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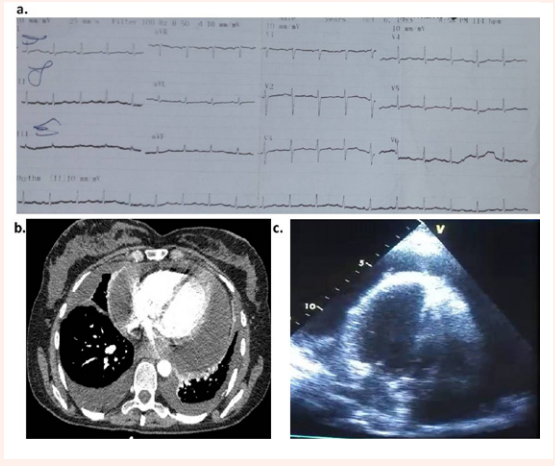

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Endometrial pipelle scratching may decrease abortion rates rather than increasing pregnancy rates in intrauterine insemination cycles

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ABSTRACT

To assess the efficacy of endometrial pipelle scratching (EPS) preceding intrauterine insemination (IUI) cycles. A total of 348 patients with unexplained infertility were enrolled into the study. 117 women with EPS were compared with 231 women without EPS and IUI. Livebirth and pregnancy rates and abortion rates were the primary outcomes. There were no difference in age, duration of infertility, basal hormone and total gonadotropin dose used throughout the cycle and endometrial thickness. In terms of pregnancy outcomes, pregnancy and livebirth rates in EPS group were17.1% and 15.7% respectively, while pregnancy and livebirth rates in Non EPS group were 23.8% and 16% respectively. Spontaneous abortion rate in EPS group was significantly lower than Non EPS group (1.7% vs. 7.8%) which was statistically significant (p = 0.021). EPS did not improve pregnancy and livebirth rates. However, abortion rates were significantly lower in EPS patients. EPS may not have any impact on the embryonic implantation but may improve the proper development of implanted embryos by modulating the local factors.

1. Introduction

IUI is an inexpesive and easily performed, common assisted reproductive technique. Pregnancy outcomes of IUI in the literature ranges from 4% to 24% per cycle (Papillon-Smith et al., 2015). IUI is often the preferred firstline treatment in; unexplained, cervical factor and mild to moderate male factor infertility (Veltman-Verhulst et al., 2016). Clinical pregnancy rates may be influenced by the method of ovarian stimulation, type and duration of infertility, sperm parameters and ovarian stimulation response, female age and endometrial scratching (Luco et al., 2014).

It is the implantation of the embryos that remains to be solved in the modern era of ART and it seems that there

is still a long way to go to understand the implantation process. It is assumed that almost ¾ embryos failed to implant during implantation period (Afsoon et al., 2014). The attractive mechanisms between embryos and the receptive endometrium are two key factors for succesful implantation to occur (Diedrich et al., 2007)

Endometrial scratching, also named as trauma, injury or biopsy, is a simple and microinvasive procedure which can be applied in outpatient conditions. Endometrial scratching can also be applied by hysteroscopy. The mechanism of action of endometrial injury may be hypothetised as; a) endometrial decidualization b) healing which leads to the immune reaction and c) endometrial maturation to improve the synchrony (Jeffrey and Goldberg, 2018).

Animal studies revealed that endometrial trauma provoke the receptivity and decidualization of the endometrium (Finn and Martin, 1972). Scratching th endometrium in either follicular phase or in luteal phase was positively correlated with the increase in the pregnancy rates in IVF (Barash et al., 2008). The luteal phase endometrial scratching carries a risk to damage the developing ealy pregnancy thus scratching in the follicular phase seems much more acceptable (Jeffrey and Goldberg, 2018). Scratching of the endometrium become a controversial issue in ART practive since some report that endometrial injury was associated with increased pregnancy rates (Zhou et al., 2008) while others prove that this procedure may have negative impact on the clinical outcomes (Karimzade et al., 2010). Endometrial scratching during IUI cycles is not extensively studied compared to IVF (Gnainsky et al., 2010; Goel et al., 2017; Senocak et al., 2017).

This study aims to present the impact of endometrial injury in the follicular phase of previous menstrual cycle on the clinical outcomes of IUI cycles in couples with unexplained infertility and mild male subfertility.

2. Materials and methods

This is a retrospective case control study conducted at the fertility clinic of Maternity Hospital, Samsun Training and Research Hospital, Turkey. 117 IUI patient files including EPS were driven from the archives and included in Group 1 while 231 IUI patient files without EPS were included in Group 2 as controls between January 2014 and June 2017. EPS for better clinical outcome were offered to IUI couples and the ones preferred EPS before IUI were compared with the ones refused to have EPS. All participated couples in both groups had basal fertility evaluations including tubal patency test, transvaginal ultrasound examination, semen analysis, a battery of basal hormonal evaluation and serological tests. Written informed consent for each patient were taken and recorded. This study was approved by the institutional review board of Medicana Samsun International Hospital by a grant number of 1997/08.03.2019.

Files not covering the basal evaluations were not included in the study. For obtaining homogenuity in both groups, only unexplained infertiliy and mild male subfertility cases were enrolled into the study.Thus IUI performed due to other factors were also excluded from the study. EPS and Non EPS applied IUI patients performed 42 hours after hCG administration were excluded from the study.

Demographic features and baseline clinical characteristics of participants were collected from the files and recorded to a database program for statistical analysis.

Ovarian stimulation, IUI luteal phase support and pregnancy confirmation by B-hCG test

All participating women had a diagnosis of unexplained infertility. Recombinant gonadotropin 75 IU (Gonal-f, Merck Serono, Switzerland) SC daily was started on day 3 of menstrual cycle and patients were monitored for follicular response. Patients were called on day 7 and day 10 for follicular monitorization. Gonadotropin stimulation continued until the leading follicle reached 17mm and a single dose of recombinant hCG 250 microgram (Ovitrelle, Merck Serono, Switzerland) injected subcutaneously 36 hours before IUI. IUI was performed under outpatient conditions in Maternity Hospital. A sterile speculum was used to visualize the uterine cervix and cervix was cleansed with isotonic solution. An IUI soft catheter (Cook catheter, Tekservis Medical, Turkey) was inserted through the cervical canal and prepared sperm was injected through the catheter in 60 seconds. A 5 more second was spent for catheter removal. Oral progesteron 100 mg (Progestan tablet, Koçak Farma, Istanbul, Turkey) thrice a day was started as luteal support. A blood test for B-hCG were recommended 15 days after IUI procedure to evaluate the pregnancy outcome.

Sperm preparation for IUI

The semen sample for insemination was analyzed for conventional semen parameters (volume, sperm count, and motility). Semen samples were collected by masturbation into a sterile semen jar in sperm giving room located in the IVF center and processed in the IVF laboratory according to the principles of discontinuous density gradient centrifugation. Semen sample was handled 2 hours before IUI procedure for preparation. Sperm findings after preparation for IUI were recorded. Then prepared sperm in an injector was handled by the patient to keep the body temperature until sample is inseminated by the following physician. A sterile soft IUI catheter was given to the patient to overcome inconvenience that may result from the absence of catheter in maternity hospital.

Sperm is incubated in CO_2 incubator for liquification for 30 minutes in order to get rid of prostaglandin-rich seminal and prostatic secretions. Then sperm is cultured by G1 medium (Vitrolife, Denmark) and sperm is

evaluated under Macler counting system and findings were recorded.Sperm was prepared by continuos density gradient centrifugation and flushed thereafter. Following the washing step, the obtained pellet was suspended in 0.5 mL G1 medium and loaded to an insulin injector .The loaded injector and a catheter for insemination were given to the partner of the patient to be used in insemination procedure.

