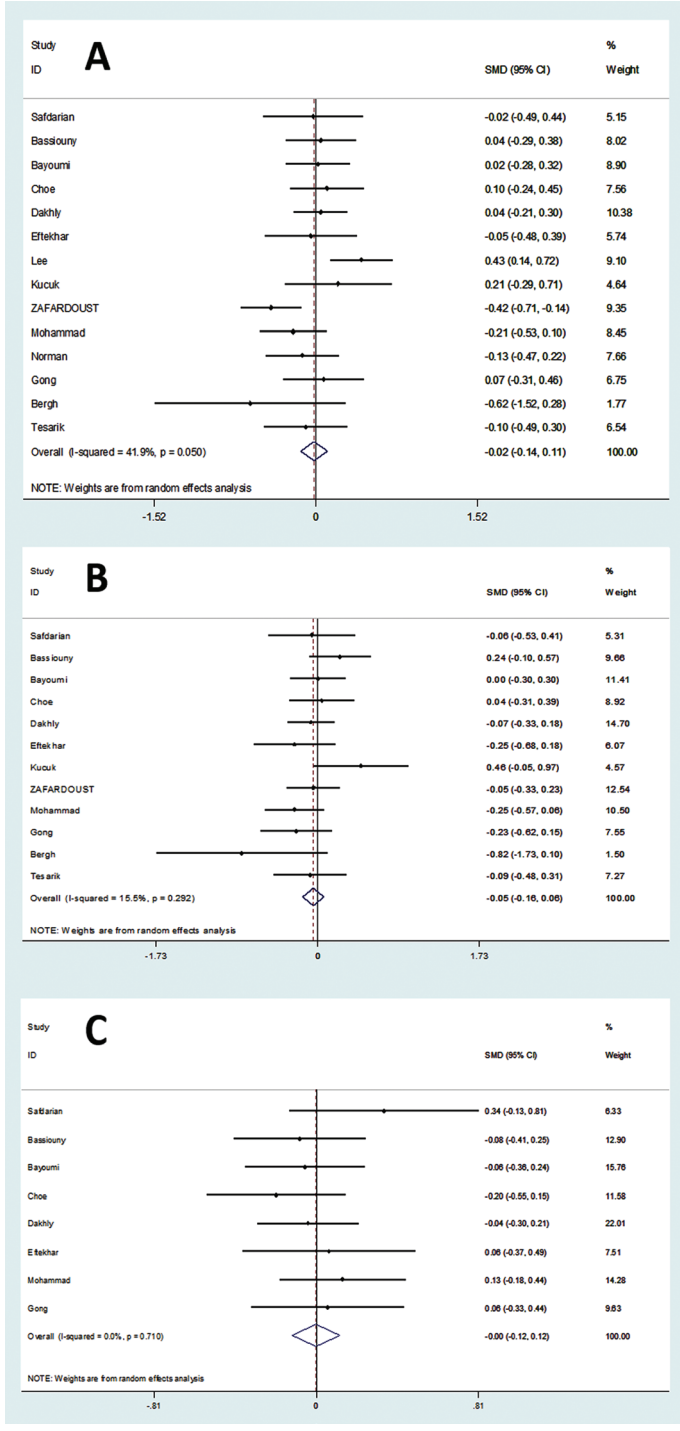

Figure 2. Forest plot for demographics; A) Age, B) BMI, C) Duration of infertility

CI: Confidence interval, SMD: Standardized mean difference, BMI: Body mass index

Figure 3A. However, significant heterogeneity was observed among these studies (I2=90.6%), as a result, the random effects model was applied.

Number of Transferred Embryo

A total of 15 studies reported the number of transferred embryo, four of which[22,29,32,35] did not provide standard deviation of data or lacked specific data required for a meta-analysis. The meta-analysis finally included 11 studies[5,16-18,23,24,26-28,30,31] for 1255 patients (621 in the GH group and 634 in the control group). The result showed a significant increase in the number of transferred embryo in the group given GH o-treatment compared to the control group (SMD, 0.80, 95% CI, 0.39, 1.21). A random effects model was applied due to the significant heterogeneity among these studies. (I2=91.4%), as shown in Figure 3B.

