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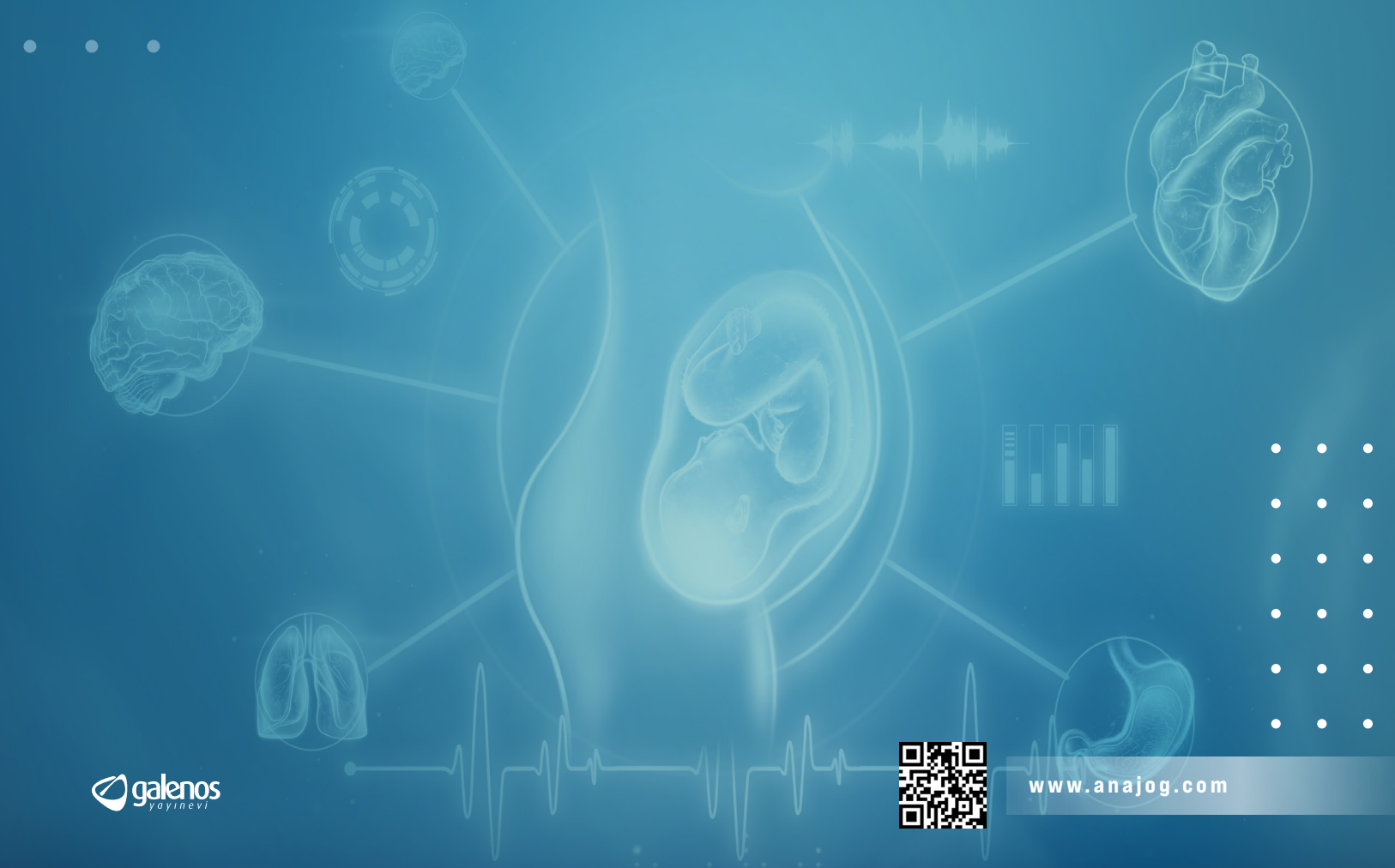

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Efficacy of Treatment of Cesarean Scar Ectopic Pregnancies with Alcohol Injection to the Gestational Sac and Dilatation Curettage with or without Systemic Methotrexate: A Case-Control Study

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ABSTRACT

Purpose: We aim to assess the effectiveness of treating cesarean scar ectopic pregnancies by injecting alcohol into the gestational sac (GS) and performing dilatation curettage with or without prior systemic methotrexate (MTX) administration.

Methods: A total of 37 patients were treated for cesarean scar pregnancy (CSP), 11 of which received systemic 75 mg MTX three days before local injection of 10% alcohol into the GS via 18G double lumen oocyte pick-up needle (Geotek, Ankara, Turkey) and 26 cases received local alcohol injection without prior MTX. Two or three days after the alcohol injection, the products of conception were removed again with a Karman cannula, and the β-hCG level was monitored weekly. After termination of CSP, the patients were followed up until they used contraception or delivered the following pregnancy.

Results: The MTX plus alcohol injection group and the alcohol injection alone group were compared. Significantly more women required Foley balloon tamponade 13 (50%), erythrocyte transfusion 13 (50%), and fresh frozen plasma infusion 9 (34.6%) in the local alcoholl injection alone group compared to the MTX plus alcohol group [n=1 (9.1%) p=0.01, n=1 (9.1%) p=0.01, n=0 p=0.02, respectively]. The mean resolution time of β-hCG was shorter in the MTX group [m=25±7.1 (18-48) and m=32.6±9.3 (22-58), p=0.01]; also; however, long hospitalization time was a disadvantage in this group. The recurrent CSP rate of 7.7% (n=2) was higher in the local alcohol injection alone group compared to nil in the MTX group. Cesarean niche surgery, abortion rate, and term pregnancy rates were similar in the two groups.

Conclusion: Although the efficacy of local alcohol injection alone is comparable to MTX plus alcohol injection, this group is at a disadvantage due to increased hemorrhage risk and the need for hemorrhage management. Local alcohol injection in combination with systemic MTX may be utilized as a good treatment option in patients.

Keywords: Cesarean scar pregnancy, methotrexate, alcohol, transvaginal ultrasound

INTRODUCTION

Cesarean scar pregnancies (CSPs) are a type of ectopic pregnancy located in a cesarean scar that has been experienced at least once before.[1,2] A gestational sac (GS) typically resides in the anterior uterine wall with a thinned myometrium between the sac and the bladder and an interruption in the anterior wall of the uterus next to the GS.[3] The prevalence of CSP has increased since the 2000s due to higher cesarean section rates,[4,5] changes in cesarean section techniques (one-layer technique, compared with the previous two-layer technique),[6] and improved ultrasound technologies for diagnosis.[7]

There are many ways to treat CSPs, as documented in the literature. These treatment modalities include expectant management, medical treatment by systemic methotrexate (MTX), medical treatment by systemic and local MTX, treatment by needle aspiration and local MTX, uterine curettage, hysteroscopy, resection of CSP through a transvaginal approach, uterine artery embolization (UAE), laparoscopy, and high-intensity focused ultrasound.[8-13] Various types of agents, such as potassium chloride (KCl), MTX, and vasopressin, have been experimented with for intragestational injection.[14-16] CSP can be a life-threatening condition if unrecognized and inadequately managed.

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Unfortunately, there is no definitive treatment with consensus in the literature.

In this study, we aim to assess the effectiveness of treating cesarean scar ectopic pregnancies by injecting alcohol into the GS and performing dilatation curettage two days apart.

METHODS

We conducted the study by retrospectively searching for patient data in the Consultant Clinic database from 2012 to 2020. Actually, all patients were followed up longitudinally between 2012-2020 in accordance with the principles outlined in the Declaration of Helsinki. Serum samples were collected at admission and during follow-up on the days specified.

During this study period, we did not aim to apply these methods on pregnancies older than ten weeks, and actually, no admissions later than ten weeks were observed in our clinic.

The diagnosis and management of CSP was conducted by the same perinatologist (EÇ). Voluson S8 5 MHz vaginal probe was used for the diagnosis of CSP. The diagnostic criteria were as follows: no content in the uterus and endocervical canal; identification of a GS and/or placenta in the area near the previous incision (hysterotomy scar or niche); a missing or slim layer of muscle tissue between the GS and the anterior uterine or bladder wall; and Doppler USG examination to determine rich peritrophoblastic blood flow around the GS.

The patients received 75 mg systemic MTX, three days before local alcohol injection if the gestational age was more than seven weeks or 49 days with positive fetal heart rate. A total of 37 patients were treated for CSP, 11 of which received systemic MTX before local injection of 10% alcohol into the GS via 18G double lumen oocyte pick-up needle (Geotek, Ankara, Turkey) under conscious sedation. Intravenous fentanyl 0.5-2 micrograms administered slowly in 25 microgram increments up to 75 micrograms were used for conscious sedation.[17] After vaginal disinfection with povidone-iodine, the needle was inserted through the anterior of the cervix between the uterus and the urinary bladder until puncturing the GS. The fluid inside the sac was aspirated first, and then 10% alcohol was injected instead of the fluid until the GS was fully dilated again. The patient was then followed up for two to three days, and the products of conception were removed again with a number 6 or seven Karman cannula (cervical dilation and manual vacuum aspiration: D&S) under conscious sedation and transabdominal ultrasound guidance. If bleeding is bright red and excessive, according to the managing perinatologist, Transamin 1 gr was administered, and/or Foley catheter size 16 was inserted into the uterine cavity; the Foley balloon was inflated with 3 mL of saline, and under transabdominal guidance, pulled back until the cesarean niche and then inflated up to 10-15 mL until the bleeding ceases. The balloon was kept in place for 24 hours and removed thereafter. All the procedures were performed in inpatient or outpatient settings according to the patient's preferences. Weekly β-hCG values were measured until the value dropped to non-pregnant reference values. The patients were followed up until the first spontaneous pregnancy after the termination of CSP. All women were then allowed to conceive spontaneously. All cases were followed until conception unless they decided to use contraception.

Statistical Analysis

Statistical analysis was used using the Statistical Program for Social Sciences (SPSS 20.0). Continuous variables between the two groups were compared using the Mann-Whitney U test. Categorical variables were compared using the chi-square test. A probability less than 0.05 was considered to be statistically significant. Continuous variables are presented in brackets as the mean and standard deviation of the mean and minimum-maximum values, and categorical variables are presented in brackets as numbers and percentages.

RESULTS

The mean age of the CSP patients was 34.2±4.2 (20-43) years, and the mean gestational age at diagnosis was 48.1±8.3 days. Twenty-two (59.5%) of the cases had one prior cesarean delivery, while 12 (32.4%) had two, and three cases (8.1%) had three cesarean deliveries before the scar pregnancy. The present CSP was achieved with intrauterine insemination in two (5.4%) and in vitro fertilization in four (10.8%) of the patients.

The distribution of selected variables and the outcome of the cases are presented in Table 1. The maternal age, gravida, parity, and number of prior cesarean deliveries were similar in the MTX plus alcohol injection group compared to the alcohol injection alone group. In accordance with selection criteria for inclusion in the MTX plus alcohol group, the mean gestational age 56±3.6 (52-61) and β-hCG at admission was higher 11605±5785 (4350-22748) compared to the alcohol injection alone group [m=44.3±3.7 (35-49), p<0.001; m=9043±5609 (1750-24857), p=0.03 respectively]. Significantly more women required Foley balloon tamponade 13 (50%), erythrocyte transfusion 13 (50%), fresh frozen plasma infusion 9 (34.6%) in the local alcohol injection group compared to MTX plus alcohol group [n=1 (9.1%) p=0.01, n=1 (9.1%) p=0.01, n=0 p=0.02 respectively]. Hospitalization time was longer in the systemic MTX plus local alcohol injection group, as most patients were hospitalized for MTX injection until local alcohol injection (Table 1). The mean resolution time of β-hCG to undetectable levels was shorter in the MTX plus local alcohol injection group compared to the local alcohol injection alone group [m=25±7.1 (18-48) and m=32.6±9.3 (22-58), p=0.01].

Follow-up of the patients and pregnancy outcome after resolution of CSP is presented in Table 2. Eleven cases had cesarean niche surgery, seven with laparoscopy, and four with laparotomy due to inability to conceive within a year or symptomatic menstrual spotting. Two cases had recurrent CSP in the Local Alcohol injection alone group compared to none in the MTX plus local alcohol injection group. A total of four (10.8%) patients had abortions, and fifteen patients (40.5%) had term pregnancies during follow-up. The distribution of pregnancy outcomes among the treatment groups was similar.

DISCUSSION

We found that local alcohol injection into the GS of the CSP by adding MTX in pregnancies more than seven completed weeks of pregnancy is effective. However, in our study, Foley balloon tamponade was needed to stop bleeding, and transfusion of erythrocyte suspension and fresh frozen plasma were more frequent in the local alcohol injection alone group.

Prenatal diagnosis of CSP is very important; it can be confused with missed/incomplete miscarriage or simply intrauterine pregnancies. This can be followed without intervention or with sharp curettage intervention, so it may cause complications such as heavy bleeding and uterine rupture.[18] Poor management of CSP can lead to severe life-threatening conditions such as hemorrhage, uterine rupture, hysterectomy, third-trimester bleeding, maternal death, and the occurrence of an abnormally invasive placenta.[19] There is no definitive consensus on the optimal CSP treatment modality and no guidelines on which patients should be treated and how. As a result, clinicians have experimented with various treatment modalities.

According to a systematic review, which evaluated 2,037 women in fifty-two studies, Treatment for CSPs should be interventional rather than medical. The review also recommended treatment options for CSP based on their efficacy and safety for clinical practice.[20]

Bağlı et al.[11] evaluated the efficacy of suction curettage (SC) as an effective treatment alternative for CSPs. Of 36 patients, 31 had favorable results with SC ± Foley balloon tamponade with a success rate of 86% (31/36). They reported that; this success is not related to the presence of an embryonic pole and fetal cardiac activity also, initial β-hCG levels, and a history of vaginal delivery. However, myometrial thickness was significantly depressed in the failed group (p=0.033).[11]

In some cases, researchers have attempted to use expectant management as a method of treatment for patients with a progressing pregnancy that eventually leads to a viable birth.[21] Silva et al.[22] published a systematic review including 47 studies on the expectant management of CSP. Miscarriage occurred in 20.1% of pregnancies, while 8.3% experienced fetal death. Only 25% of pregnancies lasted to term, while 41.8% were preterm, and 13.9% were born before 34 weeks. Also, In 52.6% of patients, a hysterectomy was performed. All cases had antenatal suspicion for placenta accreta spectrum and were later confirmed as placenta increta or percreta.[22]

It is widely known that MTX works by stopping the production of DNA at different points in the cell cycle. As a result, it causes

Table 1. Distribution of the selected clinical variables in cesarean scar ectopic pregnancies with respect to management groups

Variable	Systemic methotrexate plus local alcohol injection n=11	Local alcohol injection alone n=26	p-value
Maternal age (y)	34.1±2.6 (30-39)	34.3±4.8 (20-43)	0.56
Gravida	3.7±1.6 (2-7)	3.3±1.6 (2-10)	0.56
Parity	2.1±1.5 (1-5)	1.6±0.69 (1-3)	0.52
Prior cesarean delivery	1.4±0.68 (1-3)	1.5±0.64	0.83
Gestational age at admission (days)	56±3.6 (52-61)	44.3±3.7 (35-49)	<0.001**
Initial beta-hCG (IU/mL)	11605±5785 (4350-22748)	9043±5609 (1750-24857)	0.03**
Fetal cardiac activity	9 (81.8%)	16 (61.5%)	0.22
Transamin 1 gr	4 (36.4%)	13 (50%)	0.44
Foley baloon tamponade	1 (9.1%)	13 (50%)	0.01*
Any erythrocyte suspension	1 (9.1%)	13 (50%)	0.01*
Any fresh frozen plasma	0	9 (34.6%)	0.02*
Hospitalisation time (days)	7.7±5.4 (0-16)	3.3±3.1 (0-13)	0.01**
Resolution time(d)	25±7.1 (18-48)	32.6±9.3 (22-58)	0.01**

*: Statistically significant, chi-square test, p<0.05
**: Statistically significant, Mann-Whitney U test, p<0.05

Table 2. Follow-up of the patients and pregnancy outcome after resolution of cesarean scar pregnancy

Variable	Systemic methotrexate plus local alcohol injection n=11	Local alcohol injection alone n=26	p-value
Cesarean niche surgery	1 (9.1%)	10 (38.5%)	0.07
Future pregnancy outcome Contraceptive use Cesarean scar pregnacy Abortion Term pregancy	 5 (45.5%) 0 1 (9.1%) 5 (45.5%)	 11 (42.3%) 2 (7.7) 3 (11.5%) 10 (38.5%)	0.8

the death of cells that divide rapidly and trophoblast cells.[23] This particular mechanism makes MTX an effective treatment for a type of ectopic pregnancy known as CSP.