Endometrial Pipelle Scratching (EPS)

Mechanical endometrial trauma or scratching were performed in the follicular or proliferative phase of menstrual cycle preceeding the IUI. 12 times scratching were performed to induce the acute ingflammatory reaction on the ndometrium. Pipelle (Pipelle de Cornier, Prodimed, France) was preferred to perform endometrial scratching.

Statistical analysis

Descriptive statistics for continuous variables were expressed as mean ± standard deviation or median (minimum–maximum), and nominal variables were expressed as the number and percentage (%). Differences in mean values for each group were evaluated using the Student's t-test, and differences in median values were evaluated using the Mann–Whitney U-test. Categorical data were compared using the Chi-square distribution, with p-values of ≤0.05 considered as statistically significant. Statistical analysis was performed using SPSS for Windows version 22 software (SPSS, Inc., Chicago, IL, USA).

3. Results

A total of 348 patients presented with primary infertility due to unexplained infertility and mild male subfertility were enrolled. 117 patients whom applied EPS were included in group 1 and 231 patients whom did not applied EPS were included in Group 2 for comparisons. Each couple has one cycle of IUI.

Table 1. Baseline characteristics and serum hormone levels of participants. Numerical data presented as mean (SD) or median (25th to 75th percentile).

	EPS (n=117)	Non EPS (n=231)	p values
Age (years)	29.33 ± 6.05	30.33 ± 5.22	0.084
Infertility duration (years)	7.39 ± 4.96	6.23 ± 4.17	0.059
BMI ((kg/m2)	24 (19-28)	23 (19-27)	0.322
Type of infertility Primary Secondary	 78 (66.5%) 39 (33.5%)	 144 (62.5%) 87 (37.5%)	0.728
Basal FSH (mIU/mL)	6.75 (4.0-17.09)	6.50 (3.41-13.14)	0.205
Basal LH (mIU/mL)	4.50 (2.84-12.65)	4.75 (2.31-13.14)	0.135
Basal E2(ng/m)	36.95 (15.05-55.45	35.34 (11.30-51.92)	0.995
Basal Prolactin (ng/mL)	12.75 (6.14-34.30)	13.20 (4.55-34.55)	0.758
Basal TSH (uIU/mL)	1.27 (0.47-2.28)	1.42 (0.71-2.39)	0.438
Endometrial thickness (mm)	8.5 (5-12)	9 (6-12)	0.101
Total gonadotropin dose used	750 (450-1250)	750 (450-1250)	0.304

When the groups were compared in terms of demographic characteristics and baseline hormone levels, there were no difference in age, duration of infertility, basal hormone and total Gonadotropin dose used throughout the cycle and endometrial thickness. Baseline characteristics and hormonal parameters are presented in Table 1.

When the groups were compared in terms of pregnancy outcomes, pregnancy and live birth rates in EPS group were 17.1% and 15.7% respectively, while pregnancy and livebirth rates in Non-EPS group were 23.8% and 16% respectively. Though no statistical significance observed between the groups in terms of live birth and pregnancy rates, number of patients conceived in Non-EPS group was found higher. Spontaneous abortion rate in EPS group was significantly lower than Non-EPS group (1.7% vs. 7.8%) and the difference between the groups was statistically significant (p = 0.021). Pregnancy outcomes are presented in Table 2.

Table 2. Pregnancy outcomes for EPS versus Non-EPS IUI cycles.

	EPS (n=117)	Non EPS (n=231)	p values
Positive β-hCG test	20 (17.1%)	55 (23.8%)	0.150
Pregnacy rate	20 (17.1%)	55 (23.8%)	0.150
Live birth	18 (15.4%)	37 (16.0%)	0.879
Spontaneous abortion	2 (1.7%)	18 (7.8%)	0.021*

4. Discussion

This study aimed to show the beneficial effect of EPS in the follicular phase of previous menstrual cycle on the clinical outcomes of IUI cycles in couples with unexplained infertility and mild male subfertility. It was found that EPS was not associated with increased pregnancy rates in IUI cycles instead the number of pregnancies were lower than Non-EPS patients though not raised statistical significance. The results of this study showed that a microinvasive outpatient procedure, EPS, may have beneficial effect on the proper implantation of the embryos since the abortion rates were significantly lower in EPS group.

Endometrial scratching has long been used in many ways in the clinical practice of ART and is one of the most controversial issues in infertility practice. Simon et al investigated more than 300 publications on the use of endometrial scratching and nearly all papers were found low or poor quality (Simon and Bellver, 2014). Levin et al. (2017) retrospectively evaluated 1810 IVF cycles in which 415 cycles were with endometrial pipelle scratching while 1395 cycles were matched as controls. After completing the matching process, they compared 238 ES cycles with 238 NonES cycles and concluded that mechanical endometrial trauma has nothing to do

with improved implantation and pregnancy rates (Levin et al., 2017). A metaanalysis of 14 randomised controlled trials conducted by Nastri et al. (2015) concluded that endometrial scratching in the luteal phase of previous menstrual cycle or early follicular phase of fresh IVF cycles improves the clinical outcomes (Wise 2013; Nastri et al., 2015). Though it is widely used in IVF practice, there is a very low tendency to use endometrial scratching in ovulation induction or IUI cycles (Lensen et al., 2016). A Cochrane review of nine RCTs with 1512 patients in 2016 conducted by Lensen et al revealed that the quality of evidence fort he use of ES in IUI cycles are low or very low (Lensen et al., 2016). Vitagliano et al. (2018) reproted an update review in 2017 and by their rigorus effort, studies not peer reviewed and with poor quality were excluded and eligible 8 RCTs including 1871 cycles were enrolled into their systematic review. They concluded that ES significantly improves the clinical pregnancy rates without increasing ectopic, miscarriage and multiple pregnancy rates (Vitagliano et al., 2018). Goel et al. (2017) al found significantly high pregnancy rates in thier RCT. They assesded 284 IUI cases and eligible 144 patients were randomised into two groups equally without any dropouts and found 31.9% pregnancy rate in ES group compared to 16.7% pregnancy rate of nonES controls and concluded that ES is beneficial, cost effective and simple method that can be attached in IUI cycles (Goel et al., 2017). Radhakrishan et al., in their randomised case control study evaluated the impact of ES on the clinical outcomes in 240 patients and concluded that ES, by increasing the levels of IL-6 and adhesion molecules like E-Cadherin, may increase the clinical pregnancy rates in IUI cycles (Radhakrishnan, 2015). Gupta et al. (2015) evaluated 64 couples fort he efficiency of ES in their RCT and randomised patients equally and 32 women with 78 IUI cycles with ES were compared with 32 women with non ES. They concluded that ES has better pregnacy and ongoing pregnancy rates (Gupta et al., 2015). Şenocak et al. (2017) evaluated 80 women in their RCT and 40 women with ES by Novak curette to the posterior side of the endometrium were enrolled into group 1 while 40 women without ES were included in control group for comparisons. They concluded that though the clinical pregnancy rates and pregnancy rates were higher than controls, the difference did not reach statistical significance and they recomend more RCTs with larger patient populations (Senocak et al., 2017). Contrary to these datas, Zarei et al. (2014) in their RCTs in 144 patients concluded that the use of ES in previous menstrual cycle has nothing to do with the increased chance of pregnancy (Zarei et al., 2014). However, they add their limitations and problems in design in their study and inconvenience of their patients with their protocol. Kriplani et al. (2016) arranged a RCT and evaluated 124 women and 62 women with ES in IUI cycles were compared with 62 cycles without ES and concluded that though pregnancy rate in ES group is higher, it did not reach a statistical significance and they also recommended larger scale RCTs to document the impact of ES during IUI cycles (Kriplani et al., 2016). In this study, pregnancy rates were slightly lower than Non-EPS patients but abortion rates were found significantly lower in EPS patients which may be explained by the beneficial effect of local mediators on the implanted embryos rather than implantation of embryo itself. This outcome was not reported in the published articles on endometrial scratching in IUI cycles and should be taken into consideration.