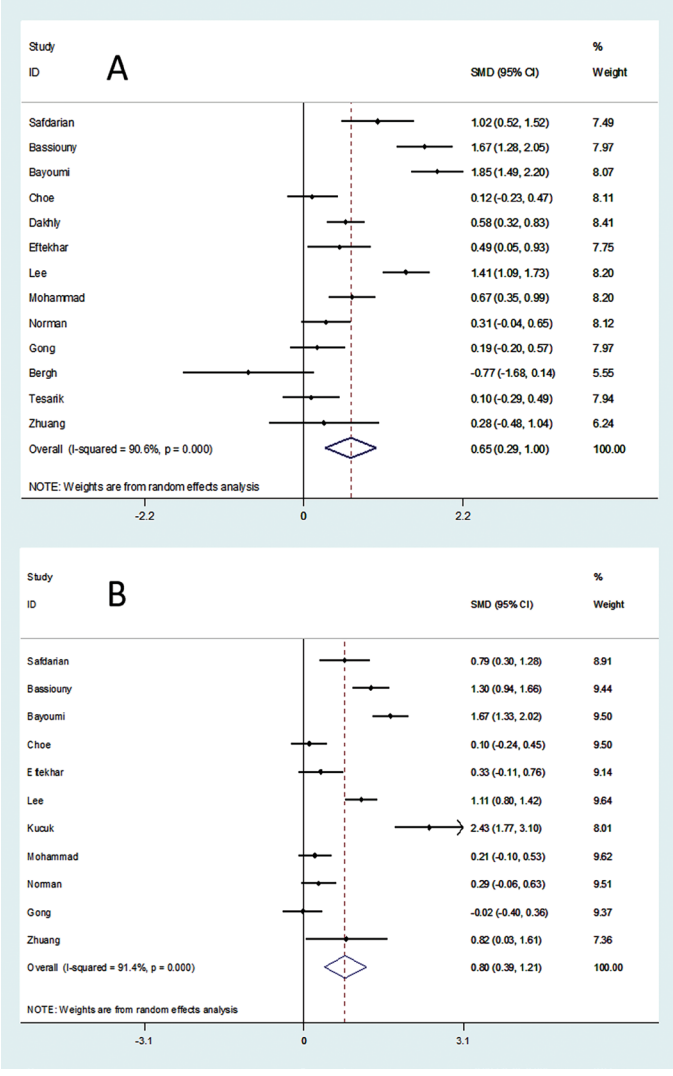

Figure 3. Forest plot for Secondary outcomes; A) Number of retrieved oocytes, B) Number of transferred embryo

CI: Confidence interval, SMD: Standardized mean difference

Total Dose of Gonadotropin

A total of 15 studies reported total dose of gonadotropin, four of which[29,32-34] did not provide standard deviation of data or lacked specific data required for a meta-analysis. The meta-analysis finally included 11 studies[5,16-18,22,24-28,31] for 1449 patients (716 in the GH group and 733 in the control group). The result indicated a significant decrease in the gonadotropin dosage with the administration of GH [SMD=-0.82, 95% CI=(-1.25, -0.39)]. A random effects model was applied due to the significant heterogeneity among these studies. (I2=92.8%), as shown in Figure 4A.

Duration of Gonadotropin Therapy

A total of 14 studies reported the duration of gonadotropin therapy, three of which[22,32,34] did not provide standard deviation of data or lacked specific data required for a meta-analysis. The meta-analysis finally included 11 studies[5,16-18,23-27,31,35] for 1478 patients (732 in the GH group and 746 in the control group). The findings showed a notable reduction in gonadotropin dosage with GH administration (SMD, -0.63, 95% CI, -1.04, -0.22). A random effects model was applied due to the significant heterogeneity among these studies (I2=91.7%), as shown in Figure 4B.