Heidar et al.[24] conducted a study on the effectiveness of systemic and/or local MTX treatment. The study evaluated four cases. A single dose of systemic MTX treatment was effective in two cases. However, in two other cases, the β-hCG level increased after a single dose of systemic MTX administration; for these cases, multiple doses of MTX were used; in addition to systemic administration, MTX was also injected into the GS. They emphasized that medical management alone can successfully treat CSP diagnosed at early gestation, with an additional injection into the sac required if primary treatment fails.[24]

Al-Jaroudi et al.[8] shared their experiences from a single center on various treatment options for CSP. These options included systemic MTX [n=14 (51.85%)]; intra-sac and systemic MTX [n=3 (11.1%)]; intra-cardiac KCl along with systemic MTX [n=2 (7.4%)]; expectant management [n=5 (18.51%)]; laparotomy wedge resection (n=1); UAE and systemic MTX (n=1). They find that first-line treatment success is 74.07% (n=20). They did not observe any side effects in the MTX group. No significant correlation was found between the time it took to resolve β-hCG and the chosen treatment methods (p=0.58).[8]

Bağlı et al.[11] retrospectively examined 36 patients with CSP treated solely by SC and found that a Foley catheter was needed in 23 patients (n=23/36, 63.8%). In our study, this rate was only 9.1% (n=1) in the group to which we added systemic MTX. Also, they reported that blood products were required in four patients (4/36, 11.1%) in their study, while only one patient (9.1%) received ERT and no fresh frozen plasma in the systemic MTX-added group of our study. In addition, they performed laparotomy on two patients due to hemodynamic instability, but it was not necessary for us. Adding systemic MTX to D&S treatment appears to reduce bleeding, treatment needs, and complication rates related to bleeding. Moreover, In a systematic review, Kanat-Pektas et al.[25] reported that the hysterectomy rates were higher in CSP cases treated with the D&S group than with the systemic MTX group (7.3% vs. 3.6%, respectively). Because MTX inhibits folic acid synthesis and new purines and pyrimidines, the synthesis of DNA and cell proliferation are destroyed. Tissues with high cell turnover, such as pregnancy products, are particularly prone to experiencing these effects, so MTX causes the death of trophoblasts. This death may cause thrombosis in the vessels feeding the product of conception, which may explain the lesser amount of bleeding if MTX is added to the treatment.

Heidar et al.[24] published four CSP patients who were treated with systemic MTX + local MTX/KCl. They didn't see any bleeding complications. Weeks later, they removed remnants of tissues by hysteroscopy.[24]

Giampaolino et al.[26] shared their experiences of 45 cases retrospectively. The patients were treated with five different approaches: Expectant management, Hysteroscopic resection, UAE + D&S, UAE and surgical laparotomic resection, systemic MTX + D&S. The group with the highest complication (profuse bleeding, hematoma, myometrial infarction), rate with a statistically significant difference was the UAE + D&S group (p≤0.001). No complications were observed in the MTX + D&S group.[26]

Like our study, bleeding-related complications were rare in the MTX + D&S group. A single dose of 50 mg MTX was administered, and after that, following 48 hours, the manual vacuum aspiration with a Karman cannula (D&S). This 48-hour period appears to be crucial for trophoblast death, leading to thrombosis. On the other hand, Huo et al.,[27] in their 11-year experience, declared that patients with a history of treatment for CSP using systemic or local and systemic MTX are more likely to develop persistent scar pregnancy and also have a higher risk of bleeding during subsequent surgery. However, they didn't combine the MTX treatment with the D&S, which could explain the disadvantages of MTX treatment.

Ge et al.[9] attempted to treat CSP using both intrachorial and systemic MTX applications, and they successfully treated 8 out of 11 patients (72.7%). However, our study showed a 100% treatment success rate. D&S was additionally required in two patients, and in one patient, UAE was needed.[9]

Although injecting MTX into the GS along with systemic MTX treatment seems like a reasonable approach to treating CSP, our study found that there were no observed side effects in patients who received systemic MTX in combination with local alcohol injection into the GS. In contrast, two patients experienced MTX side effects in that study, possibly due to an increase in the total MTX dose administered.[9]

In terms of hospitalization time, the systemic MTX plus local alcohol injection group seems to be more disadvantaged than the local alcohol injection alone group in our study (7.7±5.4 vs. 3.3±3.1, p=0.01, respectively). On the other hand, the resolution time is shorter than the local alcohol injection alone group (25±7.1 vs. 32.6±9.3, p=0.01, respectively). The use of MTX has been linked to a decrease in the time required for hCG remission and the disappearance of cesarean scar masses.[28]

Treatment choice for CSP is important not only in terms of bleeding complications but also in terms of how it affects the patient's fertility in the future. Unfortunately, very few studies in the literature have followed cases in terms of fertility outcomes.

Qian et al.,[29] 24h after UAE, compared D&S (n=33) and operative hysteroscopy (n=33) in the treatment of CSP, and they didn't find a significant difference in the intrauterine pregnancies after surgeries between the two groups (p=1.000) in the 12 months following. According to a report by Gundewar et al.,[30] three of four patients who desired to conceive were able to do so naturally after undergoing intra-sac KCl and MTX treatment. Likewise, in a study, among 13 cases treated with systemic MTX, three out of four patients who wanted to conceive were able to have a successful pregnancy.[31] Our study group that received systemic MTX showed similar results.

Qian et al.[29] found recurrent CSP was seen in one of 33 patients in the HS group, while none was in the D&S group. In a study of patients with CSP treated only with local MTX, 5 of 8 CSP cases desired subsequent pregnancies. Four healthy

pregnancies were observed, but one had recurrent CSP.[32] In our study, although the rate of recurrent CSP in patients who received local alcohol injection treatment was quite low (n=2/15), adding MTX to the treatment reduced recurrent CSP rates.

Insufficient literature data make it impossible to comment on subsequent pregnancy outcomes and recurrent CSP rates. However, our study is important because it includes subsequent pregnancy data for all treated CSP cases.

Although, in our study, there is no statistically significant difference between the groups, systemic MTX plus local alcohol injection group seems to be more advantageous in terms of future pregnancy outcomes like CSP, abortion, and term pregnancy. This may be due to the low number of patients in the systemic MTX plus local alcohol injection group.

Unfortunately, after the CSP treatment, there is insufficient data in the literature about abortion rates and the need for niche surgery, so it is impossible to comment on the effects of treatment options on these outcomes.

The literature widely discusses several factors that can affect the success of treatment methods used to treat CSP. These factors include the initial β-hCG level, fetal cardiac activity at the time of diagnosis, the location of scar pregnancy, and the thickness of the lower uterine segment myometrium. However, it is unknown which factors significantly impact the treatment's effectiveness, and there are no consensus cut-off values.

While our study's strength is that it follows our patients after CSP treatment and includes subsequent pregnancy data, the number of cases in our study is entirely satisfactory despite most publications on CSP treatment having limited case numbers.

Since not all treated patients plan pregnancy after treatment, it is pretty restrictive to comment on the effect of the treatment options on resulting in abortion or achieving term pregnancy. Although this is a limiting aspect of our study, it contains more data than many studies in the literature on this subject.

Subsequent pregnancy outcomes after treatment can be determined more clearly in larger case series in which systemic MTX is added to treatment.

CONCLUSION

In conclusion, adding systemic MTX to D&S treatment can reduce bleeding, bleeding-related treatment needs, and associated complication rates, which in turn helps lower treatment expenses.

Although MTX treatment can be effective, the fibrous tissue circumambient of the GS in systemic administration could limit exposure to the trophoblast.[33] Therefore, local administration of alcohol directly to the GS is necessary. Particularly in the later stages of pregnancy, combining a local injection of medication with aspiration is a more appropriate strategy.[34]

Local alcohol injection in combination with systemic MTX may be utilized as a good treatment option in patients where surgery is not a viable choice.

Ethics

Ethics Committee Approval: As the data was collected retrospectively, there is no requirement for approval from the ethics committee. All patients were followed up longitudinally between 2012-2020 in accordance with the principles outlined in the Declaration of Helsinki.

Informed Consent: Retrospectively study.

Authorship Contributions

Surgical and Medical Practices: B.A., Concept: H.Ş.S., Design: H.Ş.S., Data Collection or Processing: H.Ş.S., E.Y., N.A., B.A., Analysis or Interpretation: H.Ş.S., Literature Search: H.Ş.S., E.Y., N.A., Writing: H.Ş.S.

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Original Article

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Biomechanical Properties of the Umbilical Cord and Its Relationship with Perinatal Outcomes

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ABSTRACT

Purpose: The aim of this study was to determine the elasticity modulus of umbilical cord (UC) using biomechanical tests in diabetic, preeclamptic and control groups and to investigate the relationship with perinatal outcomes.

Methods: Patient data from diabetic, preeclamptic and healthy control group women, who gave birth in a single center between September and December 2019 were collected. Prenatal demographic data, pregnancy outcome, and ultrasound Doppler pulsatility index (PI) was obtained. Cord samples were taken at birth and newborn morphometric parameters were measured. The diameter of UCs were measured. The samples then underwent biomechanical testing. By calculating strain and stress, the elasticity modulus of samples were derived.

Results: There were thirty subjects in each group. Mean UC radius was significantly greater ($p<0.01$) in the diabetic group (1.03 ± 0.29 cms) compared to control group (0.86 ± 0.21 cms) and preeclamptic group (0.74 ± 0.14 cms). Median (range) elasticity modulus was highest in the preeclamptic vs. the diabetic and control groups [0.28 (0.22-0.34) vs. 0.12 (0.8-0.30) vs. 0.14 (0.12-0.34), respectively; $p<0.01$]. Increase in birth week ($r=-0.26$, $p=0.01$), birthweight ($r=-0.42$, $p<0.01$), newborn height ($r=-0.38$, $p<0.01$), and UC diameter ($r=-0.78$, $p<0.01$) were all negatively correlated with elasticity modulus. Umbilical artery Doppler PI values had weak positive correlation with elasticity modulus ($r=0.21$, $p=0.4$).

Conclusion: Morphological, mechanical and histological studies were performed on the UC. It appears that the UC its characteristics are changed in disease processes affecting pregnancy. We believe that if ultrasonographic, histological, biochemical and immunohistochemical data are combined with biomechanical data, larger serial studies may provide new parameters with which we can evaluate fetal well-being based on UC characteristics.

Keywords: Umbilical cord, preeclampsia, diabetes, elasticity

INTRODUCTION

The umbilical cord (UC) is a structure that provides the vital connection between the fetus and the mother.[1] The placental-fetal relationship is conducted through the UC. The UC continues develops from the third week of embryonic life until the twelfth week and, starting from the first trimester, the UC can be visualized through ultrasonography, generally for its entire length. The primitive umbilical ring, the precursor of the primitive UC, originates from the ventral reflection line of the amnio-ectodermal junction.[1] It is a mesoblastic structure approximately 50-60 cm long and 1.5-2 cm thick at term.[2]

In section, the UC contains two umbilical arteries (UA) transferring fetal blood to the placenta, an umbilical vein (UV) transferring oxygenated blood from the placenta to the fetus, and the amnion membrane around the outside. In addition, within the lamellar structure filling the inside of the cord, there is the tissue known as Wharton's jelly (WJ), which consists of structural support from the mesodermal formation and connective tissue.[3] The UC usually contains 10-11 full turns from the fetus to the placental insertion site.[1,2] The UC, a vital component of the fetoplacental unit, is the only structure that plays a decisive role in the beginning of extrauterine life, but is unnecessary after life begins.[2] Evidence obtained through

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clinical experience and experiments has shown that the morphology and components of the UC affect the course of pregnancy, mode of delivery and pregnancy outcome.[4-6]

As anatomical, histological, and ultrasonographic studies, and thus UC-related data, has increased, the importance of the UC has became more clear. The relationship between sonographic UC thickness and fetal growth in early trimesters has been recently published. Many groups have reported that altered UC morphology in the second and third trimesters is associated with poor perinatal outcomes, including fetal distress, fetal growth restriction, gestational diabetes, hypertensive disorders, intrapartum complications, and altered UV blood flow.[3-7] In addition, in the second trimester, the presence of a thin UC is associated with the fetus having a lower birth weight for gestational age and being more likely to show signs of distress during birth.[8,9] Studies conducted in the second trimester have concluded that the interrelationship of umbilical vessels and WJ components may affect pregnancy prognosis.[4,7-9]

Studies have shown that UC diameter and UC area is correlated with fetal macrosomia, fetal weight and fetal biometric parameters.[6,10,11] The results of these studies indicated the importance of UC morphometry in terms of possible risks to the fetus during pregnancy and birth in diabetic pregnancies.

Studies conducted with pregnant women diagnosed with intrauterine growth restriction (IUGR) and preeclampsia showed a decrease in the area of WJ and umbilical vessels as the earliest finding.[5,12] Importantly, these changes are present in the absence of fetal growth disorders and altered UA Doppler parameters. It was reported that in fetuses in pregnancies affected by preeclampsia and in IUGR fetuses, the decrease in umbilical vessel area may be due to vasoconstriction of these vessels through an altered function of locally acting factors. Since human UC vessels lack neural innervation, the action of vasoactive substances may be crucial for vascular control of the UC.

Comparison of clinical outcomes and UC thickness measured ultrasonographically and pathologically has shown that the diameter of the UC and the arteries and veins within it are important indicators of the intrauterine development of the fetus, birth and perinatal complications and general well-being.[13]

The current study aimed to identify the relationship between the diameter and biomechanical properties of the cord and perinatal outcomes, based on the assumption that the UC is a structure that surrounds the outside of the artery and vein that circulate oxygenated blood, providing nutrition to the fetus, and also creates resistance, and that such measurements can provide information for the evaluation of fetal development and fetal well-being in the intrauterine period.

METHODS

In the present study, the UCs of patients who gave birth in the 22nd-41st week of pregnancy in a single center between September 2019 and December 2019 were prospectively evaluated. After obtaining consent from the patients, UC sections were prepared. These sections were divided into three groups: samples from diabetic pregnant women, preeclamptic pregnant women and healthy pregnant women without known additional diseases. Ethical committee approval was received from the local ethics committee for the study.

The inclusion criteria were patients who gave birth to a healthy baby weighing over 500 grams between 22-41 weeks of gestation; diabetic pregnant women, pregnant women diagnosed with preeclampsia and healthy pregnant women without comorbidities; babies without genetic or systemic abnormalities; no high blood pressure (systolic <140 mmHg, diastolic <90 mmHg) in the diabetic group; normal UC morphology (two arteries and one vein); and singleton pregnancy. Exclusion criteria were multiple pregnancies; maternal infection, preterm rupture of membranes, latent labor phase, pregnancies with a single umbilical artery; and those who were diabetic and also developed preeclampsia or high blood pressure.

Demographic characteristics, physical examination results, umbilical artery findings of Doppler ultrasonography, laboratory values such as hemoglobin level and blood gas pH and birth records of the patients were recorded. Obstetric ultrasonographic examination of patients before birth were recorded and umbilical artery pulsatility index (PI) values were measured. umbilical artery blood gas sampling values at 0 minutes postpartum were noted. First and five-minute Apgar scores, birth weight and length were noted. Information on whether there was a need for neonatal intensive care unit admission in the postnatal period was noted.

During birth, 10 cm of the UC was cut in the delivery room and instantly stored in 5% formaldehyde solution. Each UC sample was soaked in formaldehyde for approximately 20 days and then taken to the biomechanics laboratory to be studied.

To determine the UC thickness, the circumference of the cord was measured, and the radius value was used by calculating r (radius) with the formula $2\pi r$. The cord area was calculated with the formula πr^2 (mm^2) and used in the elasticity modulus calculation.

Each cord was subjected to uniaxial pulling without undergoing any physical processing in the biomechanics laboratory. All cords were tested at room temperature (22 °C) at single tension, parallel to the long axis of each sample. Tensile testing was performed for each cord sample using a universal testing machine and the load was measured with a 20kN load cell. The samples were placed on both jaws of the testing device from both ends and an average distance of 2 cm was set between them. A preload tension of 5 N was applied to pre-stress the loose cords and remove any residual mis-shappeness when placed between the jaws. For stress-strain testing, samples were tested at a displacement rate of 60 mm/minute until failure. Displacement (mm) and load (maximum force, N) of the cords were obtained from the test device. A force-displacement curve was obtained for each tensile test performed.

To calculate the elastic modulus, all samples were examined by the same scientist, under the same room conditions, and at postpartum 20th day to avoid data error. The stress-strain diagram of each tested sample was obtained, and the

tangent modulus and elastic modulus were calculated. While calculating the elasticity modulus, the unit displacement value (ε) in the formula was taken as the distance (mm) extended by the cord in our tensile test. The tension (б) was taken as the force [1 Megapascal (MPa)=1 newton per square millimeter] at the moment of breaking of the cord. The elasticity module value was found for each data using the formula E=б/ε.

Statistical Analysis

Statistical evaluation was performed with SPSS, version 23.0 (IBM Corp., Armonk, NY, USA). The conformity to normal distribution was evaluated with the Shapiro-Wilk test. Numerical variables with normal distribution (age, height, fasting blood sugar value, cord diameter) are shown as mean ± standard deviation while numerical variables that do not show normal distribution (gestational age, weight, body mass index, birth length, hemoglobin, blood pressure, hemoglobin A1c (HbA1c), birth weight, umbilical artery Doppler PI, proteinuria, maximum cord force value, cord displacement, and elasticity value) are shown as median (interquartile range). Finally, categorical variables are given as frequency (%). For normally distributed numerical variables, the difference between groups was analyzed by One-Way Analysis of Variance (ANOVA) and Tukey's multiple comparison test. Non-parametric data sets were compared using the Kruskal-Wallis, ANOVA and Dunn's multiple comparison tests. The Yates and Monte Carlo chi-square test were used for categorical variables. A $p<0.05$ was considered sufficient for statistical significance.

RESULTS

Samples were taken from 90 patients, including 30 women with gestational diabetes, 30 preeclamptic pregnant women and 30 healthy pregnant women and the elastic modulus of all samples were calculated by the same scientist, under the same room conditions, and at postpartum 20th day to avoid data error.