The exact mechanism of endometrial injury is not certain but it is aimed to trigger acute inflammatory reaction and to induce the release of certain cytokines and other inflammatory molecules which mimics the implantation process and crosstalks of embryo and endometrium. Further release of macrophages and other immune cells produce an environment resembling endometrial window (Lensen et al., 2016; Ashrafi et al., 2017). This hypothesis was supported by the increased factors found in the second biopsies of women who had endometrial scratching (Gnainsky et al., 2010). Preferrence of pipelle for endometrial trauma may have additional benefit since gentle scratching triggers inflammatory response without producing any harm to the endometrium. Others like Novak curette or vacuum aspirators may produce harmful endometrial injury than expected beneficial effect (Vitagliano et al., 2018).

Here in this study, pipelle was preferred for endometrial scratching in the follicular phase of menstrual cycle precceding IUI cycle.

In conclusion, EPS did not improve pregnancy and livebirth rates and even pregnancy rates were lower than Non-EPS patients although not raised statistical significance. However, abortion rates were significantly lower in EPS patients. EPS may not have any impact on the embryonic implantation but may improve the proper development of implanted embryos by modulating the local factors. Retrospective nature of this study is a limitation and further prospective randomised studies, especially those evaluating the endometrial factors improving the clinical outcomes or decreasing the abortion rates are warranted. EPS seems beneficial in reducing the abortion rates in IUI cycles of unexplained infertility with mild male subfertility.

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Changing rates for the induction of labor over the last five decades in a tertiary center

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ABSTRACT

The aim of this study is to evaluate the changing rates for induction of labor, induction failure and obstetric characteristics of patients over the decades in a tertiary center. The data on labor inductions were retrospectively evaluated. The cases were divided into five groups: Group 1 (1976, n = 62), group 2 (1986, n = 104), group 3 (1996, n = 81), group 4 (2006, n=120) and group 5 (2016, n = 379). The rates of the induction cases, deliveries with labor induction among deliveries at ≥37th gestational week, primiparous induction cases, induction failure, the mean maternal age, gestational week at birth and birth weight were compared between the groups. The percentages of induction cases among the total number of deliveries for each year were 2.3% in group 1, 4.3% in group 2, 4.6% in group 3, 6.9% in group 4 and 20.2% in group 5, respectively (p<0.001). The rates of labor induction for deliveries at ≥ 37th gestational week were 2.4% in group 1, 4.7% in group 2, 5.4% in group 3, 8.5% in group 4 and 22.1% in group 5, respectively (p<0.001). Statistically significant differences were found between the groups for the number of primiparous induction cases, the rate of induction failure, mean maternal age, gestational week at birth and birth weight (p values were <0.001 for all). The frequency of labor induction has increased at our clinic with application at earlier gestational weeks and there have been higher induction failure rates over the decades.

1. Introduction

Induction of labor refers to techniques for stimulating uterine contractions to accomplish delivery prior to the onset of spontaneous labor (ACOG, 2009). The purpose of this practice is to achieve birth in a short time and in a controlled manner in cases where the continuation of the pregnancy is likely to constitute a high risk for the mother and the baby. Examples of high-risk conditions that require induction of labor include postterm pregnancy, premature rupture of membranes, hypertensive diseases of pregnancy, fetal death, maternal diabetes, fetal growth restriction, chorioamnionitis, oligohydroamnios and cholestasis of pregnancy (ACOG, 2009; Caughey et al., 2009). In addition, the elective induction of labor without any medical indication has increased in recent years in the world (Grobman, 2007; Caughey et al., 2009). On the other hand, giving birth with a classical caesarean incision, having undergone gynecologic surgery requiring a complete insicion in the uterus fundus, transmural myomectomy reaching the uterus cavity, history of uterine rupture, presence of active genital herpes infection, placenta / vasa previa, cord prolapse, fetal transverse

posture, invasive cervical cancer and advanced fetal stress conditions are considered as contraindications for labor induction (Caughey et al., 2009).

While the frequency of labor induction varies between countries, its prevalence was found to be 23.3% in the United States (USA) according to a recent study (Osterman and Martin, 2014). In addition to reducing complications such as stillbirth and macrosomia, induction of labor at term also has the advantages of giving opportunity for a timely and controlled delivery (Ehrenthal et al., 2011; Rosenstein et al., 2012; Mishanina et al., 2014). However, it is more widely accepted to expect spontaneous delivery in the absence of medical indications whenever possible as induction of labor may cause increased cesarean section rates, procedure related complications and higher cost (Grobman, 2014).

The Bishop score is the most commonly used method for predicting induction success (Crane, 2006). In this scoring system, the clinician evaluates the cervical dilatation, effacement, consistency, position, and the station for the presenting part of the fetus (Crane, 2006). In most of the studies, the probability of vaginal delivery increased at scores of 6 and above, while scores of 3 and below were shown to increase the likelihood of cesarean delivery (Teixeira et al., 2012; Kolkman et al., 2013; Gibson and Waters, 2015). The use of cervical ripeners (such as prostaglandin analogues, laminar japonicum, osmotic dilators, foley catheters and cervical balloons) in patients with a low probability of vaginal delivery increases the success of induction (ACOG, 2009). Following ripening of the cervix, uterine contractions are induced by oxytocin administration (Alfirevic et al., 2017). In addition, methods such as membrane stripping, amniotomy and nipple stimulation are also used with or without induction of pharmacological agents (ACOG, 2009). Tachysystole, decelerations during intrapartum fetal heart rate monitoring, maternal hyponatremia and hypotension, uterine rupture and less commonly amniotic fluid embolism are reported as side effects of labor induction (Battista et al., 2007).

Factors such as changing socioeconomic conditions, increasing medicolegal events, the limited time physicians can devote to patients, and the decreasing tolerance of the patient and the healthcare system to obstetric complications have led to radical changes in obstetric practice (Queenan, 2011; Betrán et al., 2016; Beksac et al., 2018). These changes have caused increased cesarean rates and delivery induction (Osterman and Martin, 2014; Betrán et al., 2016). Furthermore, in some studies it has been shown that delivery induction may increase the risk of cesarean delivery by approximately two fold (Luthy et al., 2004; Vahratian et al., 2005). On the other hand, increased cesarean delivery also brings increased maternal / neonatal complications and cost (Molina et al., 2015; Mylonas and Friese, 2015). Thus, Turkey also aims to increase the rate of vaginal delivery, like other countries with increased cesarean rates. One of the most important steps to be taken for this purpose is to perform appropriate induction of labor protocols and to utilize the experience of past years when cesarean rates were within reasonable limits (Beksac et al., 2018).

Our aim in this study was to evaluate the changing rates for induction of labor, induction failure and obstetric characteristics of patients over the decades in a tertiary center.

2. Materials and method

We retrospectively evaluated the data of labor inductions performed for various indications at the 37th gestational week or later in singleton, live births with vertex presentation for the years 1976, 1986, 1996, 2006 and 2016. Induction of labor procedures performed in pregnancies with fetal congenital anomalies were excluded from the study. The data of 10.477 births, which took place in the mentioned years, were examined retrospectively through patient records in the archive, and 746 patients who met the required criteria were considered as the study group. The cases were divided into five groups according to the years of labor induction: The cases were divided into five groups: Group 1 (1976, n = 62), group 2 (1986, n = 104), group 3 (1996, n = 81), group 4 (2006, n=120) and group 5 (2016, n = 379). The percentages of induction cases in the total number of deliveries for each year, the rates of deliveries with labor induction in the total number of deliveries at the 37th gestational week or later, the number of primiparous induction cases, the rate of induction failure, mean maternal age, gestational week at birth and birth weight were compared between the groups.