Peak E2 Level

A total of 14 studies reported peak E2 level. Three of which[32-34] did not provide standard deviation of data, Finally, 11 studies[5,16-18,22,23,26-28,30,35] including 1395 patients (691 in the GH group and 704 in the control group) were eligible for the meta-analysis. A significant increase in the level of peak E2 was observed in the GH group compared to the control group (SMD, 1.20; 95% CI, 0.59, 1.81), as shown in Figure 4C. However, significant heterogeneity was observed among these studies (I2=96.1%), therefore, the random effects model was applied.

Meta-regression Analysis

A meta-regression analysis was conducted to investigate whether the differences of reported outcomes in GH and control group were correlated to effectiveness of intervention in regards to cycle cancellation rate, pregnancy rate, and live birth rate. The findings of the meta-regression analysis indicated that there was no significant linear relationship between the effectiveness of intervention and any of the reported outcomes, including the number of retrieved oocytes, the total dose of gonadotropin, the duration of gonadotropin therapy, the number of transferred embryos, and the peak E2 level. Further details can be found in Table 3.

Sensitivity and Bias Analysis

The sensitivity analysis indicated that no single study or group of similar studies significantly affected the SMD and its corresponding CI, suggesting the overall findings are robust. Also, Egger's regression test, Begg's test, and funnel plot analysis were performed to detect publication bias in relation

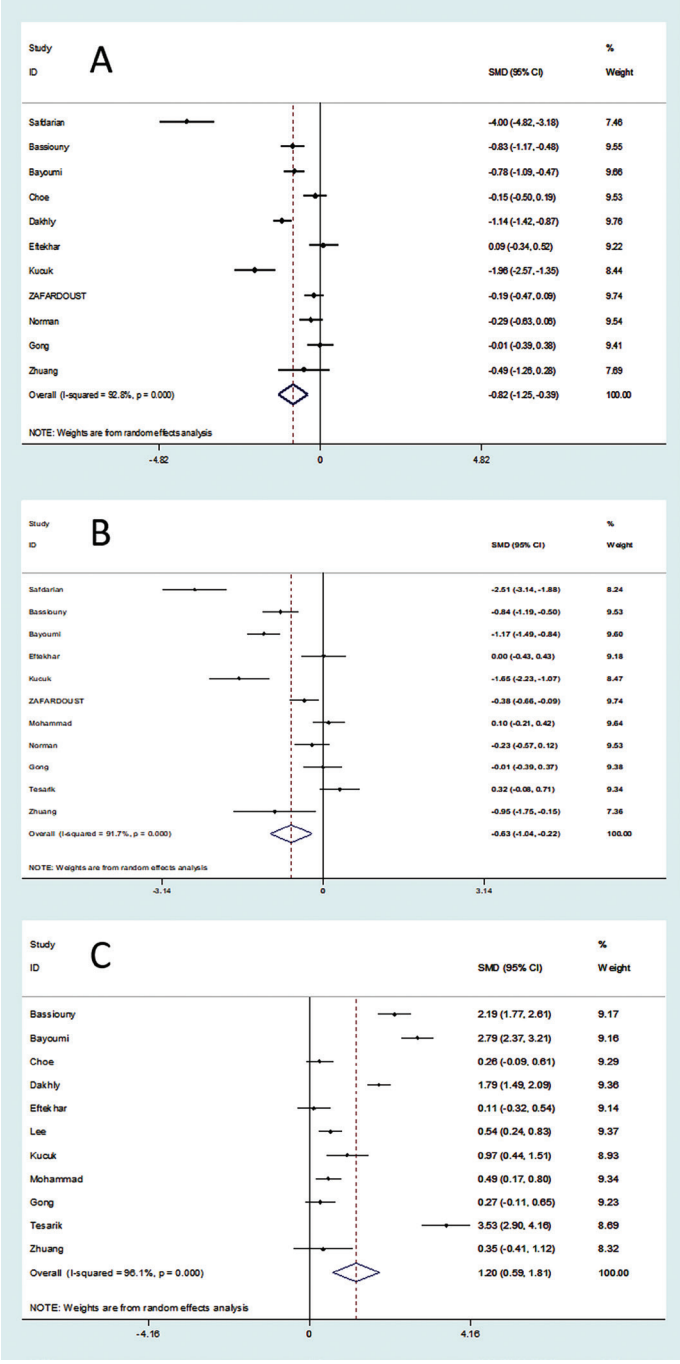

Figure 4. Forest plot for secondary outcomes; A) Total gonadotropin, B) Duration of gonadotropin therapy, C) Peak E2 level