Demographic characteristics of the women are presented in Table 1. The preeclamptic women were significantly younger than the women in the diabetic group ($p=0.01$). Gestational age at birth was significantly earlier in the preeclamptic group compared to both control group and diabetic group ($p<0.01$). Maternal weight of the diabetic group was significantly heavier in the diabetic group than the preeclamptic and control groups ($p<0.01$).

Comparisons of the fetal parameters between the study groups are given in Table 2. First minute Apgar scores, fifth minute Apgar scores, mean birth weight, and mean fetal birth length of the preeclamptic group were significantly lower than the diabetic and control groups ($p<0.01$ for all).

Comparison of UC radii, umbilical artery PI, strain, maximum stress and modulus of elasticity values are presented in

Table 1. Demographic characteristics of patients according to groups

	Diabetic (n=30)	Preeclamptic (n=30)	Control (n=30)	p-value
Age	33.1±5.1	28.9±5.3	30.7±5.6	**0.01**θ
Birth week	37 (36-38)	35 (28-36)	38 (37-39)	**<0.01**θμ
Gravida	3 (2-3)	2 (1-3)	2 (1-3)	0.13
Parity	1 (0.75-2)	0 (0-2)	1 (0-2)	0.19
Abortus	0 (0-1)	0 (0-0.25)	0 (0-0)	0.54
Living child	1 (0-2)	0.5 (0-1.2)	1 (0-2)	0.52
Height	1.62±5.84	1.61±5.50	1.63±6.40	0.32
Weight	86 (76-100)	73 (66-83)	75 (67-87)	**<0.01**θł
BMI	33.5 (23-49)	28.9 (25.3-32.1)	28.3 (24.8-34.0)	**<0.01**θł

θ: There is significant difference difference between diabetes and preeclampsia. One-Way ANOVA, Tukey test.
ł: There is significant difference between diabetes and control group One-Way ANOVA, Tukey test.
μ: There is significant difference between preeclampsia and control group. One-Way ANOVA, Tukey test.
BMI: Body mass index

Table 2. Comparison of fetal parameters between the study groups

	Diabetic	Preeclamptic	Control	p-value
Apgar first minute	8 (7-8)	7 (4-7)	8 (7-8)	**<0.01**θμ
Apgar fifth minute	9 (9-9)	8 (7-9)	9 (9-9)	**<0.01**θμ
Birth weight (g)	3178±599	2194±1042	3206±554	**<0.01**θμł
Birth length (cm)	50 (49-51)	45 (38-48)	50 (48-51)	**<0.01**θμ
Haemoglobin (g/dL)	18.4±1.4	17.1±2.8	18.4±2.2	0.09
Blood gas pH	7.35 (7.32-7.37)	7.33 (7.29-7.38)	7.37 (7.33-7.38)	0.078

θ: There is a difference between diabetes and preeclampsia. One-Way ANOVA, Tukey test.
ł: There is a difference between diabetes and control group. One-Way ANOVA, Tukey test.
μ: There is a difference between preeclampsia and control group. One-Way ANOVA, Tukey test.

Table 3. Umbilical artery Doppler PI values of the patients were compared using prenatal US images. The median (range) PI was 1.07 (088-1.28) in the preeclampsia group, 0.80 (0.73-0.94) in the diabetic group, and 0.7 (0.58-0.87) in the control group. PI was significantly greater in preeclamptic pregnant women ($p<0.01$). The smallest radius of UC was observed in the preeclamptic group. The mean value was found to be 0.74±0.14 cm. The average value of the control group was 0.86±0.21 cm, the diabetic group was 1.03±0.29 cm, and it was statistically significant that the cord radius of diabetic patients increased, and the cord was thinner in preeclampsia ($p<0.01$).

The elasticity modulus values were compared between groups. These values were found to be 0.12 (0.8-0.30) mPa/mm (in the diabetic group, 0.28 (0.22-0.34) mPa/mm in the preeclamptic group, and 0.14 (0.12-0.34) mPa/mm in the control group. A higher value for elasticity modulus indicates less flexibility and greater fragility, and this value was significantly lower in the preeclampsia group compared to the diabetic and control groups ($p<0.01$). No difference was found between the diabetic and the control groups ($p=0.84$). Maximum stress value was lowest and maximum stress value was highest in preeclamptic group compared to diabetic and control groups, but it was not significantly different.

Every unit increase in birth week ($r=-0.26$, $p=0.01$), birthweight ($r=-0.42$, $p<0.01$), newborn length ($r=-0.38$, $p<0.01$), and UC diameter ($r=-0.78$, $p<0.01$) was negatively correlated with elasticity modulus. UA Doppler PI values had a weak positive correlation with the elasticity modulus ($r=0.21$, $p=0.4$).

When correlation analysis was conducted in the preeclamptic group, birth week ($r=-0.37$, $p=0.03$), birthweight ($r=-0.542$, $p<0.01$), newborn length ($r=-0.60$, $p<0.01$), and UC diameter ($r=-0.55$, $p<0.01$) were negatively correlated with elasticity modulus. However, in this group UA Doppler PI values did not show correlation with elasticity modulus.

DISCUSSION

The UC is one of the most important parameters that indicates us the welfare of fetal life.

In biomedical and multidisciplinary studies have shown that differences from the norm of the UC and its components will have an effect on the pregnancy process and neonatal outcomes.[4-6] Intrauterine loss, gestational diabetes, preeclampsia, intrauterine growth retardation, fetal distress during birth, and the relationship between fetuses with meconium and the UC have recently attracted the attention and interest of many researchers.[4-7,14] Multisystemic diseases such as preeclampsia and diabetes remain the main focus of studies. It is undeniable that there are important conditions in the perinatal period that can negatively affect both fetal life and maternal life. As has been shown, biomolecular structures in the cord structure cause changes in the cords by affecting their histological, biomechanical and anatomical properties.[15]

Raio et al.[4] found that the rate of low birth weight and fetal distress increased depending on the gestational week in fetuses with a thin UC. They suggested that the presence of a thin UC be used as a marker for low birth weight and fetal distress. Goodlin reported that babies who underwent caesarean section due to fetal distress and also had meconium had a thinner UC.[7]

In contrast, Ghezzi and Weissman, investigated the relationship between thick UC, gestational diabetes and macrosomia and reported that the birth weight was higher in fetuses with thick UC. They suggested that the thickness of the UC increased significantly in patients with gestational diabetes compared to the control group, and that gestational diabetes should be investigated in pregnant women whose UC was measured to be thick.[6,10] In the current study, cord thickness was significantly higher in diabetic pregnant women ($p<0.01$).

Although there was a significant relationship between cord components and fetal macrosomia, no difference was observed between the diabetic group and the non-diabetic control group. Cromi et al.[10] stated that the cord area, and especially the WJ area, was larger in diabetic pregnant women than in non-diabetic pregnant women, and especially in those who give birth to macrosomic babies. Similarly, in the study conducted by Weissman and Jakobi,[6] it was concluded that there was a significant association between birth weight and cord radius in diabetic pregnant women it was suggested that by combining these two variables, macrosomic fetuses would be predicted with a high degree of accuracy.[3,6] In our study, there was a significant positive correlation between cord radius and birth weight in diabetic pregnant women ($p<0.05$).

Barbieri et al.[16] constructed reference curves for the WJ area in low-risk pregnancies between 13-40 weeks and its relationship with estimated fetal weight (EFW) was evaluated. In 2,189 low-risk pregnancies, estimated WJ area was calculated as

Table 3. Comparison of umbilical cord radii, umblical artery pulsatility index and modulus of elasticity

	Diabetic	Preeclamptic	Control	p-value
Umbilical artery PI	0.84±0.18	1.09±0.36	0.71±0.14	**<0.01**[θm]
Cord radius (cm)	1.03±0.29	0.74±0.14	0.86±0.21	**<0.01**[θǂ]
Strain (mm)	28.7±9	31±9.3	26.3±9.9	**0.1**
Maximum stress (mPa)	61±16.8	54.3±17	62±18	**0.1**
Elasticity modulus	0.12 (0.8-0.30)	0.28 (0.22-0.34)	0.14 (0.12-0.34)	**<0.01**[θm]

[θ]: There is a difference between diabetes and preeclampsia. One-Way ANOVA, Tukey test.
[ǂ]: There is a difference between diabetes and control group. One-Way ANOVA, Tukey test.
[m]: There is a difference between preeclampsia and control group. One-Way ANOVA, Tukey test.

the 10th, 50th, and 90th percentile using USG and a third-order polynomial regression procedure. EFW and WJ area measured by USG were correlated. WJ area increased linearly according to the gestational week (R^2=0.64) and stabilized from the 32nd week. There was a significant linear correlation (r=0.782) between WJ area and EFW until the 26th gestational week. In addition, it has been reported that the UC diameter increases significantly with gestational age until the 32nd-36th week, and then this value decreases.[4,6,17] In their respective nomograms, Weissman and Jakobi[6] reported this limit as the 36th week and Raio as the 34th week. In our research, a statistically significant positive correlation was found between gestational age and UC diameter in all groups.

Raio et al.[12] reported the first finding that WJ morphometric changes were present in the cord of fetuses with early-onset preeclampsia. The WJ area was smaller in the group diagnosed with preeclampsia. The most important aspect of these changes is that they are present in the absence of fetal growth disorders and altered UA Doppler parameters.[12]

In biochemical studies, changes were observed in the UC extracellular matrix of preeclamptic women. Bańkowski et al.[18] and Pawlicka et al.[19] found a significant increase in WJ, sulphated glycosaminoglycans and type III collagen and a decrease in hyaluronic acid in preeclamptic women. These findings suggest that in preeclampsia, WJ is characterized by reduced hydration. The second finding was that the cord thickness was smaller in preeclamptic women than in healthy pregnant women. In our research, cord thickness was significantly different between diabetic and preeclamptic pregnant women ($p<0.01$). The decrease in thickness in preeclamptic pregnant women compared to normal pregnant women was significant.

Antepartum fetal monitoring with UA Doppler has shown significant diagnostic effectiveness in determining fetal risk in complicated pregnancies, such as IUGR and preeclampsia. A significant relationship was observed between abnormal Doppler indices and fetal hypoxia, fetal acidosis and adverse perinatal outcomes.[20] However, its effectiveness in reducing perinatal mortality has been demonstrated in randomized clinical trials and meta-analyses. Among the tests performed to understand fetal well-being, the most effective is the antepartum fetal test.[21] In our study, we compared the UA PI value in all three groups and the PI value was significantly higher in the preeclamptic group. Bilateral correlations were examined in the preeclamptic group, and a significant negative correlation was found between PI values, and birth weight. Although in preeclamptic and diabetic groups blood gas pH value was lower than in the control group, the difference was not significant.

Ferguson and Dodson[3] performed biomechanical, histological and biomolecular tests in preeclamptic pregnant women and showed that the elasticity modulus was increased (i.e., the elasticity decreased) due to the decrease in collagen and elastin in the extracellular matrix in the cord. In the present study, there was a significant increase in elasticity modulus in preeclamptic pregnant women compared to the diabetic and control groups. This suggests that cord flexibility is reduced in preeclamptic pregnant women. Interestingly, no significant difference was found in elasticity modulus values between diabetic pregnant women and the control group.

The limitation of our study is the formaldehyde solution processing of the UC which may have caused alteration in the elasticity of the UC samples.

CONCLUSION

The UC is critical for normal fetal development during most of pregnancy. Therefore, defining the changes and differences in the cord and its multifactorial features will be beneficial in understanding fetal life. It is clear that the morphological, physical and developmental changes occurring in the UC should be investigated by multidisciplinary (including bioengineering and medicine) teams.

We suggest that predicting macrosomia in diabetic pregnant women in the future or screening for diabetes in pregnant women with a large cord radius can be done, once there is sufficient evidence to validate this approach. A limited number of patients were included in our study, and therefore we were unable to investigate the relationship between macrosomia, fetal outcomes and cord elasticity and diameter in the diabetic pregnant group. We believe that conducting studies in larger series may provide additional and more conclusive data. That the US-measured cord diameter and area, and diameters and elasticity data of the UA and veins were not included in the study are further limitations of the current study.

Larger-scale studies combining biomechanical data with ultrasonographic, histological, biochemical and immunohistochemical data may provide new insights into monitoring fetal well-being together with a better understanding of cord morphology and thus help prevent cord pathologies leading to serious fetal and maternal complications, such as preeclampsia, in terms of treatments at the molecular level, in the coming years.

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Ethics

Ethics Committee Approval: The approval of Alanya Alaaddin Keykubat University Clinical Research Ethics Committee (decision dated: 16.11.2022 and numbered: 12/05).

Informed Consent: Participation in the study was voluntary and all participants read and approved the informed consent form.

Authorship Contributions

Surgical and Medical Practices: S.K.Ö., T.Ç., A.Ç., Concept: S.K.Ö., M.Ç.K., L.A., H.D., A.Ç., Design: S.K.Ö., A.Ç., Data Collection or Processing: S.K.Ö., M.Ç.K., T.Ç., L.A., H.D., A.Ç., Analysis or Interpretation: S.K.Ö., M.Ç.K., T.Ç., H.D., A.Ç., Literature Search: S.K.Ö., M.Ç.K., T.Ç., L.A., A.Ç., Writing: S.K.Ö., L.A., H.D.

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Original Article

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Role of High Endometrial Natural Killer Cell Concentration in Patients with Recurrent Miscarriage

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ABSTRACT

Purpose: Lymphocyte subpopulation distribution and activation at the time of the window of implantation are likely to play a critical role in pregnancy loss. This study was planned to evaluate the prevalence of natural killer (NK) cells in the mid-secretory endometrium of women with recurrent miscarriage (RM) versus fertile controls.

Methods: The study group comprised 35 women with a history of two or more spontaneous abortions and 12 healthy fertile women as a control group. Mid-secretory endometrial tissue samples were obtained with a pipeline catheter, and endometrial NK cell phenotypes were determined by flow cytometry.

Results: While other endometrial lymphocyte populations remained constant, uterine NK cells in women with RM increased in the secretory phase. CD16+NK cell expression levels in women with RM were significantly higher than that of the fertile controls (0.57 vs. 0.08; $p<0.005$, respectively). However, decreased expression of CD4+ and CD4+3 cells and reduced ratio of CD4+/CD8+ were observed in women with RM.

Conclusion: Significantly increased levels of CD16+ in the endometrium of women with RM suggest that NK cells may have a significant role in the etiology of RM.

Keywords: Natural killer, recurrent miscarriage, abortion

INTRODUCTION

The American Society for Reproductive Medicine defines recurrent miscarriage (RM) as more than two consecutive pregnancy losses, while the World Health Organization (WHO) defines it as ≥ three miscarriages.[1-3] RM affects approximately 2.5% of women.[4] Although there is no underlying cause can be determined, many of the couples do not give term birth. A growing body of evidence points toward an immunological component of implantation failure. Pregnancy constitutes an immunological paradox since it implies that a fetus antigenically distinct from the mother is accepted by her immune system from embryo implantation to delivery. Immune balance between the mother and fetus is essential for the survival of an allogeneic fetus in the uterus. Natural killer (NK) cells are the primary immune cells that support a healthy pregnancy and have been linked to successful reproduction as a safety consideration.[5] NK cells are derived from hematopoietic progenitor cells that express the surface marker CD56,[6] which induces lymphangiogenesis, spiral artery remodeling, and trophoblast invasion.[7,8] In peripheral blood, there are two major types of NK cells: 90% are $CD56^{dim}$ CD16+ NK cells, and 10% are $CD56^{bright}$ CD16- NK cells.[9,10] However, the phenotype of uterine NK (uNK) cells, primarily $CD56^{bright}$ CD16- cells, is prevalent in the endometrium during the luteal phase and the early stages of pregnancy. Recent data indicate the presence of a subset of uNK cells, termed endometrial NK cells (eNK), with a yet-to-be-determined role.[11] This subset of cells might form a precursor of the uNK cells, given their similarity to classical uNK cell phenotype.[12] uNK cells are one of the most dominant leukocyte populations in the endometrium and account for 30% of cells during the window of implantation.[13,14]

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They play a role in immune response modulation, tissue repair promotion and vascular remodeling regulation. NK cells interact with trophoblast cells to have a role in initiating and maintaining pregnancy.[15-17]

One type of lymphocyte essential to adaptive immunity is the T cell. T cells play a role in the endometrium's local immune responses, specifically in controlling inflammation and immunological tolerance.[18] A balance between Th1 and Th2 is essential for fetal survival in the maternal uterus. For example, maternal tolerance to the semi-allogeneic fetus is thought to be predominantly related to the anti-inflammatory Th2 phenotype.[19,20] However, proinflammatory Th1 immune response is crucial for trophoblast invasion and parturition.[21,22] Additional data indicates that uNK is predisposed to release proinflammatory cytokines similar to Th1-type cytokines while reducing the anti-inflammatory Th2-type cytokines required to keep a pregnancy healthy.[23]

In women with RM, high numbers of uNK cells in endometrial biopsies taken in the late secretory phase correlated positively with the formation of blood and lymphatic vessels, spiral arteriole smooth muscle differentiation, and extent of endometrial edema. It is postulated that this exposes implanting blastocysts to excessive NK cell cytotoxicity and oxidative stress, leading to embryonic loss.[24] King et al.[25] have demonstrated that the NK percentage is >18%, which is highly specific for women with RM. Elevated NK cells play a pivotal role in RMs.[26,27] Alteration in the numbers or activity of uNK cells can, therefore, lead to reproductive failure and pregnancy complications. Owing to the wide variety of NK cell tests available to women with RM, we planned to determine whether NK cell concentrations in the late-secretory endometrium of RM women differ from those of fertile women.