The method of labor induction was determined by the experience of the clinicians, the clinical characteristics of the cases, and the findings of vaginal examination. The labor induction protocol of our institution is oxytocin infusion (ACOG, 2009). In this study, induction failure was defined as the absence of vaginal delivery in spite of the applied induction methods and consequent delivery with caesarean section (ACOG, 2009).

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS, version 22.0, for Windows, Armonk, NY: IBM Corp). The Kolmogorov-Smirnov test was used to evaluate the normal distribution of the data. Normally distributed data are presented as mean and standard deviation. Since maternal age, gestational age at birth and birth weight values were found to be normally distributed, these parameters were compared using one-way ANOVA test among the groups. The homogeneity of the variances was assessed by the Levene test. Independent-samples t test was used to compare parametric variables between the groups. Categorical variables were compared using chi-square test. The significance level with a p value of <0.05 was determined. Written informed consent was obtained from all the patients, and the study was approved by the institutional ethics committee of Hacettepe University. No funding was used for this study.

Table 1. The comparision of study groups in terms of mean maternal age, gestational week at birth, birth weight and induction characteristics.

	1976 (n=62)	1986 (n=104)	1996 (n=81)	2006 (n=120)	2016 (n=379)	P value
Maternal age (years) (mean±SD)	24.94±3.94	27.50±4.54	27.10±5.39	30.30±5.30	29.40±5.61	<0.001a
Gestational week at birth (mean±SD)	39.90±2.42	39.02±2.30	38.33±1.62	37.55±2.85	37.09±3.53	<0.001a
Birth weight (g) (mean±SD)	3251.94±535.42	3366.92±453.28	3140.74±490.62	3129.00±42.84	3018.30±714.64	<0.001a
Percentages of the induction cases in the total number of deliveries (%)	2.3%	4.3%	4.6%	6.9%	20.2%	<0.001b
Rates of deliveries with labor induction in the total number of deliveries at 37th gestational week or later	2.4%	4.7%	5.4%	8.5%	22.1%	<0.001b
Percentage of primiparous induction cases (n,%)	42 (67.7%)	38 (36.5%)	52 (64.2%)	49 (40.8%)	207 (54.6%)	<0.001b
Rate of induction failure (n,%)	5 (8.1%)	17 (16.3%)	28 (34.5%)	55 (45.8%)	194 (51.2%)	<0.001b

3. Results

The mean maternal age of all patients included in the study was 28.66 ± 5.48 years. In addition, the mean gestational week at birth and mean birth weight of all the patients in the study were 37.80 ± 3.15 weeks and 3313.46 ± 646.92 g, respectively.

The percentages of induction cases in the total number of deliveries for each year were 2.3% in group 1, 4.3% in group 2, 4.6% in group 3, 6.9% in group 4 and 20.2% in group 5, respectively. Additionally, there were a total of 8827 deliveries at the 37th gestational week or later and the rates of labor induction in these deliveries were 2.4% in group 1, 4.7% in group 2, 5.4% in group 3, 8.5% in group 4 and 22.1% in group 5, respectively (Fig. 1 and Table 1).

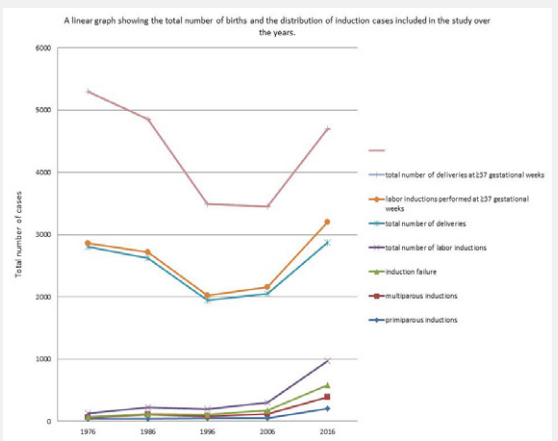

Fig. 1. A linear graph showing the total number of births and the distribution of induction cases included in the study over the years.

A total of 2671 deliveries were performed at our institution in the year 1976. Of the 62 total induction procedures in group 1, 42 (67.7%) were applied to primiparous patients and 5 of these inductions failed (8.1%). When we evaluated the year 1986 (group 2), 38 induction procedures (36.5%) were applied to primiparous patients and 17 induction failures (16.3%) were reported in a total of 2396 deliveries. In the year 1996 (group 3), 52 of the 81 total induction procedures (64.2%) were applied to primiparous women and 28 (34.5%) of them had induction failure in a total of 1752 deliveries. Additionally, in the year 2006 (group 4), 49 of the 120 total induction procedures (40.8%) were applied to primaparous women and 55 (45.8%) of them had induction failure in a total of 1754 deliveries. Finally, in 2016 (group 5), 207 of the total 379 induction applications among 1904 deliveries were applied to primiparous pregnancies (54.6%) and 194 (51.2%) induction failures were reported (Fig. 1 and Table 1).

Statistically significant differences were found between the groups for the percentages of induction cases in the total number of deliveries for each year, the rates of deliveries with labor induction in the total number of deliveries at the 37th gestational week or later, the number of primiparous induction cases, the rate of induction failure, mean maternal age, gestational week at birth and birth weight (p values were <0.001 for all).

Mean maternal age was 24.94 ± 3.94 years for group 1. Statistically significant differences were found between group 1 and the other groups when pairwise comparisons were conducted (p values were 0.003, 0.016, <0.001 and <0.001 for group 2, group 3, group 4 and group 5, respectively). In addition, mean maternal age was 27.50 ± 4.54 years for group 2. There was no statistically significant difference between groups 2 and 3 (p=0.609), although statistically significant differences were found between groups 2, 4 and 5 (p<0.001 for both). Mean maternal age in group 3 was 27.10 ± 5.39 and there were statistically significant differences between groups 3, 4 and 5 (p <0.001 for both values). Finally, the mean maternal age values in groups 4 and 5 were 30.30 ± 5.30 and 29.40 ± 5.61 years, respectively, and there was no statistically significant difference between the two groups (p = 0.154).

The mean gestational week at birth was 39.90 ± 2.42 weeks in group 1. When pairwise comparisions were performed, statistically significant differences were found between groups 1, 3, 4 and 5 (p values were 0.002, <0.001 and <0.001, respectively). However, there was no

statistically significant difference between groups 1 and 2 (p=0.069). The mean gestational week at birth in group 2 was 39.02 ± 2.30 weeks. There was no statistically significant difference between groups 2 and 3 (p = 0.126). On the other hand, statistically significant differences were found between groups 2, 4 and 5 (p <0.001 for both). Furthermore, the mean gestational week at birth in group 3 was 38.33 ± 1.62 weeks and there was no statistically significant difference between groups 3 and 4 (p = 0.076). However, there was a statistically significant difference between groups 3 and 5 (p = 0.001). For groups 4 and 5, the mean gestational week at birth was 37.55 ± 2.85 and 37.09 ± 3.53 weeks, respectively, and there was no statistically significant difference between the two groups (p = 0.137).