CI: Confidence interval, SMD: Standardized mean difference

to the number of retrieved oocytes. Both Egger's regression test and Begg's test did not reveal any evidence of publication bias (p>0.1), and funnel plot analysis yielded a symmetric plot for the number of retrieved oocytes (Figure 5). Therefore, giving no indication of publication bias.

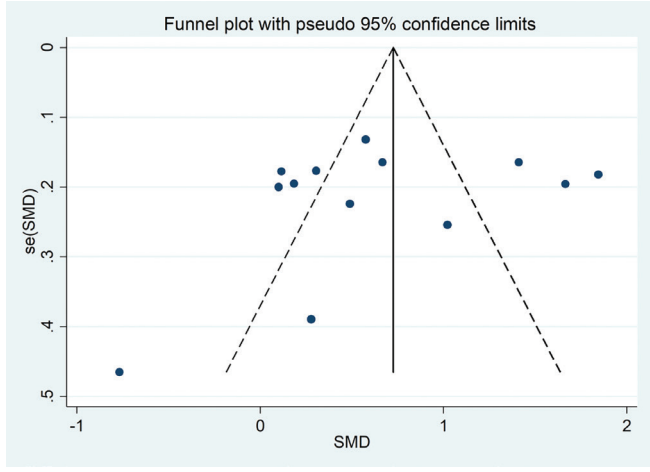

Figure 5. Funnel plot of the studies represented in the meta-analysis

SMD: Standardized mean difference

Discussion

This systematic review and meta-analysis included 18 studies involving 1870 participants. The findings revealed a significant correlation between the usage of GH and various factors, such as the number of retrieved oocytes, embryo transfer, total dose of gonadotropin, duration of gonadotropin therapy, and peak E2 level. However, there was no significant association between the improvement in these factors and the success of IVF, including LBR, pregnancy rate, and cycle cancellation rate. Several studies, consistent with our findings, demonstrated a concurrence regarding the number of retrieved oocytes[5,16-18,22-24]. GH is believed to play a crucial role in enhancing ovarian function by stimulating follicular growth, gametogenesis, increasing estrogen production, and promoting oocyte maturation[16,18,23]. It facilitates the growth of small follicles, increasing gonadotropin sensitivity, and reduces follicular degeneration and atresia before ovulation, thus improving ovulation[18]. Additionally, GH stimulates the synthesis of IGF-1 by influencing granulosa cells, which mediates the action of GH[24]. Lower doses of GH have shown more favorable responses on the ovary[30]. Although some studies did not support this outcome and did not report a significant relationship in terms of oocyte retrieval[29,31].

Another aspect investigated in this study was the number of transfer embryos, which exhibited a significant improvement, consistent with several other studies[18,22-24,30]. Nonetheless, conflicting results were reported in some studies[29,31].

Our study observed a correlation between the usage of GH and a decrease in the dose and duration of gonadotropin use, supported by several studies[5,16,17,22,31]. GH concentration in follicular fluid plays a role in enhancing ovarian response to gonadotropin[22]. Together with IGF-1, GH enhances the function of FSH, leading to a reduction in the required dose

and duration of gonadotropins[22,32]. However, this effect was more pronounced in patients with poor ovarian response, whereas patients with normal ovarian response required higher doses of gonadotropin, resulting in a less favorable response[32]. Taking a lower dose of GH has been reported to increase the need for gonadotropin and reduce ovarian response[30], while a higher dose of GH has the opposite effect[5]. Another mechanism that may explain this effect is the acceleration of ovarian follicle development and earlier oocyte production during GH administration, thereby reducing the need for higher gonadotropin doses[31].