METHODS

Study Subjects

Thirty-five women who were admitted to the Departments of Obstetrics and Gynecology of the Kocaeli University School of Medicine for RM treatment were included in the study. The study group comprised women with a history of two or more spontaneous abortions (n=35). They were recruited into the study at the point of being admitted for further evaluation of miscarriage etiology. Women in the RM group had normal ovulatory hormone profiles and routine pelvic ultrasound, and all semen analyses were reported as being within normal limits. Women in the control group (n=12) were previously fertile and were attending a variety of procedures, mainly intrauterine device application.

This study protocol was approved by the local ethical review board at Kocaeli University Medical Faculty and was designed and carried out by the Declaration of Helsinki. All patients entered this study only after a signed informed consent for the use of the endometrium was obtained. After a detailed questionnaire with demographic and socioeconomic data was filled, all women with RM underwent hysteroscopy (H/S) and hysterosalpingography (HSG) for uterine anomaly and fallopian tubal patency evaluation. Patients with uterine anomalies after H/S and HSG were excluded from the study. The men underwent at least one semen analysis according to the WHO criteria. The women underwent day-3 hormonal assessment to evaluate their ovulatory cycles, thyroid function, prolactin, and androgen levels. The ovarian reserve was detected by measuring serum follicle stimulation hormone level and antral follicle count on the third day of the menstrual cycle. Resumption of ovulation was defined by measuring mid-luteal progesterone. The couples in whom one of the partners presented an anomaly in one or more of the above tests and a history of diabetes mellitus, thrombophilia, hyperhomocysteinemia, and abnormally high HbA1C levels were excluded from this study.

Endometrial Sampling

All patients, independent of the group, were selected for the present study based on consistent histological findings, menstrual history, and serum progesterone levels. Endometrial samples were obtained with a pipeline catheter (Vel-Med) from all the participants in the mid-luteal phase of the cycle during the implantation window (cycle days 20-24). The endometrial tissue was divided into two sections; one was fixed in 10% formalin and embedded in a paraffin block. The other was washed three times with a sterile saline solution to remove blood and stored at -80 °C for future analysis. The mid-luteal phase was calculated as the 7 to 9 days after the ultrasonographic confirmation of ovulation and was confirmed by endometrial histological dating and serum progesterone levels. All menstrual cycles studied in the current study were ovulatory according to mid-luteal serum progesterone >10 ng/mL. Endometrial dating was performed by an independent pathologist experienced in gynecological pathology. Paraffin-embedded sections of 4 mm were stained with hematoxylin and eosin and periodic acid-Schiff stain. Then, these specimens were evaluated according to the histopathological criteria of Noyes et al.[28] An out-of-date biopsy was defined as a lag of ≥3 days between the chronological and the histological day.[29]

Flow Cytometry

We measured NK cell concentrations in endometrial biopsy specimens using flow cytometry. Findings derived from the peripheral NK cells in infertile women may not represent what happens at the feto-maternal interface.[30] We, therefore, did not measure blood NK cells. There has yet to be a consensus in the published literature about the timing for NK cell testing. Although uNK cells were mostly measured in the late luteal phase in the studies, there is cycle-to-cycle variation in the number of uNK cells.[31] Therefore, endometrial biopsy was collected from women during the implantation window (cycle days 20-24) in the present study.

Human endometrium tissue was obtained from 35 women with RM and 12 fertile women. Collected endometrial samples were centrifuged at 4,000 rpm for 10 min at four °C following a heat inactivation at 56 °C. After removing the transport medium from endometrium tissues, they were washed three times with Hanks balanced salt solution (HBSS) containing 5% penicillin/streptomycin on a Petri dish and minced into small pieces. Endometrial stromal and glandular cells were

isolated by digesting with 0.5% collagenase type II in Ca^{2+}, Mg^{2+} free HBSS at 37 °C for 5 min. Following gentle pipetting, the suspension was left to form the sediment for 5 min, and the upper part of the suspension was transferred into a separate tube for centrifugation at 400 g for 5 min. The supernatant was removed. The pellet was resuspended in RPMI medium, including 10% patient serum and 0.5% penicillin/streptomycin (stroma 1). This process was repeated four times (stroma 2-4) by adding collagenase solution to the primary sediment for sequential digestions. The contents of all stroma tubes were collected together. RPMI medium, including 10% serum from the patient and 0.5% penicillin/streptomycin, was added, and after centrifugation for 5 min at 400 g, the supernatant was discarded. The pellet of stromal cells was seeded into 25 cm^2 culture flasks. After stroma 4, the suspension was left to settle for 15 seconds. The upper part was collected and resuspended in RPMI with 10% patient serum and 0.5% penicillin/streptomycin. After brief centrifugation, the pellet containing glands was seeded into 25 cm^2 culture flasks. Cells were harvested and seeded into four healthy petri dishes, about $5x10^5$ cells per well.

Immunophenotype analysis by FACS

Endometrial cells were subjected to flow cytometry analyses. Cells were harvested and resuspended in a culture medium at 10^6 cells/mL concentration. Flow cytometry was performed using the flow cytometry instrument FACS Calibur (BD Biosciences, San Jose, CA, USA). The data were analyzed with Cell Quest software (BD Biosciences), and the forward and side scatter profiles were gated out of debris and dead cells. Immunophenotyping of endometrial cells was performed with antibodies against the following human antigens: CD45, CD14, CD45+14, CD3, CD4, CD8, CD8+3, CD16+56, CD3+16+56, CD5, CD10, CD19, CD103, CD22, CD20, CD57, cCD68, CD8, CD4+3, CD4. All of the antibodies were obtained from BD Biosciences.

Proportions of uNK cells and other lymphocyte subpopulations were determined as number (n) and percentage (%) of lymphocytes in each sample. The distribution of categorical variables between the two groups was tested with a chi-squared test. If continuous variables show a normal distribution, they are presented as mean and SD; otherwise, all results are expressed as median (range). The t-test or Mann-Whitney U test assessed statistical differences between groups. $P<0.05$ was considered significant.

Statistical Analysis

The data was analyzed by using the Statistical Package for Social Sciences software 13.0 for Windows package software (SPSS, Inc., Chicago, IL, USA).

RESULTS

Each group's demographic and laboratory characteristics were presented in Tables 1 and 2. The average age and body mass index of the group of women with RM was not significantly different from that of the fertile controls. Karyotype analysis of women with RM was normal. The mean gestational age occurring miscarriage was 7.17 weeks. The average number of miscarriages was 3.65. One patient with RM underwent elective cerclage at 12th gestational weeks. Of the endometrial samples used for endometrial dating analysis, all fertile endometrium samples were in the secretory phase, while 31 infertile endometrium samples were in the secretory phase, three was in the proliferative phase, and one was diagnosed with simple endometrial hyperplasia without atypia.

While other endometrial lymphocyte populations remained constant, uNK cells in women with RM increased in the secretory phase. CD16+NK cell levels in RM women were significantly higher than that of the fertile controls (0.57 vs. 0.08; p=0.005 respectively). However, decreased expression of CD4, CD4+3 cells, and decreased ratio of CD4/CD8 were observed in women with RM. The two groups had no significant differences concerning human leukocyte antigen G (HLA-G) levels (1.34 vs. 1.41; p=0.57, respectively). Day-3 hormone levels of groups were similar except for estradiol (E2) levels. Women with RM had significantly higher E2 levels than fertile controls (p=0.003). Blood folic acid and homocysteine levels of women diagnosed with RM were normal. The thrombophilia panels of RM women were heterogeneous and presented in Table 3. Because the thrombophilia panel is heterogeneous

Table 1. Baseline characteristics of the patients for each group [values are n, mean ± (standard deviation)]

Characteristic	RM (n=35)	Control (n=12)	p-value
Age (y)	31.87±5.5	32.5±4.4	0.7
BMI (kg/m^2)	25.2±5.6	25.7±2.9	0.79
Smoking (mean)	8.6 (3%)	8.3 (1%)	>0.05
Gravida [mean (min.-max.)]	3.4 (2-10)	1.5 (2-6)	0.004
Parity [mean (min.-max.)]	0.3 (0-3)	1 (2-4)	0.08
Abortus [mean (min.-max.)]	3.08 (2-8)	0-0	0.09
Alive [mean (min.-max.)]	0.28 (0-2)	2.08 (2-4)	0.01

RM: Recurrent miscarriage, BMI: Body mass index, min.-max.: Minimum-maximum

Table 2. Evaluation of the expression of immune parameters and comprasion between the RM and control group

	RM n=35 Mean (min.-max.)	Control n=12 Mean (min.-max.)	p-value
CD45	30.6 (82.76-0.52)	32.8 (65.27-8.37)	0.68
CD14	0.56 (6.8-0)	0.27 (0.93-0)	0.91
CD45+14	1.13 (5.12-0)	0.62 (1.28-0.12)	0.09
sCD3	15.10 (40.8-0.41)	16.10 (36.7-4.58)	0.55
CD4	5.19 (15.88-0)	6.19 (13.95-1.12)	0.28
CD8	13.55 (61.8-0)	8.25 (22.35-1.39)	0.55
CD8+3	7.08 (21-0)	6.26 (21.74-0.81)	0.9
CD4+3	1.83 (10.76-0)	6.73 (12.54-3.40)	0.012
CD4 count	85.5 (274-0)	140.9 (341-7)	0.05
CD8 count	157.6 (730-0)	195.6 (449-9)	0.25
CD16+CD56	4.95 (22.32-0)	4.77 (12.16-0.32)	0.63
CD3+16+56	0.39 (2.07-0)	0.21 (0.63-0)	0.22
CD5	13.71 (30.48-0.22)	17.8 (44.07-5.43)	0.37
CD10	21.2 (76.19-0)	28.9 (69.1-4.04)	0.17
CD19	1.9 (36.41-0)	1.4 (6.1-0)	0.3
CD103	6.06 (16.72-0.07)	7.6 (15.46-2.8)	0.17
CD22	2.03 (35.16-0)	1.3 (5.11-0)	0.39
CD20	1.9 (30.74-0)	0.55 (4.41-0.14)	0.30
CD57	3.31 (16.27-0.07)	1.6642 (5.09-0)	0.24
CD16	0.57 (4.01-0)	0.08 (0.68-0)	0.005*
cCD68	2.06 (13.81-0)	6.2 (62.11-0.02)	0.69
CD4	18.32 (55.53-0)	29.38 (39.19-8.61)	0.01*
CD8	30.50 (69.23-0)	41.73 (59.11-9.01)	0.17
CD4/CD8	0.7475 (6.7-0)	0.7950 (1.14-0.13)	0.03
HLA-G	1.34 (9.62-0.12)	1.41 (6.28-0.13)	0.57

*: Statistically significant, $p<0.05$, independent samples t-test.
RM: Recurrent miscarriage, HLA-G: Human leukocyte antigen G

Table 3. Thrombophilia mutation in recurrent spontaneous group

Thrombophilia mutation	n (%)	Homozygous	Heterozygous
Factor V leiden	24 (68.6%)	0	4 (31.4%)
Factor V	26 (74.3%)	0	2 (5.7%)
MTHFRc	11 (31.4%)	6 (17.1%)	15 (42.9%)
MTHFR 1298C	9 (25.7%)	4 (11.4%)	15 (42.9%)
Prothrombin	26 (74.3%)	0	2 (5.7%)
Factor 13	18 (51.4%)	2 (5.7%)	8 (22.9%)
Fibrinogen	12 (34.3%)	5 (14.3%)	11 (31.4%)
HPA	0	5 (14.3%)	23 (65.7%)

MTHFR: Methylenetetrahydrofolate reductase, HPA: Human platelet antigen

in our study population, it cannot be the primary underlying mechanism of RM.

DISCUSSION

To test the hypothesis that local uNK cells acting in a dysregulated way could lead to miscarriage, we compared NK cell expression by the eNK cell population in RM women and fertile controls. We have shown that CD16+ expression on endometrial samples isolated from RM women during the secretory phase was significantly higher than in fertile women. Fukui et al.[26] reported that uNK cells play an essential role in implantation and that an increase in cytotoxic peripheral and uNK cells can affect reproductive performance. A study has shown that women with RM have a significantly higher NK percentage than fertile controls.[25] They also demonstrated that an NK percentage of 18% was highly specific for women with RM and defined 12.5% of women with RM as having an elevated NK cell percentage.

In humans, uNK cells are associated with the synthesis of immunoregulatory cytokines, which promote physiological angiogenesis and placental growth.[32,33] These cells accumulate around uterine spiral arteries, indicating their potential role in modulating trophoblast invasion and vascular remodeling. Therefore, higher expression levels of CD16+NK cells in unexplained infertile women may exert an unfavorable influence on embryo attachment by overproduction of cytokine and growth factor secretion, which affects placental development and vascular growth.[34] Moreover, high numbers of CD16+uNK cells in endometrial samples may cause defective spiral arteriole formation and trophoblast invasion, which inhibits embryo implantation and may cause early embryonic demişe.[25] CD16 levels were higher in the RM group. In addition, HLA-G expression was similar in the two groups.[35] In 1996, Lachapelle et al.[36] compared uNK cells in RM with fertile controls by flow cytometry analysis that described no significant difference in the overall number of uNK cells; however, there was a noticeably increased percentage of CD56+ cells that also expressed CD16 in RM patients, indicating a critical function for NK subsets in the pathophysiology of miscarriage. Kuon et al.[37] evaluated 130 women compared idiopathic RM patients and showed a higher prevalence of >300 uNK cells/mm^2 than controls (34.5% vs. 5.9%, p=0.02). In 88% of controls and 62% of RM patients, uNK cells were detected within the range of 40-300/mm^2. In a study by Zargar et al.,[38] peripheral NK cells (CD16+ and CD56+) were higher in the RM group than the control group.

CD4 and CD4+3 expression and CD4/CD8 ratio in endometrial cells were significantly lower in RM women compared with fertile women in the secretory phase of the menstrual cycle. The mechanisms responsible for the decline in CD4 and CD4+3 leukocyte numbers and CD4+/CD8+ ratio in the endometrium of RM women are unclear. The human uterus is an immune-modulated site that keeps apart the implanted semi-allogenic embryo from the harmful maternal immune response. A well-regulated cytokine network is crucial for normal immune reactions. Pro and anti-inflammatory immune responses are both postulated to be required for gestation.[39] Therefore, a decline in the lymphocyte subpopulation may disturb the balance between Th1 and Th2, which is essential for fetal survival. Studies showed no significant difference between the two groups in the T-cell count.[40]

The normal value of NK cell levels favoring or "permitting" implantation and the employed testing methodology vary between studies.[41] Our results are consistent with the results of some studies in literature but incompatible with others. Different assessment methods of NK cell numbers or percentages across the studies can be a significant source of this difference. Such contradictory results may be due to genetic and phenotypic differences in populations from different regions of the world or differences in measurement methods.

Literature evaluation revealed 13 studies comparing NK cell levels in women with RM versus controls. Six of the 13 studies evaluated peripheral NK cells, and seven evaluated uNK cells. Meta-analysis of the six studies by Seshadri and Sunkara that evaluated uNK cells expressed as a percentage of the endometrial cells in women with RM versus controls showed no significant difference between the two groups.[42] In contrast, another study that expressed uNK cells as numbers reported significantly higher levels in women with RM compared with controls.[43] Interestingly, a meta-analysis of the four studies that evaluated peripheral NK cell levels expressed as percentages showed a significant difference between women with RM versus controls.[42] The systematic review contained sixty articles comparing the CD56+ uNK level in women with RM to controls, which revealed that, in a subgroup analysis of endometrial samples, women with RM had substantially higher levels.[44] Another possibility may be statistical heterogeneity across the studies, and there is no consensus about the elevated level of NK cells. Other factors such as diurnal variation of NK cells, maternal stress, hormonal effect, exercise, time of day, parity of women, and expression of NK cells as numbers or percentages may explain the difference among the studies.[43,45] Inconsistency among the study results may vary depending on laboratory techniques, sampling methods, and the study population's selection.

Many questions remain regarding the origins, functions, and regulation mechanisms of human lymphocyte subpopulation in the etiology of women with RM because detailed, gestational time-course studies are not feasible, and endometrial sampling occurs after pathology is recognized.[46] uNK cells are transient cells endowed with angiogenic, lymphogenic properties and secretory activities that participate in the early optimization of maternal care of the fetus before birth. However, it is essential to understand that NK is not the only cell that reflects specific immune responses to pregnancy. It has been shown that T and B lymphocytes, macrophages, and NK cells are recruited into the endometrium during the mid-secretory phase of the cycle in preparation for the onset of implantation. Whatever the results, it must be remembered that RM is a heterogeneous problem. Not all women with RM will have an NK cell-related problem, and of those who do, a variety of NK cell-related problems are possible, as defined in our study. Due to the

complexity of the innate immune system, one variable and one measure cannot predict reproductive outcomes. Further studies are needed to explore the lymphocyte population's underlying role and mechanisms of action in women with RM. Targeted immunotherapy may be guided by the results of well-designed functional studies in the future, which might provide insight into the direction of uNK's effect on women experiencing reproductive issues.

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Ethics

Ethics Committee Approval: This study protocol was approved by the local ethical review board at Kocaeli University Medical Faculty and was designed and carried out by the Declaration of Helsinki.