The mean birth weight was 3251.94 ± 535.42 g for group 1, and when pairwise comparisions were performed, a statistically significant difference was present only between groups 1 and 5 (p values were 0.260, 0.301, 0.214 and 0.006 for groups 2, 3, 4 and 5, respectively). The mean birth weight for group 2 was 3366.92 ± 453.28 g, and there were statistically significant differences between groups 2, 3, 4 and 5 (p values were 0.017, 0.005 and 0.001, respectively). On the other hand, the mean birth weight for group 3 was 3140.74 ± 490.62 g, and no statistically significant differences were found between groups 3, 4 and 5 (p values were 0.892 and 0.098, respectively). Finally, the mean birth weight values for groups 4 and 5 were 3129.00 ± 642.84 g and 3018.30 ± 714.64 g, respectively, and there was no statistically significant difference between the two groups (p = 0.08).

4. Discussion

The incidence of labor induction has gradually increased from the end of the 1980s to the 2000s (Osterman and Martin, 2014). According to the results of a study conducted in the USA, the frequency of induction, which was 9.5% in 1990, reached the highest level of 23.8% in 2010, and then slightly decreased to 23.3% in 2012 (Osterman and Martin, 2014). The groups most contributing to the decline observed in recent years are the patients within weeks 36, 37 and 38 of gestation (Osterman and Martin, 2014). Recent studies have shown that newborns delivered between 370 and 386 gestational weeks, defined as early term, also carry increased risk of neonatal morbidity (Clark et al., 2009; Dietz et al., 2012). In this regard, the American College of Obstetricians and Gynecologists (ACOG) recommends avoiding elective induction of labor before the 39th gestational week (ACOG, 2013).

In this study, the frequency for induction of labor increased from 2.3% in 1976 to 20.2% in 2016, over these years. This finding is consistent with the current literature (Osterman and Martin, 2014). On the other hand, the important point is that the rate of 6.9% in 2006 increased dramatically in a decade to 20.2%. Our data are dissociated from the USA study at this stage. The frequency for induction of labor, which was at its highest value in the same years, showed a slight decline in recent years. On the contrary, a significant increase was reported in our study. Many factors may be taken into consideration with regard to this tendency. First of all, cesarean section rates, which have risen rapidly in our country, especially at the beginning of the 2000s, should be held responsible for the low rate of inductions in 2006 (Töre et al., 2009). On the other hand, elective cesarean section applications were prohibited by law in 2012 (Ozyuncu et al., 2019). This law has probably led to an increase in labor induction procedures in the following years. The presence of such a law for caesarean section is unique to Turkey.

This study demonstrated that the mean gestational week for induction of labor and birthweight has decreased over the years. The mean gestational week at birth, which was 39.90±2.42 in 1976, decreased to 37.09 ± 3.53 in 2016. Physicians were waiting until further weeks of gestation for induction of labor in the past in our clinic. However, earlier gestational weeks were preferred for labor induction in the last decades. Mean birthweight values also decreased over the years among the labor induction patients, which was consistent with the changes in obstetrics practice worldwide. The mean birthweight, which was 3251.94 ± 535.42 g in 1976, declined to the lowest value of 3018.30 ± 714.64 g in 2016. When we interpret these findings, we can conclude that there is a tendency for induction of labor at earlier gestational weeks in our clinic in the last decades, contrary to some other countries (ACOG, 2009; Osterman and Martin, 2014).

Mean maternal age also increased over the years. While it was 24.94±3.94 in 1976, the values increased to 30.30±5.30 and 29.40±5.61 in 2006 and 2016, respectively. This shows us that there is an increasing trend in the maternal age of labor induction patients. When the parity of the patients were compared, the primiparous induction rate was highest in 1976 (67.7%) and lowest in 1986 (36.5%). The distribution of primiparas in groups did not show any particular pattern. Presumably, this distribution was influenced by changing clinical practices and physicians' preferences.

The most striking finding of our study is that induction failure has increased significantly over the years. This rate, which was 8.1% in 1976, increased to 51.2% in 2016, increasing every decade step by step. In addition to the status of the cervix in predicting induction failure, parity, gestational week, rupture of membranes, body mass index, maternal height, baby's weight and placental insufficiency are also important factors (Crane, 2006; Canda et al., 2010; Gibson and Waters, 2015). Studies in the literature did not indicate such high failure rates despite the increase in induction of labor frequency (Yeast et al., 1999; Heffner et al., 2003; Wolfe et al., 2011). We believe that this high rate is due to the intense social and

legal pressure on physicians. The physicians probably prefer cesarean section with the smallest suspicious condition encountered during the induction of labor in order to protect themselves from medicolegal problems. The main strength of our study was the inclusion of data consisting of five decades experience in the same clinic. On the other hand, the limitations of our study were that it did not contain induction of labor indications and neonatal results, especially due to the limitations of data in the past decades.

In conclusion, the frequency of labor induction has increased at our clinic with application at earlier gestational weeks and there have been higher induction failure rates over the decades.

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Antiproliferative and apoptotic effects of Onobrychis albiflora extract on HCT-116 cells

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ABSTRACT

In this study, the cytotoxic effects of the extracts of methanol: water (80:20) prepared from the above-ground parts of the varieties of Onobrychis albiflora Hub.-Mor., Onobrychis argyrea Boiss. subsp. argyrea, Onobrychis galegifolia Boiss. and Onobrychis tournefortii (Willd.) Desv. species to HCT-116 cells were investigated. With cytotoxicity analysis that with these species inhibitor concentrations (IC50) which resulted in a 50% reduction in the proliferation of HCT-116 cells were identified. In continuation of the study; the antiproliferative and apoptotic effects of Onobrychis albiflora extract on HCT-116 cells were evaluated by Caspase 3, Annexin V / PI Apoptosis / Necrosis analysis, Apopxin Green and 7-AAD Apoptosis / Necrosis analysis. The antiproliferative, apoptotic and necrotic effects of Onobrychis albiflora extract on HCT-116 cells also were determined.

1. Introduction

The use of natural resources by humans for medical purposes was always the case since the existence of humanity. Human beings have benefited from natural resources, especially plants, to protect their health and to cure their diseases. Medicinal plants are of utmost importance as they are used as medicines themselves, as well as being the source of new medicines. Nearly 50% of the approved drugs used in the field of cancer in the last 30 years are produced directly or indirectly from natural products (Veeresham, 2012).

Paclitaxel, docetaxel, vinblastine and vincristine are just some of them (Cragg et al., 2005; Lee and Xiao, 2005; Cragg et al., 2012; Lee and Xiao, 2012). The fact

that plants contain compounds with different biological effects, and that some plant components have multiple effects and that plants are an unlimited source for new drug molecules are the most important reasons for their widespread use in the development of new drugs that can be used in the treatment.

Onobrychis Miller is a genus of perennial plants of the Fabaceae family. Onobrychis species, which have been cultivated since ancient times, are widespread in the Near East Flora, including our country. It is a plant which is more resistant to cold and drought and it is more fertile in arid, calcareous soils which are not suitable to other plants (Acikgoz, 2001).

Both in this respect and in terms of having high nutritional value, it is common for our country to be used as fodder plant especially in Eastern Anatolia Region. The genus Onobrychis is distributed in Europe, America, West Asia and North Africa with more than 160 species. In our country, 55 species belonging to the genus Onobrychis are grown naturally and approximately 50% are endemic (Avci et al., 2016). In these plants species; O. argaea, O. elata, O. argyrea and O. tournefortii have entered to the List of Medicinal and Aromatic Plants of Turkey (Acikgoz, 1998). Although there are some studies on antioxidant and anticancer effects of Onobrychis species, there is no study on the anticancer effect of O. argyrea and O. albiflora which we used in our study.

According to World Health Organization 2014 data, 8.8 million people died due to cancer worldwide. Colon cancer is the fourth highest incidence in the world, with the third highest mortality rate (WHO, 2014).