GH induces changes in the hormonal profile of patients, with one of the most notable effects being the increase in peak E2 levels, supported by several studies[5,17,23]. Elevated E2 levels indicate an increase in the number of follicles stimulated by GH and, consequently, an increase in the number of produced oocytes[17]. It is proposed that GH may enhance the chance of pregnancy by elevating the E2 level in follicular fluid[5,23]; however, this outcome was not observed in our study.

Theoretically, GH can influence the pregnancy rate through various mechanisms, such as improving oocyte quantity and quality, enhancing embryo quantity and quality, increasing the number of transferable embryos, and promoting implantation potential[5,16,17,22]. GH may improve the success of embryo implantation by enhancing endometrial blood supply and increasing endometrial receptivity[30]. Even though a study by Safdarian et al.[24] reported a notably higher clinical pregnancy rate, although there was no significant difference in LBR. Nevertheless, our study, along with several others, supports the notion that improving these factors does not significantly impact the outcomes of IVF[16-18,22,28].GH consumption affect the quality and quantity of oocyte production but does not have significant correlation with pregnancy rate and LBR[16,17,22]. While the administration of a high dose of GH in the late luteal phase demonstrated an increase in the pregnancy rate in Kucuk et al.'s study[5], that was not statistically significant.

Overall, the effectiveness of GH is influenced by various factors, including the hormonal background, patient characteristics, and GH dosage[24]. GH deficiency may be a necessary condition for its effectiveness in IVF[24]. The timing of GH administration during the cycle is another influential factor[28]. It has been reported that the effect of GH administration is more pronounced in PORs compared to normal responders, particularly in terms of qualitative response rather than quantitative response[28]. Furthermore, increasing age leads to a decrease in GH, making older women with GH deficiency an ideal group to receive GH supplementation[22].

Study Limitations

This study encompassed diverse ethnic groups, thereby enhancing the generalizability of the results to other populations. However, our study had some limitations, too. Included studies had used various criteria for diagnosing POR, although the dominant method was the Bologna criterion. Also, some studies had small populations, which indicates the requirement of future studies with larger populations.

Conclusion

In summary, this systematic review and meta-analysis investigated the relationship between GH usage and factors related to IVF. The findings indicate a significant association between GH and improved outcomes such as the number of retrieved oocytes, transferable embryos, total gonadotropin dosage, duration of gonadotropin therapy, and peak E2 levels. However, despite these positive effects, there was no significant impact on the success of IVF in terms of LBR, pregnancy rate, and cycle cancellation rate. The effectiveness of GH appears to depend on individual factors and the timing and dosage of administration. Further research is needed to clarify its role in IVF outcomes and identify the specific patient groups that may benefit most from GH supplementation.

Ethics

Authorship Contributions

Concept: F.Z., N.D., Design: F.Z., A.A., N.D., Data Collection or Processing: Q.B., A.A., R.K., Analysis or Interpretation: F.Z., Q.B., Literature Search: R.P., F.A.M., Writing: F.Z., Q.B., R.P., A.A.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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DOI: 10.4274/tjod.galenos.2024.c001
Turk J Obstet Gynecol 2024;21:219

The study titled 'Effect of using magnifying loupe glasses on lymphocele formation and surgical outcomes in gynecologic oncology,' published in the Turkish Journal of Obstetrics and Gynecology 2019;16:255-9, was supported by the Scientific Research Commission of Süleyman Demirel University (Project No: BAP 4812-TU1-16). The authors thank for the financial support."

"The study titled 'Surgical risk assessment for gynecological oncologic patients,' published in the Turkish Journal of Obstetrics and Gynecology 2019;16:158-63, was supported by the Scientific Research Commission of Süleyman Demirel University (Project No: BAP 4813-TU1-16). The authors thank for the financial support."