Informed Consent: All patients entered this study only after a signed informed consent for the use of the endometrium was obtained.

Authorship Contributions

Surgical and Medical Practices: N.Y., Ş.Y.K., A.Ç., E.Ç., Concept: N.Y., E.Ç., Design: N.Y., E.Ç., Data Collection or Processing: Ş.Y.K., N.Y., E.Ç., Analysis or Interpretation: G.G., E.Ç., Literature Search: E.Ç., Writing: E.Ç.

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Original Article

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The Effect of Two Different Embryo Culture Media on Birthweight of the Offspring

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ABSTRACT

Purpose: To investigate the effect of medium type on neonatal birthweight in singleton, term infants conceived following assisted reproductive technology (ART).

Methods: The records of 352 patients who gave birth after in vitro fertilization (IVF) treatment from January to December 2014 in the Eurofertil IVF Center using a time-lapse embryo culture system were analyzed. Data analysis was performed using two available culture media: Vitrolife (n=267) and MediCult (n=85).

Results: The mean birthweight of the infants from Vitrolife cultures was 3006 grams, though it was 3128 grams for the offspring from MediCult (p=0.154). There was no significant difference between neonates born using either medium in terms of birthweight.

Conclusion: There was no association between neonatal birthweight after ART and the *in vitro* embryo culture media used in this population. These findings are important in order to assess human embryo *in vitro* culture safety. To evaluate if culture media affects prenatal outcome and development, more randomized, controlled studies would be needed.

Keywords: Birthweight, embryo, fertilization in vitro

INTRODUCTION

The most frequently used treatments for subfertility are in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Over the past forty years, significant advancements and enhancements have been made to assisted reproductive technology (ART).[1] From early simple balanced salt solutions to later complex culture systems containing multiple components like phosphate, vitamins, amino acids, lipids, trace elements, and other biomolecules, the medium underwent a gradual optimization process.[2] Without knowledge of the optimal microenvironment for embryo development *in vivo*, we are unable to ascertain the most suitable circumstances for cultivating embryos in the laboratory.

It has been extensively documented that newborns conceived through ART are more prone to preterm birth and low birthweight when compared to infants born naturally.[3] This has been attributed to both inherent individual features and particular characteristics of the IVF procedure.[4] In addition, other well-known factors that influence neonatal outcome are maternal body mass index (BMI), smoking and alcohol consumption.[5] Moreover, there may be a correlation between gender and elevated birthweights; males tend to have greater birthweights than girls. Furthermore, both parity and gestational age are associated with an increase in birthweight.[6]

During preimplantation development, there is a process termed "epigenetic reprogramming" that involves deleting gametic alterations and establishing embryonic epigenetic modifications. ART has the potential to modify these modifications, including the potential influence of the diverse composition of the culture media employed during *in vitro* embryo development.[7] Different reports indicate that the culture media utilized in IVF embryo culture have distinct impacts on perinatal outcomes. In addition, the success rate and treatment results of IVF/ICSI operations depend on culture media choice. Selection of embryo culture medium affects embryo quality and conception rates.[8]

Since the inception of IVF as an ART, various culture systems have been used for the cultivation of human embryos. For optimal development, the chemical composition of the media used *in vitro* to cultivate human embryos is critical. There is no growth medium that perfectly replicates the conditions *in*

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vivo, so embryos that are grown in a laboratory are always stressed in some way. The embryo culture medium used in IVF laboratories may have an impact on birthweight and other outcomes for infants.[9] Animal studies have shown that particular components of the culture media, and changes in the concentration of these components, may lead to changes in birthweight of offspring.[10]

An important randomized controlled trial (RCT) observed a mean disparity of 158 g in birthweight between two distinct media sources.[11] The substantial value of the evidence gathered from RCTs supports the hypothesis that IVF culture media affect the in utero development, weight, and adiposity of the neonate at the age of nine.[12]

The impact of medium type on birthweight in human IVF remains mostly unknown. Furthermore, a recent systematic review revealed that there are few randomized studies comparing the clinical outcomes of various culture media, and those that do have unsound methodology.[13,14] Thus, this topic remains plagued by unanswered questions, and the debate continues. Given the significant predictive value of birthweight for long-term health consequences, it is important to determine whether particular culture media are associated with adverse fetal outcomes that may have enduring effects in maturity.

In this study the effects of two different commercial embryo culture media (MediCult, Denmark vs. Vitrolife, Sweden) on birthweight were investigated.

METHODS

Subjects

In the present study, the records of patients who gave birth after IVF treatment at the Eurofertil IVF Center from January to December 2014, using a time lapse embryo culture system, were analyzed by the end of 2015. The research protocol was approved by the Ethics Review Committee of Bahçeşehir University.

Maternal age, maternal BMI, paternal age, cycle type (agonist, antagonist, Femara, thaw, natural cycle), estradiol (E2) levels and gonadotropin intake status and sperm preparation methods were evaluated for both media. Pregnancy and infant characteristics (gestational age, newborn gender, twin pregnancy rates and mean birthweight) were compared between the two media. Only the data from the initial IVF treatment cycles of all patients treated at our center during the research period were selected for the present study. Patients requiring preimplantation genetic diagnosis and pregnancies resulting in significantly abnormal birthweight (<2500 g and >4500 g) were excluded. Among the cases receiving IVF treatment during the study period only those cases having clinical pregnancy diagnosed with transvaginal ultrasound were included.

Laboratory Protocols

Embryos derived from a fresh IVF/ICSI cycle were cultured either in MediCult (Jyllinge, Denmark) or Vitrolife (Gothenburg, Sweden). Subsequently, the laboratory's routine insemination protocols were followed for the IVF and ICSI procedures. Fertilized oocytes were placed in microwells (Mannheim, Germany) with 35 mL culture medium and 1.4 mL oil. A time-lapse monitoring system with an incubator and optical microscope automatically captured images until transfer. Five focal planes were used to record embryo growth every 10 minutes.[15] An assessment of the morphology of the embryos was conducted 68-72 hours post-inoculation, focusing on cell count, fragmentation and symmetry. The criteria for scoring were fragmentation, grade, and cell number. Good quality 72-hour embryos were distinguished by the presence of 7-9 mononucleated blastomeres of equal size, with fragmentation below 10%.

Embryo Transfer Protocols

The number of embryos to transfer was determined based on each patient's age, medical history, and the characteristics of the available embryos. Embryo transfer (ET) was performed on days 2, 3, or 5 after oocyte retrieval, depending on embryo quantity and quality. The selection of embryos for transfer was based on their developmental quality, determined by assessing their morphokinetics and ultimate morphological appearance. The serum ß-human chorionic gonadotropin (β-hCG) level was assessed 12 days post ET. An ultrasound was conducted 14 days following a positive β-hCG test to determine the presence of gestational sac(s). The assessment of clinical pregnancy occurred between 7 and 14 days after the identification of the gestational sac, through the detection of fetal cardiac heartbeat.

Statistical Analysis

The data were subjected to statistical analysis utilizing SPSS, version 23.1 (IBM Inc., Armonk, NY, USA). After fresh ETs, the mean values of patient baseline characteristics were analyzed across the two categories of culture media using chi-square testing for categorical and continuous variables and Student's t-tests for continuous variables. A significance level of two-sided p-values below 0.05 was regarded as statistically significant.

RESULTS

During the study period there were 815 couples receiving IVF treatment. The clinical pregnancy rate was 50.7% (414/815). There were 47 (11.3%) cases of abortion before 20 weeks of gestation, nine cases lost to follow-up due to telephone or address change and six cases of intrauterine deaths, leaving 352 patients with live birth for analysis. There were 312 singleton births and 41 sets of twins.

Based on the culture medium employed, Table 1 shows the key maternal, paternal, and treatment characteristics of the two groups. Age, BMI, paternal age, cycle type, sperm preparation method, and gonadotropin utilized in the treatment were all criteria that did not show a significant difference between the two groups that were given cultured medium. In Table 2, cycle type (agonist, antagonist, Femara, dissociative, natural cycle), E2 levels and gonadotropin uptake status and sperm preparation methods were evaluated for both media. It has been shown that cycle type, gonadotropin intake and sperm preparation method are significant in terms of outcome ($p<0.05$).

Table 1. Demographic data of the parents

Variable	Group 1 (Vitrolife) (n=612)	Group 2 (MediCult) (n=203)	p-value
Maternal age (years)	31.2±4.9	30.8±5.1	0.3
Maternal BMI (kg/m^2)	23.8±4.1	24.4±3.9	0.06
Paternal age (years)	33.1±5.2	32.7±5.3	0.34
Basal FSH	7.0±2.6	7.1±2.3	0.62
Previous ICSI cycles	1.7±1.3	1.9±1.4	0.06
Cause of infertility			
Unexplained	202 (33)	71 (35)	0.2
Male factor	269 (43.9)	73 (36)	-
PCOS	37 (6)	14 (6.8)	-
Endometriosis	55 (8.9)	18 (8.8)	-
Tubal factor	36 (5.8)	20 (9.8)	-
Azoospermia	13 (2.1)	7 (3.4)	-

The data are presented as n (%) or mean ± SD from the mean.
BMI: Body mass index, FSH: Follicle stimulating hormone, ICSI: Intracytoplasmic sperm injection, PCOS: Polycystic ovary syndrome, SD: Standard deviation

Table 2. Cycle characteristics and pregnancy outcome

Variable	Group 1 (Vitrolife) (n=612)	Group 2 (MediCult) (n=203)	p-value
Cycle type			
Agonist	9 (1.4)	1 (0.4)	<0.01*
Antagonist	459 (75)	119 (58.6)	-
Letrazole only	7 (1.1)	1 (0.4)	-
Thaw cycle	134 (21.2)	81 (39.9)	-
Natural cycle	3 (0.4)	1 (0.4)	-
E2 (pg/mL)	2175±941	2075±1325	0.2
Gonadotropin (450 IU/0.75 mL)			
Recombinant FSH	325 (53.1)	132 (65)	<0.01*
Urinary FSH	18 (2.9)	8 (3.9)	-
FSH+LH	269 (43.9)	63 (31)	-
Sperm preparation method			
Swim up	466 (76)	131 (64.5)	<0.01*
Gradient	74 (12)	16 (7.8)	-
Sperm wash	72 (11.7)	56 (27.5)	-
MII oocytes aspirated	10.8±5.4	10.2±4.7	-
Clinical pregnancy rate	318 (51.9)	96 (47.2)	0.2
Spontaneous abortion rate	36 (5.9)	11(5.4)	0.8
Lost to follow-up	7 (1.1)	2 (0.9)	0.8
Take home baby rate	267 (43.6)	85 (41.8)	0.6

***p<0.05**
The data are presented as n (%) or mean ± SD from the mean.
FSH: Follicle stimulating hormone, LH: Luteinizing hormone, E2: Estradiol, MII: Metaphase II, SD: Standard deviation

Table 3. Pregnancy outcome and infant characteristics according to culture media used

	Group 1 (Vitrolife) (n=267)	Group 2 (MediCult) (n=85)	p-value
Gestational age (weeks)	37.19±0.19	37.89±0.17	0.045*
Gestational age category			
<32 (very preterm)	21 (8.0)	2 (2.4)	0.14
32 to 37 (preterm)	38 (14.5)	10 (11.9)	-
≥37	202 (77.5)	72 (85.7)	-
Newborn gender			
Male	141 (47.9)	40 (43.4)	0.71
Female	153 (52.1)	52 (56.6)	-
Twin	33 (12.3)	9 (10.5)	0.50
Not twin	234 (87.6)	76 (89.4)	-
Mean birthweight of singletons (g)	3006±44	3137±58	0.13
Mean birthweight of twins (g)	2285±106	2323±126	0.86
Birthweight category			
<1500 g (very low)	15 (5.6)	1 (0.1)	0.35
<2500 g (low)	34 (12.7)	10 (11.7)	-
2500-4000 g (normal)	210 (78.6)	72 (84.7)	-
>4000 g (high)	8 (2.9)	2 (2.3)	-

*p<0.05

The relationship between medium used and birthweigth is shown in Table 3. Mean birthweight of the singleton offspring from pregnancies using Vitrolife was 3006 g while it was 3137 g in pregnancies using MediCult (p=0.154). Similarly, the mean twin birthweight was 2285 g for twins in the Vitrolife group and 2323 g for twins in the MediCult group (p=0.86). There was no significant difference in mean birthweight between the births using either medium type.

Table 3 shows parameters of delivery and offspring depending on the culture medium used. Parameters compared include gestational age, gender of the offspring, and mean birthweight. The only significant difference was found for gestational age which was significantly older in the MediCult group.

DISCUSSION

This retrospective study demonstrated that the birthweight of the offspring was not significantly affected by using either Vitrolife or MediCult, two distinct embryo culture media for IVF.

Birthweight is widely recognized as a prevalent indicator used for perinatal outcome assessment, which has been integrally linked to morbidity and mortality.[16] Embryonic development during IVF is dependent upon culture medium. Thus, many IVF centers have considerd the impact of *in vitro* embryo cultivation on neonatal birthweight. Nevertheless, there is variation among manufacturers regarding the composition and concentrations of nutrients present in various culture media.[13]

In terms of human IVF, the effect of medium choice on birthweight remains incompletely understood. Studies by Nelissen et al.[8] and Dumoulin et al.[9] showed that the kind of embryo culture medium used has an important effect on early embryonic development, fetal growth and birthweight of the baby. Dumoulin et al.[9] showed that singletons produced fresh

cleavage Day 2/3 ETs and grown in Vitrolife medium were heavier than those created through Cook medium culture. Using a small-scale cohort study involving both fresh and frozen ETs, he further supported his findings.[8] The disparity in growth became apparent as early as the second trimester of pregnancy and persisted for a minimum of two years after birth. Since then, research comparing various culture media has both confirmed and refuted these earlier conclusions.[8] Furthermore, birthweight after embryo culture in three different media and birthweight following spontaneous conception were compared in a relatively recently published Norwegian study, and the results revealed the birthweight as well as the placental weight differed between different culture media.[17] On the other hand, Eaton et al.[18] recently showed that there was no significant relationship between the embryo culture medium used and birthweight. Similarly, other studies using the same and other commercially available culture media did not find any changes in birthweight that were linked to using different embryo culture media.[18-20]

The results of the present study also suggest no significant association between culture medium and birth weight, in keeping with many earlier studies.[18-22] There remain the studies which have reported significant differences in birthweight in IVF neonates which have been attributed to the use of different culture media.[8,9,17]

Consistent with the results reported by Eaton et al.,[18] the current study's findings also indicate that birth weight and the culture medium employed did not correlate significantly in the 198 singleton deliveries analyzed. The results obtained in the investigation published by Vergouw et al.[19] were similar. Comparing two distinct culture media, the analysis of 358 singletons born after a fresh single ET and 159 singletons born after a frozen-thawed single ET revealed no significant difference in birth weight.

It has been reported that the use of Vitrolife medium resulted in a greater birth weight when compared to Cook culture media.[9] Upon analyzing 110 live singleton births from Vitrolife and 78 singleton births from Cook, the researchers discovered that the birth weight of the first group was significantly greater, both when adjusted for gender and gestational age. Notably, maternal and paternal weight, as well as height and weight, were significantly greater in the Vitrolife group than in the Cook group in this study. Nevertheless, Dumoulin et al.[9] conducted a retrospective analysis that encompassed singleton births occurring following fresh IVF-ICSI cycles, including those at >20 weeks gestation. This allowed for a comparison of neonates across a broader range of gestational ages.[9]

We felt it reasonable to compare MediCult and Vitrolife media because they differ in several components. MediCult incorporates synthetic serum replacement in the fertilization, cleavage, and blastocyst media. Vitrolife medium contains fructose, lactate, non-essential amino acids, and EDTA, whereas MediCult does not contain these components. Furthermore, the formulation of Vitrolife include methionine, hyaluronan, lipoic acid, and EDTA. The amino acid makeup of both blastocyst media is largely comparable, with the exception of arginine. Arginine is found in Vitrolife blastocyst medium, but not in MediCult blastocyst medium. Furthermore, both blastocyst media share four vitamins, but MediCult blastocyst medium additionally includes D-biotin, folic acid, and niacinamide. MediCult blastocyst medium includes inositol and ethanolamine, whereas Vitrolife blastocyst medium is fortified with hyaluronan.[23] The birthweight of infants raised as embryos in either of the two medium types did not vary, despite the significant component differences between two media. In this regard, the protein source in the culture media is also a significant factor since studies have indicated that its quality varies significantly across groups and manufacturers, and it has also been proposed that the protein source affects live birth rate and birthweight.[24] We hypothesize that the almost equal amounts of protein present in both culture media may be the reason for the lack of birthweight differences.

When analysing the culture media-mean birthweight relationship, many other factors should be taken into account. In the Cook and Vitrolife study groups, variations in parental characteristics, including maternal age, maternal and paternal BMI, maternal parity, and maternal smoking, may have affected the outcomes of the research conducted by Dumoulin et al.[9] and Nelissen et al.[8] In the present study maternal and paternal age and maternal BMI did not significantly differ between groups.[12]

Our retrospective study is limited in its design, a RCT would have been more informative. Another limitation of the present study is the heterogenous study populations and the differences in samples numbers for each medium. Thirdly, the influence of culture media on blastocyst transfer was not been investigated. The contentious impact of extended *in vitro* culture on birthweight was not considered.[25] Although this study only evaluated two types of culture media that are available, the findings can be integrated with other research that examine different commercially available culture media. This will expand the understanding of how various media types impact newborn outcomes.