Many previous studies have been identified that, Onobrychis contains large number of flavonoids, tannins, benzofuran derivatives and other chemicals (Marais et al., 2000; Karamian and Asadbegy, 2016). According to Phytochemical studies on the genus Onobrychis it was found that the species contained compounds with an anticarcinogenic effect like that flavonoids; afzelin (Zhu et al., 2015), quercetin (Hashemzaei et al., 2017), vitexin (Scarpa et al., 2017), myricetin (Zheng et al., 2017), benzofuran derivatives; ebenfuran-III (Halabalaki et al., 2000) and 2 arylbenzofuran (Katsanou et al., 2007); phenolic acids; cinnamic acid (Zhu et al., 2016), gallic acid (Pang et al., 2017), vanylic acid (Anbalagan et al., 2017), caffeic acid (Tyszka et al., 2017), coumaric acid (Roy et al., 2016), ferulic acid (Eitsuka et al., 2016).

The cytotoxic effects of ferulic acid and coumaric acid on human colorectal cancer cell line HCT-15 were tested and both compounds were shown to be effective in killing colorectal cancer cells (Roy et al., 2016). In a previous study on CaCo-2 colon cancer cells, the pro-apoptotic effects of vitexin have been evaluated and it has been shown that vitexin inhibits the proliferation of CaCo-2 cells as a result of the activation of Caspase 9, 8 and 3 (Scarpa et al., 2017). In another study, HT-29 colon cancer cells were treated with cinnamic acid and MTT analysis revealed that the IC50 value was ~1 mM In addition, Bcl-2 expression was decreased and apoptosis was induced in cells (Zhu et al., 2016). In this study, the cytotoxic effects of O. argyrea, O. galegifolia, O. tournefortii and O. albiflora extracts on HCT-116 colon cancer cells were investigated.

2. Materials and methods

Plant material

Onobrychis species were collected from the localities given in Table 1 from Sivas. Identification of species made by Dr. Mehmet Tekin who is Assoc. Prof. in Trakya University, Faculty of Pharmacy, Department of Pharmaceutical Botany and it is stored in the Herbarium of Cumhuriyet University. Dried and powdered aerial parts of the plant (30 g) were extracted with 300 ml of methanol: water (80:20) at room temperature during 8 hours for 3 days by continuous stirring. Each extract was filtered and concentrated to dryness under reduced pressure and low temperature (40–50 °C) on a rotary evaporator to yield crude extracts. Concentrated extracts weighed 10 mg, homogenization of the blender in 1 ml distilled water. The obtained homogenates were centrifuged at 1500 rpm for 15 minutes at 4 °C. The pellet and supernatant portions were separated; the supernatants used as stock solution were sterilized by filtration. Serial dilutions were prepared in medium.

Table 1. Onobrychis species were collected from the localities from Sivas.

Species	Localities	Collector and Herbarium No
O. albiflora	Sivas:Sincan -Kangal, 3. Ian roadside 1220 m, 39028' 01,9" N; 370 50' 34,S" E	M. Tekin, 1291
O. arevrea subsp. arwea	Sivas Zara - Divrigi. 55 Ian from Divrigi1691 m, 39036' 58)" N ; 37044' 28,4" E	M. Tekin, 1293
O. galegifolia	Sivas: Dlvrigi-Slncan.vz-S Ian from Sincan. 1312 m, 390 30' 21,7" N; 370 49' 45,4" E	M. Tekin, 1294
O. toumefortii	Sivas: Sivas-Hafik. around the village of Emre, 1317 m, 390 49' 37,S" N; 37017' 05)" E	M. Tekin, 1290

Cell culture

Studied with Human colon cancer (HCT-116) and human embryonic kidney (HEK-293) cell lines. Cultivation for HCT-116 cells were used McCOY's 5A medium (Multicell) containing, 1% antibiotic / antimycotic solution (Multicell), 10% Fetal Bovine Serum (South America Origin, Biosera), 5% L-Glutamine. And cultivation for HEK-293 cells were used high glucose DMEM medium (Multicell), containing 1% antibiotic / antimycotic solution (Multicell), 10% Fetal Bovine Serum (South America Origin, Biosera).

Cytotoxicity measurement

The cultivation of the cells was carried out to 96-well plates at 0.5 x 10^5 cells per well and allowed to stand for 24 hours to be confluent. Then, the media was aspirated on the wells. Different concentrations of the extracts dissolved in distilled water (5 mg/ml, 4 mg/ml, 3 mg/ml, 2 mg/ml, 1 mg/ml and 0.5 mg/ml) with equal amounts of media on HEK-293 and HCT-116 cells added. Triplicate was performed for 24 and 48 hours for all concentrations. At the end of incubation periods, the medium on the cells was aspirated. The MTT (Roche) agent was added at a rate of 1:20 (MTT agent: medium) and incubated at 37°C and 5% CO2 for 4 hours. Following incubation, MTT was aspirated. 1:1 (medium: DMSO) solvent solution was added and incubated for 1 hour in the dark with the orbital shaker. Cells exposed to Triton ™ X-100 (Sigma-Aldrich) were used as positive controls. The absorbance of the color change was measured by spectrophotometer at 570 nm. The inhibitory concentration (IC50), statistics and graphics of the extracts which resulted in a 50% reduction in the 24 and 48 hours proliferation of both cell lines were recorded using the GraphPad Prism 6 program.

Annexin V-FITC early apoptosis and necrosis analysis

In this study, Annexin V-FITC Early Apoptosis Detection Kit (Cell signaling technology, Cell Proliferation Kit I) was used. 1x106 HCT-116 cells cultured in a T25 flask were incubated for 24 hours with an IC50 dose of the extract dissolved in distilled water of O. albiflora. At the end of incubation, the cells were removed by trypsin (Multicell) and centrifuged at 500 g for 5 min and the supernatant was removed after centrifugation. The cell pellet was separated into 96 μl aliquots by mixing with 1X Annexin V binding buffer contained in the kit. 1 μl of Annexin V-FITC conjugate and 12.5 μl Propidium Iodide (PI) were added to each 96 μl aliquot. It was incubated on ice for 10 minutes in the dark. Final volume was added to 250 μl by adding 1X Annexin V binding buffer. For PI 560 nm and for FITC 488 nm flow cytometry (BD Influx Cell Sorter) was analyzed with appropriate laser channels.

Apopxin green and 7-AAD apoptosis / necrosis analysis

Apopxin green, 7-AAD and Cytocalcein Violet Apoptosis / Necrosis Kit (Abcam, ab176749) were used in our study. 1x106 HCT-116 cells cultured in a T25 flask were incubated for 24 hours with an IC50 dose of the extract dissolved in distilled water of O. albiflora. At the end of incubation, the cells were removed by trypsin (Multicell) and centrifuged at 500 g for 5 min and the supernatant was removed after centrifugation. The cell pellet was mixed with 200 μl assay buffer contained in the kit. 2 μl of Apopxin Green indicator, 1 μl 7-AAD (7-aminoaktinomycin D) and 1 μl CytoCalcein Violet 450 agents were added to the mixture. Incubations of 1 hour at room temperature were achieved. Before flow cytometry analysis, it was further diluted with 300 μl assay buffer. Apopxin Green Indicator using the FL1 channel (Ex/Em = 490/525 nm), 7-AAD using the FL3 channel (Ex/Em=550/650 nm), and CytoCalcein Violet USİNG450 Ex/Em=405/450 nm was analyzed by flow cytometric. HCT-116 cells that were not exposed to O. albiflora extract were used as the control group.