Comparing the published studies on the impact of culture media on birthweight is challenging due to variations in study designs and research objectives. Moreover, human studies provide unique challenges in analysis when compared to animal studies. One of the major limitations is the frequent and consecutive use of various culture media. The majority of studies have employed a retrospective approach, using various culture media in consecutive time periods. This introduces a high likelihood of bias, as it is difficult to account for all variations in treatment protocols and population factors. Birthweight studies sometimes lacked crucial data on confounding factors, such as maternal smoking, parity, socio-economic status, sex distribution, and gestational age. We believe that other variables in our study population have the potential to influence the outcomes. Performing a multivariate regression analysis would have enhanced the validity of our hypothesis.

CONCLUSION

In summary, we found no correlation between the weight of neonates following ART and the choice of culture media,

MediCult versus Vitrolife, used for *in vitro* embryo culture. These results add to the evidence regarding the safety of *in vitro* culture of human embryos. They also offer reassuring data in comparison to previous studies that demonstrated an association between particular culture media and increased birth weight. Our findings are in keepig with previous studies that have demonstrated consistent birth weights, regardless of the embryo culture media used. While these findings offer some comfort, it is necessary to conduct bigger, randomized, controlled studies to determine whether various culture media have any impact on the prenatal outcome of the babies and their subsequent development.

Ethics

Ethics Committee Approval: The research protocol was approved by the Ethics Review Committee of Bahçeşehir University.

Informed Consent: Retrospectively study.

Authorship Contributions

Surgical and Medical Practices: E.E., Concept: H.Ö., Design: H.Ö., Data Collection or Processing: E.E., Analysis or Interpretation: E.E., Ö.A., H.Ö., Literature Search: Ö.A., Writing: E.E., Ö.A.,

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Original Article

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PAX2 and Bcl-2 Expression in Early Stage Endometrioid Adenocarcinoma Compared to Endometrial Hyperplasia and the Relationship with Other Prognostic Factors

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ABSTRACT

Purpose: To determine the tissue expression of Bcl-2 and PAX2 in samples from patients with endometrial hyperplasia and early stage endometrial cancer endometrioid endometrial adenocarcinoma and the relationship with other prognostic factors.

Methods: Patients with early stage endometrial cancer endometrial adenocarcinoma (the cancer group) and with benign endometrial hyperplasia (the hyperplasia group) diagnosed at a single center between 2009 to 2012 and followed up for at least five years were included. Immunohistochemical staining for Bcl-2 and PAX2 was performed. Microcystic, elongated and fragmented pattern (MELF) numbers and ratios were determined by histopathological re-evaluation of slides from samples from the cancer group. Control and patient groups were compared in terms of Bcl-2 and PAX2 scores. Comparison of Bcl-2 and PAX2 scores in relation to different prognostic factors, such as tumor size, myometrial invasion, lymphovascular stromal invasion, myometrial invasion pattern and in tissues neighboring the cancer tissues were performed.

Results: Mean PAX2 expression scores (0.58±1.3 vs 4.3±1.06, $p<0.01$) were significantly lower in cancer group (n=57) compared to the hyperplasia group (n=34). Similarly, mean Bcl-2 expression scores were significantly lower in the cancer group (2.2±1.6 vs 4.06±1, $p<0.01$). MELF pattern was positive in 60% of the cases in cancer group compared to 10.6% in the hyperplasia group ($p<0.01$). Median Bcl-2 scores were higher in MELF positive cases (M=3) compared to MELF negative cases (M=2, $p=0.04$). In addition, median PAX2 scores were higher in MELF positive cases compared to MELF negative cases [2 (range here) vs 0 (range here), $p=0.007$].

Conclusion: Bcl-2 and PAX2 appear to be important in differentiating early stage endometrial cancer endometrioid cancer tissue from hyperplastic endometrial tissue. Their expressions were found to be similar in different prognostic subgroups.

Keywords: Endometrial cancer, Bcl-2, PAX2, gene expression

INTRODUCTION

Adenocarcinoma of the endometrium is the most common malignancy of the female genital system. It is the sixth most common malignancy in women, after breast, colorectal cancers, lung cancer, cervix uteri cancer and thyroid cancer and 4.5% of women are diagnosed with endometrial cancer.[1] Poor prognostic factors for this cancer are advanced surgical stage, advanced age, histological type, advanced tumor grade, presence of myometrial invasion, presence of lymphovascular area invasion, positive peritoneal cytology for cancer cells, and increased tumor size.[2]

Endometrial cancer is detected at stage I in 75% of patients.[3] The survival rate in stage I tumors has been reported as 85-90%. However, patients with early-stage and good prognosis who do not require adjuvant therapies, such as radiotherapy or chemotherapy, have approximately 10-15% recurrence risk during follow-up. These patients respond poorly to adjuvant chemotherapy and some patients are lost due to illness. Unfortunately, the treatment of relapses is challenging. There is a need for easy-to-use, cheap, and rapid prognostic markers to demonstrate poor prognosis in the evaluation of these patients.

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Recently, the development of investigation of genomic factors, especially immunohistochemical evaluation of changes in the expression of transcription factors, seem to be promising in determining poor prognosis and for the detection of patients in need of adjuvant therapy.[4] Two immunohistochemical methods have been studied recently which are based on the detection of changes in the expression of the protein products of the *PAX2* and *Bcl-2* genes.[5,6]

The *PAX2* gene is a nuclear transcription factor of the paired box gene family, which is necessary for the development and differentiation of epithelial and mesenchymal components of the urogenital system. Embryonic PAX2 expression is involved in the development of kidney, ureters and renal collecting duct cells, and in women the endometrium, and in men the development of vas deferens and epididymis. Decreased PAX2 expression has been reported to be associated with cervical and endometrial malignant transformation.[7] Interestingly, PAX2 has been found to act as a tumor suppressor during cell proliferation and regeneration in endometrial epithelial cells. Decreased PAX2 expression is associated with endometrial cancer.[8]

Another immunohistochemical marker of the *Bcl-2* gene is one of the main components of the mitochondrial pathway and it plays an anti-apoptotic role in cells.[9] *Bcl-2* may be the most hormone-dependent gene in endometrium and *Bcl-2* gene family members have a significant effect on carcinogenesis. Overexpression of the *Bcl-2* gene results in breast, colon, thyroid, and endometrial cancers. In recent years, there has been evidence that Bcl-2 expression plays an important role in cancer progression and prognosis.[10,11]

The aim of this study was to determine the expression of *PAX2* and *Bcl-2* genes in grade 1 endometrial endometrioid cancer compared to endometrial hyperplasia and the relationship of the levels of expression with poor prognostic factors for endometrial cancer survival.

METHODS

Patients with grade 1 endometrial endometrioid adenocarcinoma attending a single tertiary university center between 1st January 2009 and 31st December 2012 were reviewed for five-year survival rate up to 2018 and constituted the cancer group in this study. Patients diagnosed with benign hyperplasia on histopathological examination of endometrial samples formed the hyperplasia group. The patients' data were collected from the gynecology and pathology files. Specimens of all patients from both groups were re-examined in the light of the current literature, biopsies, and clinical and radiological data. A written informed consent was obtained from each patient. The study was conducted in accordance with the principles of the Declaration of Helsinki This study was supported by the local research projects coordination unit as a scientific research project and approved by the Kocaeli University Non-Interventional Research Ethics Committee (dated 19.10.2016 and numbered KÜ GOKAEK 2016/ 287).

The study retrospectively reviewed demographic features of patients, including age, the presence of diabetes, hypertension, and history of accompanying malignancy or relevant family medical history. Pathology reports were also examined to extract surgical stages, tumor size, peritoneal cytology, lymphovascular space invasion, and lymph node metastasis. Patients who received chemotherapy, radiotherapy, or brachytherapy after surgery were identified.

Microcystic, elongated, and fragmented pattern (MELF) ratios and MELF numbers were determined by re-evaluation of the preparations in cancer group. Histopathological findings of surrounding endometrial tissue and invasion patterns in the presence of myometrial invasion were reviewed.

The cancer and hyperplasia groups were compared in terms of Bcl-2 and PAX2 scores. Cross sections with a 4-micron thickness which derived from selected blocs were investigated by using positively charged lamella. After deparaffinizing with xylene, the sections were gradually rehydrated by immersion in changing concentration of ethanol solutions. Antigen retrieval was performed at 100 ˚C for 4 minutes in sodium citrate buffer solution (pH=6). Endogenous peroxidase activity was prevented by immersing the sections in 3% hydrogen peroxide (H_2O_2) solution for 15 minutes. The sections were then crosslinked for 20 hours with human anti-Bcl-2 antibodyat 1/50 dilution (Bcl-2alpha Ab-1 clone, LabVision) and human anti-PAX2 at 1/20 dilution (PAX2, Zeta) antibodies. After incubation, the primary antibody was visualized using streptavidin-biotin complex (UltraVision Detection System Large Volume Anti-Polyvalent, HRP, LabVision) and chromogen (AEC Substrate System, LabVision). Immunohistochemical studies and staining specimens were evaluated by histopathologists. Immunoreactivity was scored semi-quantitatively based on the intensity and distribution of staining for Bcl-2 and PAX2. The staining intensity of the tumor was compared to normal endometrial gland and noted as "increased, decreased, or same" when present. Coloring for the intensity of staining in the gland epithelium were given 0 points for non-staining, 1 point for weak staining, 2 points for moderate staining, and 3 points for strong staining. For the prevalence of staining in the gland epithelium was given 0 for non-staining, 1 point for 1-33% of the gland epithelium, 2 points for 34-66% of the gland epithelium, 3 points for 67-100% of the gland epithelium. The scores given for each case were collected and a final score was determined between 0 and 6 (without 1 point). Also, tumor staining in the surrounding endometrial tissue was categorized as 'increased', 'decreased', or 'unchanged'.

Statistical Analysis

Data were evaluated using SPSS for Windows, version 29 (IBM Inc., Armonk, NY, USA). Nominal variables were evaluated with Pearson's chi-square or Fisher's Exact test. Bcl-2 and PAX2 immunohistochemical staining scores between the control group and cancer group were compared with the Mann-Whitney U test. The between group comparison of Bcl-2 and PAX2 with other prognostic factors was evaluated by Mann-Whitney U and Kruskal-Wallis tests. The relationship between continuous variables with abnormal distribution were evaluated by the Spearman Correlation test and the Pearson correlation test was performed in continuous variables with normal distribution. A $p<0.05$ was considered statistically significant.

RESULTS

There were 57 women in the cancer group and 34 women in the hyperplasia group. Demographic data of the patients are shown in Table 1. Parity, smoking, and diabetes frequency were similar in the cancer and hyperplasia groups. However, the patients in the cancer group were significantly older and more likely to be postmenopausal and hypertensive.

In the cancer group the treatment method consisted of a total abdominal hysterectomy and bilateral salpingo-oophorectomy in all cases. Pelvic lymph node dissection was carried out in 29 (50.8%) patients, while pelvic and para-aortic lymph node dissection was performed in 13 (22.8%) patients. In 28 (49.1%) patients, lymph node dissection was not carried out due to the tumor being limited in the endometrium and the myometrial invasion being less than ½. For patients who underwent lymph node dissection, the number of lymph nodes removed ranged from 0 to 37, with a mean of 15±7.7. In our cohort, 84.2% were stage 1A and there was remission without additional treatment. During five years of follow-up, 2 (3.5%) died due to non-cancerous chronic disease, resulting in 96.4% survival rate and there was one case of recurrence (1.75%).

On review of the pathology results, 35 (61.4%) patients had a tumour size >1 cm and 22 (38.5%) patients has a tumor size <1 cm. Examination of the peritumoral endometrium showed 27 (47.4%) with endometrial atrophy, one (1.75%) with benign hyperplasia, 25 (43.9%) with complex atypical hyperplasia, one (1.75%) with endometrium showing features of progesterone effect and one (1.75%) with secretory endometrium.

The tumor tissue invasion pattern showed an infiltrative pattern in 18 (31.6%), 26 (45.6%) patients with an expansile pattern, one (1.75%) patient with an expansible-infiltrative pattern, and one (1.75%) patient with a destructive pattern. In addition, the MELF pattern was observed in 11 patients. Ten patients had lymphovascular invasion and 60% of them were positive for MELF pattern compared to 10.6% (n=5/47) of the cases without lymphovascular invasion (p<0.01).

The comparison of Bcl-2 and PAX2 scores in cancer vs hyperplasia groups are given in Table 2. Both the PAX2 and Bcl-2 scores were significantly lower in the cancer group compared to the hyperplasia group. Bcl-2 and PAX2 immunohistochemical staining scores with respect to tumor features are presented in Table 3. MELF pattern was positive in 60% of the cases in cancer group compared to 10.6% in the hyperplasia group (p<0.01). Median Bcl-2 scores were higher in MELF positive cases compared to MELF negative cases (p=0.04). Median PAX2 scores were higher in MELF positive cases (M=2) compared to MELF negative cases (p=0.007). Interestingly, Bcl-2 and PAX2 scores were similar in lymphovascular stromal invasion positive and negative cases, in cases with different myometrial invasion depth and different tumor size. Bcl-2 scores were similar in different myometrial invasion patterns and hyperplastic or atrophic tissue surrounding the cancer tissue. PAX2 scores were significantly higher in tumors exhibiting an infiltrative-type myometrial invasion pattern compared to expansive pattern (p=0.03). In the cancer group, hyperplastic tissue surrounding the cancer tissue had a higher PAX2 score compared to surrounding atrophic tissue (p=0.03). Although positive or negative LVSI and MELF pattern showed no significant difference in Bcl-2 staining for tumor and surrounding endometrium, Bcl-2 staining was significantly increased in the presence of more than half depth myometrial invasion (p=0.04) (Table 4). PAX2 staining was found to be similar both in tumor tissue and the surrounding endometrium in terms of LVSI and MELF pattern regardless of being positive or negative, myometrial invasion and invasion pattern (Table 5).

Table 1. Demographic data of patients with endometrial cancer and endometrial hyperplasis

Parameters	Cancer group (n=57)	Hyperplasia group (n=34)	p-value
Mean ± SD age, (years)	58.7±8.5	45.5±7.25	<0.01
Mean ± SD parity	3.3±1.87	3±1.39	0.709
Smoking, n (%)	5 (8.8)	2 (5.9)	0.617
Diabetes, n (%)	21 (36.8)	11 (32.4)	0.664
Hypertension, n (%)	35 (61.4)	13 (8.6)	0.03
Menopause status, n (%)			
Premenopausal	1 (1.8)	16 (47.1)	<0.01
Perimenopausal	15 (26.3)	11 (32.4)	
Post-menopausal	36 (63.2)	7 (20.6)	
SD: Standard deviation			

DISCUSSION

The results of the present study suggest that Bcl-2 and PAX2 expression scores can be used to differentiate early stage, well differentiated endometrioid endometrial cancers from

Table 2. Comparison of Bcl-2 and PAX2 scores in the endometrial cancer vs endometrial hyperplasia groups

Variable	Cancer group (n=57)	Hyperplasia group (n=34)	p-value
PAX2 score			
Median (min-max)	0.5 (0-5)	4 (2-6)	<0.01
Mean ± SD	0.58±1.3	4.3±1.065	
Bcl-2 score			
Median (min-max)	2 (0-6)	4 (0-6)	<0.01
Mean ± SD	2.2±1.6	4.06±1	
SD: Standard deviation, min-max: Minimum-maximum			

Table 3. Bcl-2 and PAX2 immunohistochemical staining scores with respect to tumor features

Tumor features	Bcl-2 score Median (min-max)	p-value	PAX2 score Median (min-max)	p-value
Tumor size				
Less than 1 cm (n=22)	2 (0-6)	0.59	0.5 (0-4)	0.98
More than 1 cm (n=35)	2 (0-5)		0	
Myometrial invasion				
No invasion (n=10)	2 (0-6)	0.83	0.6 (0-4)	0.36
<half depth (n=38)	2 (0-6)		0 (0-4)	
>half depth (n=9)	3 (0-4)		0 (0-5)	
LVI				
Positive (n=10)	2 (0-4)	0.73	0 (0-5)	0.26
Negative (n=47)	2 (0-6)		0 (0-4)	
MELF				
Positive (n=11)	3 (2-4)	0.04	2 (0-5)	0.007
Negative (n=46)	2 (0-6)		0 (0-4)	
Myometrial invasion pattern				
Expansive (n=28)	2 (0-6)	0.48	0 (0-4)	0.03
Infiltrative (n=19)	3 (0-4)		1 (0-5)	
Surrounding endometrium				
Hyperplastic (n=28)	3 (0-5)	0.35	1 (0-4)	0.03
Atrophic (n=29)	2 (0-6)		0 (0-5)	

LVI: Lymphovascular invasion, MELF: Microcystic, elongated and fragmented pattern, min-max: Minimum-maximum

Table 4. Bcl-2 staining comparison between the tumor and the surrounding endometrium

	Bcl-2 comparison			p-value
	Increased n (%)	Decreased n (%)	Sam n (%)	
LVI				
Negative	2 (4.3)	43 (91.5)	2 (4.3)	0.384
Positive	1 (12.5)	6 (75)	1 (12.5)	
MELF				
Positive	1 (9.1)	9 (81.9)	1 (9.1)	0.688
Negative	2 (4.5)	40 (90.9)	2 (4.5)	
Myometrial invasion				
Negative	1 (10)	9 (90)	0	0.04
<half depth	1 (2.8)	34 (94.4)	1 (2.8)	
>half depth	1 (11.1)	6 (66.7)	2 (22.2)	
Invasion pattern				
Infiltrative	1 (5.9)	13 (76.5)	3 (17.6)	0.07
Expansive	1 (3.8)	25 (96.2)	0	
Surrounding endometrium				
Hyperplastic	1 (4.1)	23 (95.9)	0 (0)	0.187
Atrophic	2 (7.7)	21 (80.8)	3 (11.5)	

LVI: Lymphovascular invasion, MELF: Microcystic, elongated and fragmented pattern

Table 5. PAX2 staining comparison of tumor tissue to surrounding endometrium

	PAX2 comparison		p-value
	Increased n (%)	Decreased n (%)	
LVI			
Negative	0 (0)	47 (100)	0.14
Positive	1 (12.5)	7 (87.5)	
MELF			
Positive	1 (9.1)	10 (90.9)	0.2
Negative	0 (0)	44 (100)	
Myometrial invasion			
Negative	0 (0)	10 (100)	0.08
<half depth	0 (0)	36 (100)	
>half depth	1 (11.1)	8 (88.9)	
Invasion pattern			
Infiltrative	1 (5.9)	16 (94.1)	0.211
Expansive	0 (0)	26 (100)	
Surrounding endometrium			
Hyperplastic	0 (100)	24 (100)	0.332
Atrophic	1 (3.8)	25 (96.2)	

LVI: Lymphovascular invasion, MELF: Microcystic, elongated and fragmented pattern

endometrial hyperplasia. Endometrial cancer is the most common malignancy of the female genital system. Reduction of cervical cancer rates by population screening programs has increased the clinical importance of endometrial cancer. In the United States, 65,950 new cases of endometrial cancer are estimated to occur in 2022, with 12,550 resulting deaths.[12] In 2016, a multicenter study from Turkey reported endometrial cancer as the fourth most common cancer among women.[13]

Stage is the most important prognostic factor in endometrial cancer. In a cohort study published by Jeppesen et al.,[14] 5-year life expectancy in early-stage endometrial cancers was 87.5% for stage 1A, 77.5% for stage 1B, and 69% for stage 2. In the present study, 84.2% of the patients were found to be stage 1A and the disease resulted in remission without additional treatment. During five years of follow-up, there was a 96.4% survival rate and no patient died as a result of their endometrial cancer.