Analysis of fluorometric caspase 3

Caspase 3 (Abcam39383) kit was used in our study. HCT-116 cells were cultured in 96 well plates to each well containing 0.5x1050 cells. After the cells were confluent, they were incubated with O. albiflora for 24 hours with an IC50 dose of 24 hours. The contents of the wells were aspirated after incubation. 50 μl of lysis buffer was added to each well. After incubation for 10 minutes on ice, 50 μl of 2x (containing 10 Mm DTT) assay buffer and 5 μl of 1Mm DEVD-AFC substrate were added. It was incubated again at 37°C for 2 hours. Caspase 3 fluorescence values of the cells were recorded with the Ex / Em: 400 nm / 505 nm spectrophotometer (SpectraMax i3x, Molecular Devices). HCT-116 cells and cells exposed to Triton ™ X-100 (Sigma-Aldrich) were used as negative and positive control groups.

3. Results

Cytotoxicity measurement

On the HCT-116 cells of the herbs, the graphs of the viability curves (Figs. 1 and 2), which were formed as a result of the 24 and 48 hours incubation, were shown and the table for the IC50 values was created (Table 2). Accordingly, as a result of 24 hours incubations, the IC50 value for O. albiflora extract was 0.001396 g/ml (Fig.1A), 0.003287 g/ml for O. argyrea (Fig.1B), for O. galegifolia 0.002707 g/ml (Fig.1C) and 0.002048 g/ml for O. tournefortii (Fig.1D).

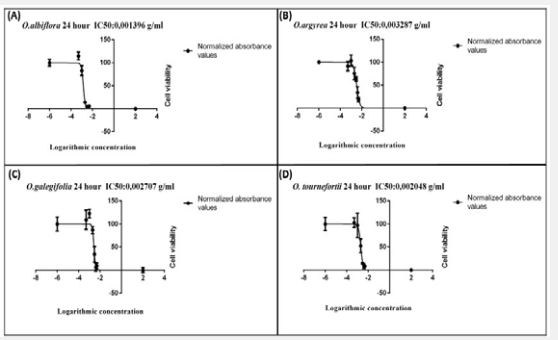

Fig. 1. The graphs of the viability curves which were formed as a result of the 24 and 48 hours incubation.

The analysis was repeated to determine the viability levels of HCT-116 cells incubated with extracts for 48 hours. As a result, the IC50 value was determined for O. albiflora extract 0.001322 g/ml (Fig. 2A), 0.001217 g/ml for O. argyrea (Fig. 2B), 0.002755 g for O. galegifolia g/ml (Fig. 2C) and O. tournefortii for 0.002236 g/ml (Fig. 2D).

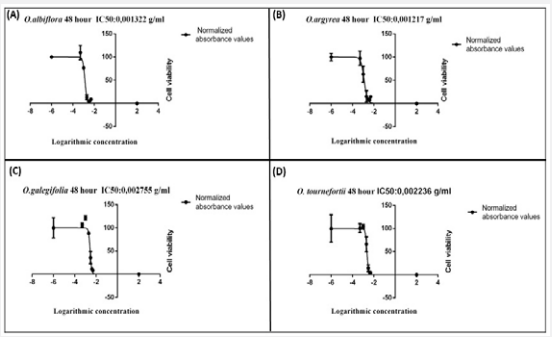

Fig. 2. The graphs of the viability levels of HCT-116 cells incubated with extracts for 48 hours.

Table 2. IC50 values of the HCT-116 cells of the herbs after 24 and 48 hours incubation.

	HCT-116	
	24 hours IC50 (mg/ml)	**48 hours IC50 (mg/ml)**
Onobrvchis albiflora	1.396	1.322
Onobrvchis ealeeifolia	2707	2.755
Onobrvchis tournefortii	2.048	2.236
Onobrvchis arzvrea	3.287	1.217

HEK-293 cell line was used to evaluate the effects of herbs on healthy cells. For 24 hours of incubation of the same doses of herbal extracts on HEK-293 cells, the IC50 value was determined for O. albiflora extract was 0.001649 g/ml (Fig. 3A) and 0.003749 g/ml for O. argyrea (Fig. 3B), 0.002563 g / ml (Fig. 3C) for O. galegifolia and 0.0009797 g/ml for O. tournefortii (Fig. 3D).

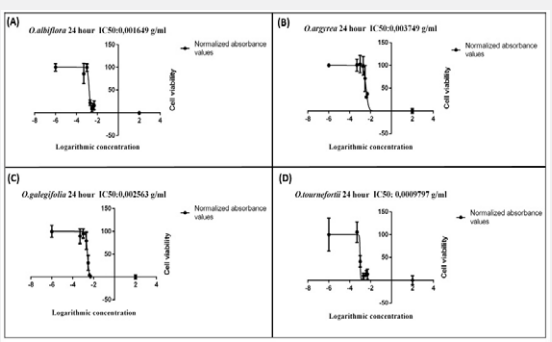

Fig. 3. The graphs of the viability levels of HEK-293 cells incubated with extracts for 24 hours.

When exposed to the extracts for 48 hours, the IC50 values was detected 0.001601 g/ml (Fig. 4A) for O. albiflora extract, 0.003611 g / ml (Fig. 4B) for O. argyrea, 0.003989 g/ml (Fig. 4C) for O. galegifolia, 0.001269 g/ml (Fig. 4D) for O. tournefortii. HEK-293 cells in 24 and 48 hours incubation of the herbs were determined as a result of the table for the values of IC50 (Table 3). As a result of MTT analysis, the most effective herb on HCT-116 cells was O. albiflora.

Table 3. IC50 values of the HEK-293 cells of the herbs after 24 and 48 hours incubation.

	HEK-293	
	24 hours IC50 (mg/ml)	**48 hours IC50 (mg/ml)**
Onobrvchis albiflora	1.649	1.601
Onobrvchis ealeeifolia	2.563	3.989
Onobrvchis tournefortii	0.9797	1.269
Onobrvchis argyrea	3.749	3.611

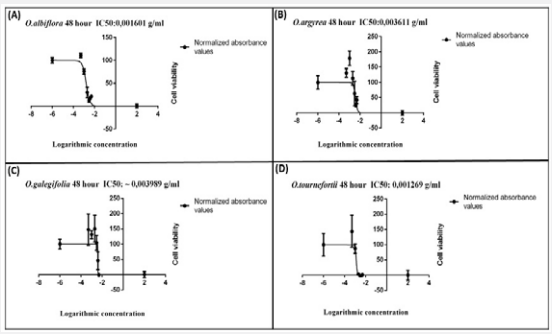

Fig. 4. The graphs of the viability levels of HEK-293 cells incubated with extracts for 48 hours.

Annexin V-FITC early apoptosis and necrosis analysis

The apoptotic effect of O. albiflora on HCT-116 cells was analyzed by flow cytometry using Annexin V-FITC and phosphatidylserine (PS). The distribution of apoptotic and necrotic cells belonging to the experimental group was shown (Fig. 5).

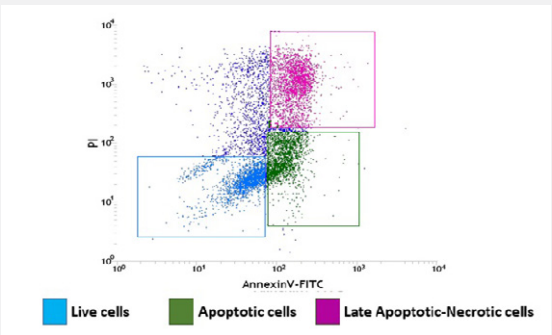

Fig. 5. The graphs of live, apoptotic, late apoptotic cells in the experimental group.

Accordingly, the ratio of apoptotic cells was 25.95 %, the ratio of late apoptotic-necrotic cells was 31.21 %, and the percentage of living cells was 24.18 % (Table 4). In addition, flow cytometry analysis with Annexin V-FITC and PS was performed in the control group HCT-116 cells, which were not exposed to any dose of the herbs (Fig. 6). While the percentage of living cells in the control group was 80.6 %, the rate of apoptotic cells was 8.56 % and the rate of late apoptotic-necrotic cells was 6.35 % (Table 5).