Many genetic mutations, resulting in PTEN inactivation or changed E-cadherin expression, or mutations in *p53* or *K-ras* have been studied in endometrial cancer.[15-19] There are conflicting results regarding Bcl-2 expression in endometrial tissues, but the general view is that Bcl-2 expression is decreased in endometrial cancer.[19,20] This view was supported by our findings with Bcl-2 expression significantly decreased in endometrial cancer patients compared to patients with endometrial hyperplasia.

In 2015, Stewart and Crook reported that loss of PAX2 expression in endometrial cancer was associated with poor prognosis.[21] In the same year, Joiner et al.[22] examined PAX2 expression in relation to World Health Organization (WHO) and endometrial intraepithelial neoplasia (EIN) classification of endometrial neoplasms and found no correlation between WHO 1994 classification and PAX2 expression. However, a decrease in PAX2 expression was associated with EIN status in patients. These authors suggested that decreased PAX2 expression can be used as a marker for progression to malignancy.[22] Kahraman et al.[23] examined the expression of PAX2 in hyperplastic and cancerous endometrial tissues in 2012 and reported that PAX2 expression was increased in cancerous tissue, which is in contrast to our findings. Quick et al.[24] studied PAX2 expression in patients with EIN and found that the reduction of PAX2 expression was valuable in the diagnosis of EIN, premalignant lesions that are precursors of endometrial cancer. In the present study, PAX2 expression was significantly reduced in cancerous tissues compared to healthy control tissues. However, when compared with other prognostic factors, there was no significant difference in those, with the exception of MELF pattern. Increased PAX2 expression was found in MELF positive cases. Increased Bcl-2 expression scores were associated with MELF pattern, which is one of the criteria that has been considered pathologically in recent years. MELF pattern was also found to be more frequent in cases with lymphovascular stromal invasion. In the present study, Bcl-2 and PAX2 scores were higher in MELF-positive patients.

Lymphovascular invasion is associated with recurrence and mortality in early stage grade 1 tumors, regardless of histologic types of endometrial cancer. In a study by Hanson et al.,[25] the rate of lymphovascular invasion was reported as 2% in grade 1 tumors, 5% in superficial myometrial invasion, 42% in grade

3 tumors and 70% in deep myometrial invasion. In the present study, lymphovascular invasion was not present in any patients without there also being myometrial invasion. Furthermore, lymphovascular invasion was found in 10.5% with less than half the depth of myometrial invasion but present in 66.7% of patients with myometrial invasion deeper than half the depth of the myometrium.

Tumor size is known to be one of the factors affecting prognosis in endometrial cancer. Senol et al.[26] reported that tumors smaller than 2 cm had a lower risk of lymph node spread (4%), whereas this rate increased to 15% in tumors larger than 2 cm. If the tumor covers the entire uterine cavity, the risk of lymph node metastasis was as high as 35%. Similarly, 5-year survival rate was 98% for tumors smaller than 2 cm, 84% for tumors larger than 2 cm, and 64% for tumors filling the entire uterine cavity.[26] In the present study, there were 22 patients with tumor size smaller than 1 cm, and 35 patients with tumor size larger than 1 cm. When Bcl-2 scores and PAX2 expression were compared with respect to tumor size, there was no significant difference.

Myometrial invasion is a prognostic factor that changes stage in endometrial cancer and also decreases life expectancy with lymph node metastasis.[27] Kaku et al.[28] in a study of non-invasive or superficial invasive tumors reported that the 90% 5-year survival rate was reduced to 60% when there was deep myometrial invasion. In the light of this, we compared myometrial invasion and Bcl-2 scores. However, we found no difference in Bcl-2 and PAX2 expression and myometrial invasion status.

Our findings should be considered in the light of the following, which may be considered limitations. There was a high rate of 5-year survival in patients with grade 1 endometrioid adenocarcinoma, there is good prognosis with this diagnosis, there were no patients who died primarily from endometrial cancer, and only one patient had recurrence of the disease.

CONCLUSION

Recently, developing genomic factors, especially immunohistochemical evaluation of changes in expression of transcription factors has been suggested as being useful when considering prognosis and for identifying patients who require adjuvant therapy. Recently, two immunohistochemical methods, based on the detection of changes in the protein expression of the *PAX2* and *Bcl-2* genes have been described. While the results of the present study suggest that Bcl-2 and PAX2 protein expression scores appear to be useful for separating cancer tissue from benign hyperplastic tissues, more studies should be performed before these biomarkers can be incorporated in routine diagnosis. However, these developments look promising for the future clinical management of endometrial cancer and may be used to determine the requirement for both surgical planning and post-surgical adjuvant methods by evaluating endometrial specimens using immunohistochemical techniques.

Ethics

Ethics Committee Approval: Approval for this study was received from Kocaeli University Non-Interventional Research Ethics Committee (dated 19.10.2016 and numbered KÜ GOKAEK 2016/ 287).

Informed Consent: A written informed consent was obtained from each patient.

Authorship Contributions

Surgical and Medical Practices: M.K., L.A., A.Ç, Concept: M.K., H.D., B.A., A.Ç, Design: M.K., B.A., A.Ç, Data Collection or Processing: M.K., M.Ç.K., L.A., H.D., Analysis or Interpretation: M.K., M.Ç.K., H.D., A.Ç, Literature Search: M.K., M.Ç.K., L.A., B.A., Writing: M.K., L.A., H.D., B.A., A.Ç.

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Case Report

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Ultrasound Guided Monofetal Aspiration and Cerclage Management of Heterotopic Cervical Pregnancy in In Vitro Fertilization Twins

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ABSTRACT

This article reports the management of monofetal heterotropic cervical pregnancy in a twin pregnancy after in vitro fertilization treatment. A 23-year-old female patient with a twin dichorionic diamniotic pregnancy was diagnosed to have a six-weeks old live heterotropic cervical pregnancy and a concomitant intrauterine live pregnancy. She underwent abdominal ultrasound-guided transvaginal monofetal aspiration of the cervical heterotopic pregnancy treatment using a Karman cannula no 6. A double cervical Shirodkar suture was placed to stop active bleeding from the endocervix. After placing the Shirodkar suture on isthmic region and Mc Donald suture on distal cervix, a three cm intrauterine hematoma occurred and resolved within three weeks. The patient delivered uneventfully at 39 weeks of gestation through vaginal route. In such cases, it is possible to surgically terminate the heterotropic pregnancy through transvaginal aspiration under ultrasound guidance and stop cervical bleeding via double cerclage.

Keywords: Heterotropic cervical pregnancy, in vitro fertilization, shirodkar cervical suture

INTRODUCTION

Heterotropic pregnancy is the rarest type of all ectopic pregnancies. Although its frequency is 1/30.000, this rate has increased to 1% with the use of assisted reproductive techniques.[1] It is a dangerous type of pregnancy that can cause mortal consequences for the mother and intrauterine healthy pregnancy.[2] It may cause vaginal bleeding in the early period of pregnancy and may or give any symptoms. Heterotropic pregnancies can be diagnosed with transvaginal ultrasound early weeks of gestation.[3] Cervical heterotropic pregnancies have a treatment challenge unlike laparoscopic removal of tubal heterotropic pregnancies and cervical ectopic pregnancies where no concerns of a remaining embryo exist.[4] Reported management methods for heterotropic cervical pregnancies are uterine artery embolization, ultrasound-guided KCL or methotrexate injection and evacuation of pregnancy by hysteroscopy.[5]

In this case, we report the method of termination of heterotropic cervical pregnancy detected in the early period with transvaginal ultrasound-guided aspiration and cervical cerclage to prevent bleeding with term delivery of the intrauterine gestation.

CASE REPORT

A 23-year-old woman admit with the diagnosis of unexplained infertility. She was married for 4.5 years with no history of surgery, pelvic infection and her hysterosalpingography was normal. There was no pregnancy despite four cycles of clomiphene citrate use. At her first in vitro fertilization (IVF) attempt short agonist protocol with a total of 2400 unit of rFSH and eight days of 0.25 mg triptorelin was used. Trigger was performed on 8th day of treatment with 250 mcg rhCG. Two day 3 embryos were transferred under transabdominal ultrasound-guidance to the supra-isthmic region, 1 cm below the fundus of the uterin cavity. At 10 days after transfer, bHCG was positive. Estradiol 4 mg and progesterone in oil intramuscular was used for luteal support until 10th week of gestation.

The pregnant admitted to us with vaginal bleeding. Two sacs were observed at routine transvaginal ultrasound performed at 6 weeks of gestational age (Figure 1). One of the fetuses was observed in the normal uterine cavity, the crown-rump length was 6 mm, the sac was reguler and the fetal heartbeat was 121 bpm/m. the other fetus was visualized as located in the cervical canal with transvaginal ultrasound. It's crl was 6.2 mm and heart beat was 122 bpm/m. Its gestational sac was regular, too.

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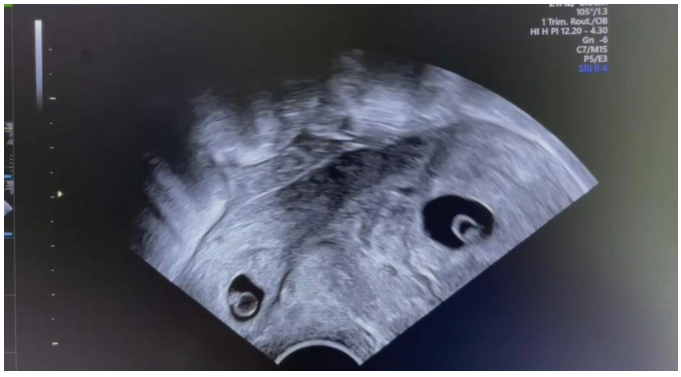

Figure 1. Monofetal heterotopic pregnancy in twin pregnancy after in vitro fertilization

In speculum examination, dilatation of the cervix was observed. Cervical length was measured as 15 mm to the cervical sac in transvaginal ultrasound. We recommended the patient to evacuate the cervical pregnancy and cerclage for the remaining fetus.

After cervicovaginal cleaning with povidone iodine under intravenous sedation, the anterior part of the cervix was held with the Allis clemps. There was evidence of cervical dilatation as no.8 bougie. The cervical sac was aspirated with the Karman cannula no.6 under transabdominal ultrasound guidance. Cervical pregnancy cleared completely. Shirodkar double cerclage suture was used to prevent possible cervical bleeding. One of the shirodkar double cerclage sutures was put on the isthmic region by using mersilene tape and the other by no.1 vicryl on the distal cervix. 1 g of cefazolin was administered intravenously to the patient during the procedure.

The patient was discharged the next day and she used 50 mg intramuscular progesterone and 4 mg estradiol daily. When she came to the control after one-week, cervical length was measured as 32 mm on transvaginal ultrasound. A hematoma area of approximately 3*2 cm by 4 cm was seen in the cavity around the remaining fetus.

The patient was informed about the current situation and it was explained that the treatment should be continued with the same drugs and doses and followed-up weekly until ten weeks of gestation. The hematoma resolved in three weeks and the patient delivered via vaginal route 39 weeks of gestation uneventfully. Our patient gave informed consent for this case report.

DISCUSSION

While the incidence of cervical pregnancy is between 1/6000 and 1/8000, both this rate and the incidence of cervical ectopic pregnancy have increased with the use of assisted reproductive techniques.[6] After the IVF procedure that resulted in pregnancy, the incidence of heterotropic cervical pregnancy is 1% to 3%.[7] There is no common view on the causes of ectopic pregnancy after IVF. Some common features such as cervical abnormality or previous curettage were encountered in patients with ectopic pregnancy.[8] In our case, there was no previous surgery or an abnormality detected in hysterosalpingography.

Some studies have shown that the embryo transfer methods used can also cause abnormal placement.[9] We performed our embryo transfer in the supraisthmic region, approximately 1 cm below the fundus.

As in our case, a rapid and safe increase in bHCG is observed in these pregnant women because of multiple pregnancy.

These pregnant women usually present with abnormal vaginal bleeding. Although rare, abdominal pain and spontaneous abortion may also be seen. Vaginal bleeding and other complications can reach life-threatening stage.[6] Patients rarely have any symptoms. The definitive diagnosis is made with a gestational sac located in the cervical canal in transvaginal ultrasound. Doppler ultrasonography can be used to confirm the diagnosis in the area where cervical pregnancy is suspected. Doppler sonography shows increased blood flow in the suspected pregnancy area.[10]

In our case, the pregnant woman had a complaint of painless vaginal bleeding. When the bleeding started, the pregnancy was 5 weeks and 6 days according to the last menstrual date. In the transvaginal ultrasound performed, fetal findings compatible with cervical localized 5 weeks and 6 days were observed, too. Intrauterine pregnancy was also compatible with the last menstrual period. both sacs were regular and there was no bleeding area.

Cervical pregnancy should be terminated immediately after diagnosis. In the literature, there is no common opinion about the method of termination of cervical pregnancy.

The most important aim is not to endanger the life of the pregnant and to ensure the continuation of a healthy intrauterine pregnancy.

Cervical pregnancy can be terminated surgically or pharmacologically treatment. The most commonly used pharmacological agents are methotrexate and potassium chloride. potassium chloride is injected into the sac under ultrasound guidance only. Methotrexate can be given both systemically and locally, it should not be preferred because pregnancy in the cavity will also be affected when given systemically. Other fluids that will create positive oncotic pressure, such as hyperosmolar glucose and sodium chloride are also used as an alternative.[11,12]

Dilatation and evacuation (D&E), aspiration, cerclage, extraction with forceps, foley catheter insertion, electrocauterization are other methods used to terminate cervical pregnancy. There is no consensus on which ones should be used to ensure the continuation of a healthy pregnancy.[13] Surgically, ligation of the hypogastric artery, uterine artery embolization and hysteroscopic cervical pregnancy removal are among the methods used.[7]

In 2022, Fan et al.[14] reported a succesful aspiration for removal of cervical pregnancy under ultrasound guidance, their aim was protecting the intrauterine pregnancy. They also used a gauze with tranexamic acid in order to cease the bleeding. Bleeding was ceased after 40 min later and blood loss was around 40 mL.

Honda et al.[15] reported their treatment as following: in order to prevent hemorrhage vazopressin was injected afterwards

curretage performed following by methotrexate injection with intent of halt residual trophoblast evolution .

Prorocic and Vasiljevic[7] described a cervical heterotopic pregnancy together with twin intrauterine pregnancy. In order to sustain the twin intrauterine pregnancy they used 2 DEXON sutures bilaterally on the cervix at the level of fornix vaginae performed a transvaginal ultrasound guided aspiration followed by hypertonic sodium chloride injection to the gestational sac. They removed the cervical pregnancy and reported no side effects after the treatment.[7]

In 2021 Schivardi et al.[16] reported the first usage of Micro Wave Ablation in a cervical heterotopic pregnancy with an intrauterine gestation. They used transabdominal guidance and insterted the antenna inside the cervical sac in a transvaginal manner and performed the ablation. They prevented the intrauterine pregnancy and didn't describe any bleeding nor uterine contractions.[16]

In this case, we did not want to use any chemical agent in order to preserve the normal pregnancy in the cavity. We avoided surgical intervention. We aspirated the cervical pregnancy by vacuum curettage. Cerclage was applied to prevent the other pregnancy from being aborted.

Antibiotic therapy and progestrone was given to prevent possible infection and vaginal bleeding. In thıs case, the pregnancy is now 10 weeks and continues in a healthy way with a minimal bleeding area around the sac.

CONCLUSION

In this case we report successful surgical termination of heterotropic pregnancy with ultrasound guided double cerclage. On the other hand the best approach in these rare cases are yet to be determined.

Ethics

Informed Consent: Our patient gave informed consent for this case report.

Authorship Contributions

Surgical and Medical Practices: G.A., E.Ç., Concept: G.A., Design: G.A., Data Collection or Processing: G.A., E.Ç., Analysis or Interpretation: E.Ç., Literature Search: G.A., Writing: E.Ç.

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Case Report

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Case Report: Clitoral Epidermoid Cyst Related to Female Genital Mutilation as a Long-term Complication

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ABSTRACT

Female genital mutilation (FGM) has been performed at least 200 million women around all over the world and this practice bring up different short- and long-term complications. We report a case of a patient with FGM history and large clitoral epidermoid cyst as a long-term complication related to FGM. The FGM practice has been criminalized and eradicated by law in many countries and it is considered as a human rights violation. Nineteen-year-old nulligravid woman from Somali presented with a genital mass. The patient has a Type 3a female mutilation history with the excision of clitoral hood when she was 5 years old. She has difficulties of walking, wearing trousers and voiding dysfunction because of the large genital mass which has been gradually progressed during last 2 years. External genital examination revealed 7-8 cm mobile, painless mass that originated from clitoris and covered by stretched clitoral and labial epidermis, clitoris was lateralized, vaginal introitus was open 2-3 cm. Magnetic resonance imaging scan suspected a benign cyst without any invasion signs. We suspected the vulvar endometrioma during transperineal ultrasound examination with ground-glass image and hypoechogenic view. We performed the total excision of the clitoral mass, clitoral reconstruction and labioplasty. Pathological findings resulted as an epidermoid cyst. FGM may cause vulvar mass including epidermoid cyst as a long-term complication. Clitoral reconstruction might be necessary for Type 3a FGM cases.

Keywords: Female genital mutilation, epidermoid cyst, clitoral mass

INTRODUCTION

According to World Health Organization (WHO), Female genital mutilation (FGM) is any procedure that involves the partial or total removal of external genitalia for non-medical reasons.[1] Approximately 200 million women had undergone FGM procedures in more than 30 countries around the world. This procedure is commonly performed due to religious and cultural tradition.[2] FGM is considered as violence against basic human rights.[3] This procedure is commonly performed in inefficient sterilized and inadequate equipped places that usually ends up with many early complications.[4] Women who have undergone FGM may suffer long-term compilations such as dysmenorrhea, dyspareunia, urinary obstructive pathologies, vulvar and clitoral abscesses and cysts, vaginismus, vaginal stenosis, lack of sexual satisfaction.[5]

WHO classifies FGM into 4 major groups. Type 1 is excision of the prepuce with or without excision of the clitoris. Type 2 is excision of the prepuce and clitoris with partial or total excision of the labia minora. Type 3 is excision of part or all of the external genitalia and stitching/narrowing of the vaginal opening (infibulation). Type 4 includes all other procedures with the aim of tightening or narrowing, such as pricking, piercing, or incision of the clitoris or labia; cauterization by burning of the clitoris and surrounding tissues; scraping of the vaginal orifice; cutting of the vagina.[6] Among these types the third type is the most extreme and catastrophic method.

CASE REPORT

A 19-year-old single nulligravida woman from Somali presented with slowly progressed genital mass that has grown gradually in last 2 years causing significant discomfort. She had undergone female genital cutting procedure when she was 5 years old in Somali. The procedure had been performed by a non-medical traditional practitioner at home without any analgesic.

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Perineal examination revealed 7 cm mobile, non-tender, rounded cystic mass partially obstructing urethral meatus, stretching the labia minora, originated from previously performed FGM scar on clitoris (Figure 1). During the FGM procedure the clitoral body was removed partially and eventually lateralized and the vaginal introitus was left open with 2-3 cm opening. Magnetic resonance imaging scan suspected epidermoid cyst with no invasion sign. We suspected the vulvar endometrioma during transperineal ultrasound examination with ground-glass image and hypoechoic view. Under general anesthesia the cyst was totally excised without rupturing. The cyst filled with dark brown keratinous material. After the cyst excision previously lateralized clitoris was moved to the normal anatomical area with reconstructive method and labioplasty was performed by removing the excess enlarged labial and clitoral skin. The patient did not have any functional or anatomical complaints at the first week and third month postoperative follow-up (Figure 2). Patient satisfaction was measured by the patient global impression of improvement method and it was noted scale 6. "Much worse" before the surgery and was improved to scale 1. "Very much better".[7] According to the female genital self-image scale (FGSIS) patient's perception of her own genital appearance recovered from 9 points up to 27 points of FGSIS.[8] The patient has been informed about the case report process and she has given consent for her case to be published in the scientific journal.

DISCUSSION

In the literature there have been reported only few cases clitoral giant cysts secondary to FGM as a long-term complication.

One of these cases has been reported a 40-year-old multipara woman with FGM history and 11x10.6 cm cyst weighed 1.9 kg which was significantly massive clitoral inclusion cyst.[9]

Figure 1. Clitoral cyst

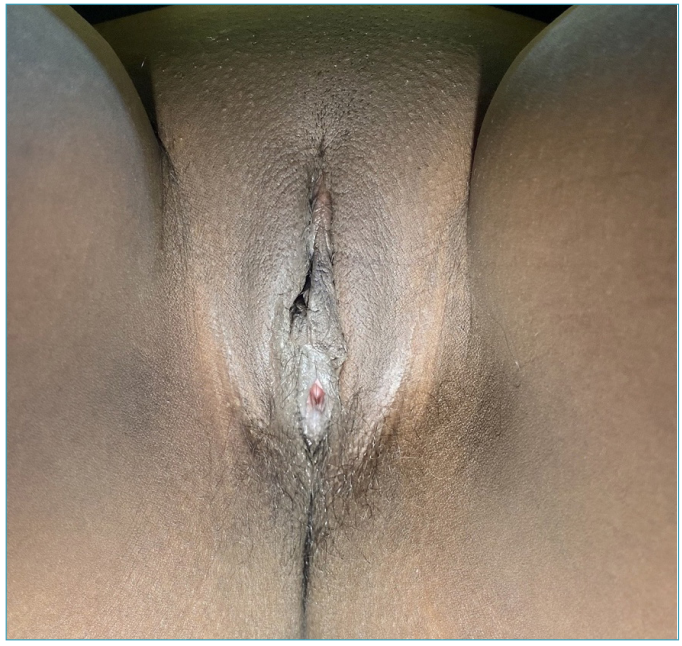

Figure 2. Postoperative 3rd month

Another case has been reported a 19-year-old woman circumcised at birth and as a late complication she had enlarging vulvar mass that arose after the onset of puberty.[10] Other reports are about two women aged 39 and 27 with childhood circumcision history and epithelial inclusion cyst of clitoris.[11]

Foldes and Louis-Sylvestre[12] published 453 patients who had undergone ritual childhood surgeries and later requested reconstructive clitoral repair surgery. This study claimed the repair surgery with preservation of clitoris provides promising sexual satisfaction and cosmetic results.[12]

FGM procedures are still performed as a part of a religious and cultural traditions all over the world as it brings up early and late complications. The UN has banned the FGM procedures since 2012 to eliminate this practice.[13] FGM is outlawed in the United States as the new immigrants might be imprisoned up to 5 years who attempt or arrange FGM for their daughters.[14]

Clinicians are facing more and more long-term complications of FGMs due to increasing immigrants and globalization of cultural traditions.

Although female genital cutting is an important cultural and religious tradition for some nations and societies, determining the medically expected short-term and long-term complications would be beneficial and discussing more issues and cases will be profitable to create perception of avoiding FGM procedures for themselves and for their daughters.

CONCLUSION

Vulvar or clitoral epidermoid cysts should be kept in mind as a long-term complication following FGM Type 3a cases. Reconstruction of normal perineal anatomy should be aimed.

Ethics

Informed Consent: The patient has been informed about the case report process and she has given consent for her case to be published in the scientific journal.

Authorship Contributions

Surgical and Medical Practices: G.A., T.D., O.D., M.Y., Concept: G.A., T.D., O.D., M.Y., Design: G.A., T.D., O.D., M.Y., Data Collection or Processing: G.A., O.D., Analysis or Interpretation: G.A., O.D., M.Y., Literature Search: T.D., Writing: G.A., T.D., M.Y.

Conflicts of Interest: The authors declare that there is not any conflict of interest regarding the publication of this manuscript.

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Case Report

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A Rare Case Report: Atypical Endometrial Hyperplasia and Combination of Placental Site Nodule and Treatment Follow-up

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ABSTRACT

Placental site nodules are benign, non-neoplastic lesions, usually seen incidentally in the pathology results of curettage materials, cervical biopsies or hysterectomy materials of women of reproductive age. It originates from intermediate trophoblasts at the implantation site. Although it is a benign lesion, its histopathological distinction from trophoblastic and other malignant neoplasms is important. A 32-year-old female patient, who had given vaginal birth twice before and who had no history of abortion, consulted with the complaint of vaginal bleeding lasting for a month. In transvaginal ultrasonography, the endometrium was 17 mm, the irregularity and thickness increase. Beta human chorionic gonadotropin (β-hCG) test result was negative. Fractional curettage (F&C) was performed. It was reported as "Hyalinized stroma fragments (consistent with placental site nodule) showing atypical endometrial hyperplasia and trophoblastic cell proliferation". Primolut-N three times a day was prescribed. The patient wanted to keep her fertility the for continuing with medical treatment was decided. A new medicine, Megace 160 mg twice a day was prescribed. The control pathology result was reported as "Hyalinized stroma fragments showing trophoblastic cell proliferation (consistent with placental site nodule). The lesion in 4 focus, the largest of which was 2 mm and the others were 1 mm in diameter and showed P63 and PLAP positive staining with the applied immunohistochemical method. Ki67 proliferation index is 2-3%". Close follow-up was planned for the patient every 6 months. Our case report is the first in the literature in terms of the association of atypical endometrial hyperplasia and placental site nodule. In a female of reproductive age who presents with the complaint of excessive vaginal bleeding that does not follow pregnancy, β-hCG should be checked, previous pregnancy or miscarriage history should be questioned, in case of negative results, a benign lesion, a placental site nodule, should be included in the differential diagnosis.

Keywords: Placental site nodule, atypical endometrial hyperplasia, vaginal bleeding, fractional curettage, female

INTRODUCTION

Placental site nodule is included in the group of rare trophoblastic tissue proliferations in the classification of the World Health Organization and the International Society of Gynecological Pathologists.[1] Placental site nodules originate from small, well-circumscribed, nodular chorionic-type intermediate trophoblasts embedded in the hyalinized stroma.[2] Most of the cases are detected incidentally during curettage or biopsies and patients usually present with irregular uterine bleeding, recurrent spontaneous abortion, abnormal cervical smears, postcoital bleeding and rarely infertility. It is a benign lesion, but due to its histological appearance, it can be confused with malignant neoplasms such as trophoblastic and squamous cell carcinoma.[3]

CASE REPORT

A 32-year-old female patient with Gravidity 2 Parity 2, who had given vaginal birth twice before and who had no history of abortion, consulted to our obstetrics and gynecology clinic with the complaint of vaginal bleeding lasting for a month. The patient with a known diagnosis of Type 2 Diabetes Mellitus and using Glifor 850 mg 2x1 had no previous operation or history of smoking. There was no feature in the family history. On speculum examination, the collum was multiparous and bleeding. In transvaginal ultrasonography (TVUSG), the endometrium was 17 mm, the irregularity and thickness increase in the fundus part was evaluated in favor of endometrial polyp. Bilateral ovaries were normal. Complete blood count (CBC), coagulation, biochemistry, tumor markers, Beta human chorionic gonadotropin (β-hCG) were requested

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from the patient. The patient's CBC, biochemistry and tumor markers were within the normal range; β-hCG was negative. Pap smear was taken and the result was reported as "negative for intraepithelial lesion or malignancy". Fractional curettage (F&C) was performed. The pathology result was reported as "Hyalinized stroma fragments (consistent with placental site nodule) showing atypical endometrial hyperplasia and trophoblastic cell proliferation". As treatment patient given Primolut-N 3x1. Hysterectomy operation was offered, but patient wanted to keep her fertility the for continuing with medical treatment was decided. Megace 160 mg 2x1 treatment was started. The patient was called for monthly liver function tests control. The control F&C was planned after 5 months. The patient's results of liver function tests were within normal ranges. Pipelle endometrial sampling was taken from the patient because the endometrium was irregular in TVUSG. The pathology result was reported as "endometrium under the influence of gestagen". It was planned for the patient to continue on Megace 160 mg 2x1 treatment. The control F&C was performed after 4 months of treatment. The pathology result was reported as "Hyalinized stroma fragments showing trophoblastic cell proliferation (consistent with placental site nodule), shedding in the surrounding endometrium and irregular proliferation findings; The lesion was observed in 4 focus, the largest of which was 2 mm and the others were 1 mm in diameter. The lesion showed P63 and PLAP positive staining with the applied immunohistochemical method. Ki67 proliferation index is 2-3%" (Figure 1). Close follow-up was planned for the patient every 6 months. Our study is patient-approved and has patient consent.

DISCUSSION

Placental site nodules originate from small, well-circumscribed, nodular chorionic-type intermediate trophoblasts embedded in the hyalinized stroma.[2] Benign proliferation of intermediate trophoblasts is placental site nodule, and malignant proliferation is placental site trophoblastic tumor.[4] Today, information about placental site nodule is limited to a small number of case series and case reports. In these studies, it was generally shown to be associated with placental site tumor or placental site trophoblastic tumor.[2,4] However, the placental site nodule seen with atypical endometrial hyperplasia is presented as a case report for the first time in the literature. High β-hCG values are observed in placental site trophoblastic tumor and choriocarcinoma, and it is used under doctor's follow-up.[2] In our case, the β-hCG value was negative. It has helped to recognize this distinction. Immunohistochemically, low Ki67 index and PLAP positivity are also important findings in the differential diagnosis of placental site nodule.[2,3] In our case, P63 and PLAP showed positive staining. Ki67 proliferation index is 2-3%. In a study, 40% of placental site nodules were found in the endocervix and 56% in the endometrium. It has been reported that it is rarely observed in the fallopian tube due to a previous tubal pregnancy.[2,5,6] In most of the cases, there is a history of therapeutic abortion or cesarean section.[7] In our case, the difference from the other cases presented in the literature was that there was no history of therapeutic abortion or cesarean section.

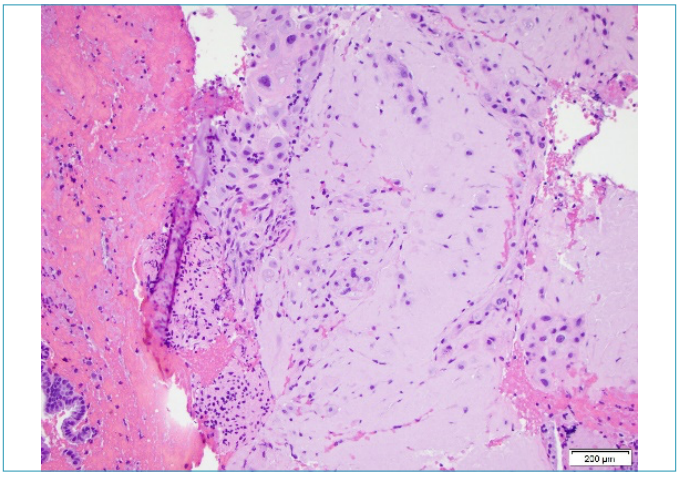

Figure 1. Placental site nodule pathology

Placental site nodule is usually seen after intrauterine pregnancies, although it has been reported that the interval can be as long as 1 month to 8 years, the average duration is 36 months.[1,8] Our case is included as a rare case report since last time the patient gave birth was 5 years ago.

Placental site nodules are benign, non-neoplastic lesions. In the study of Huettner and Gersell,[9] two recurrent placental site nodules occurred in the follow-ups of the patients, and no trophoblastic disease or gynecological malignancy development was detected in any of the patients. In our case, control F&C every 6 months after diagnosis, β-hCG follow-up and clinical follow-up were recommended.

CONCLUSION

Although our case report is the first in the literature in terms of the association of atypical endometrial hyperplasia and placental site nodule, it is among the rare cases in many respects.

In a female patient of reproductive age who presents with the complaint of excessive vaginal bleeding that does not follow pregnancy, β-hCG value should be checked, previous pregnancy or miscarriage history should be questioned, and in case of negative results, a benign lesion, a placental site nodule, should be included in the differential diagnosis.

Ethics

Informed Consent: Our study is patient-approved and has patient consent.

Authorship Contributions

Surgical and Medical Practices: A.G.Z., C.T, Concept: A.G.Z., C.T., Design: A.G.Z., C.T., Data Collection or Processing: A.G.Z., C.T., Analysis or Interpretation: A.G.Z., C.T., Literature Search: A.G.Z., C.T., Writing: A.G.Z., C.T.

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