Table 4. The Ratio of the late apoptotic-necrotic, anapoptotic and live cells in the control group HCT-116 cells.

	Cell population	% Value
All cells	7.167	100 %
Late Apoptotic-Necrotic	2.237	31.21 %
Apoptotic	1.860	25.95 %
Live cells	1.733	24.18 %

Table 5. The Ratio of the late apoptotic-necrotic, anapoptotic and live cells in the control group.

	Cell population	% Value
All cells	18.857	100 %
Late Apoptotic-Necrotic	1.198	6.35 %
Apoptotic	1.615	8.56 %
Live cells	15.199	80.60 %

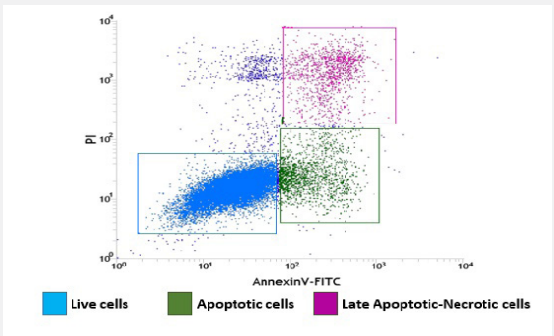

Fig. 6. The graphs of live, apoptotic, late apoptotic cells in the control group HCT-116 cells.

Apopxin green and 7-AAD apoptosis / necrosis analysis

Apopxin green, 7-AAD and CytoCalcein violet 450 were agents that include staining of early stage apoptosis cells, late apoptotic-necrotic cells and live cells. Created the peak ratio diagrams for the CytoCalcein violet 450 (respectively, Fig. 7A and 7B), Apopxin green indicator (respectively, Fig. 8A and 8B) ve 7-AAD (respectively, Fig. 9A and 9B).

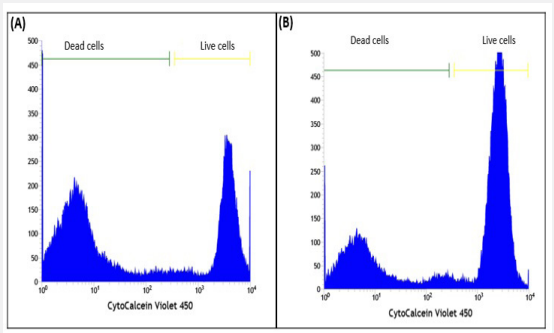

Fig. 7. The graphs of dead and live cells for the CytoCalcein violet 450.

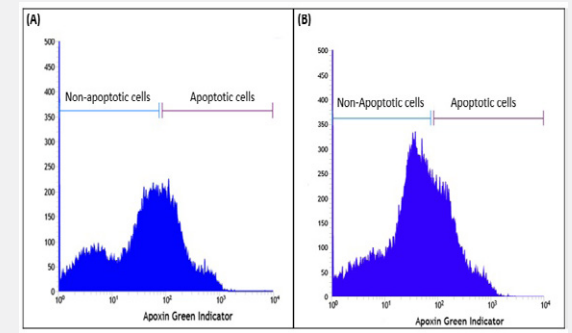

Fig. 8. The graphs of dead and live cells for the Apopxin green indicator.

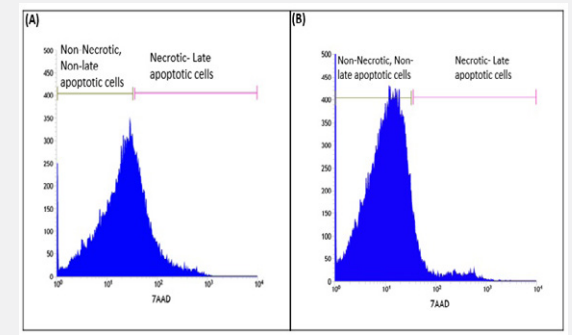

Fig. 9. The graphs of dead and live cells for the 7-AAD.

According to this, 39.82 % of the experimental group cells were alive (Table 6), while 66.29 % of the control cells were alive (Table 7). When we evaluated the ratio of apoptotic cell numbers, while this rate was recorded as 35.33 % (Table 8) in experimental group cells, 32.8% (Table 9) of the control group cells were determined as apoptotic. Necrotic-late apoptotic cell ratios were 33.86 % in the experimental group (Table 10) and 9.47 % in the control group (Table 11). Also combined peak rate diagrams of all results were generated (Fig. 10, 11, 12).

Table 6. Live and dead cells in the experimental group.

	Cell population	% Value
Live cells	14.496	39.82 %
Dead cells	21.660	59.61 %

Table 7. Live and dead cells in the control group.

	Cell population	% Value
Live cells	27.128	66.29 %
Dead cells	13.555	33.12 %

Table 8. Apoptotic and non-apoptotic cells in the experimental group.

	Cell population	% Value
Non-apoptotic cells	22.727	62.55 %
Apoptotic cells	12.839	35.33 %

Table 9. Apoptotic and non-apoptotic cells in the control group.

	Cell population	% Value
Non-apoptotic cells	27.002	65.98 %
Apoptotic cells	13.169	32.18 %

Table 10. Necrotic-late apoptotic cell ratios in the experimental group.

	Cell population	% Value
Non-necrotic, non-late apoptotic cells	23.471	64.59 %
Necrotic-late apoptotic cells	12.305	33.86 %

Table 11. Necrotic-late apoptotic cell ratios in the control group.

	Cell population	% Value
Non-necrotic, non-late apoptotic cells	36.759	89.93 %
Necrotic-late apoptotic cells	3.874	9.47 %

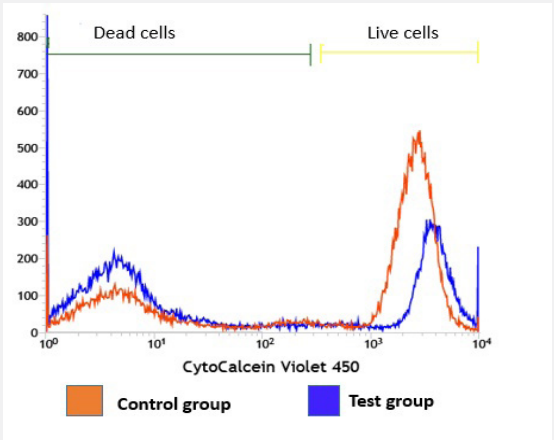

Fig. 10. The graphs of dead and live cells for the CytoCalcein violet 450 in the test and control groups.

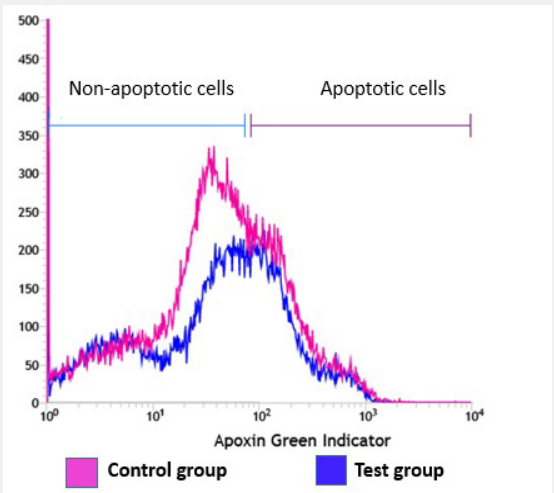

Fig. 11. The graphs of dead and live cells for the Apopxin green indicator in the test and control groups.

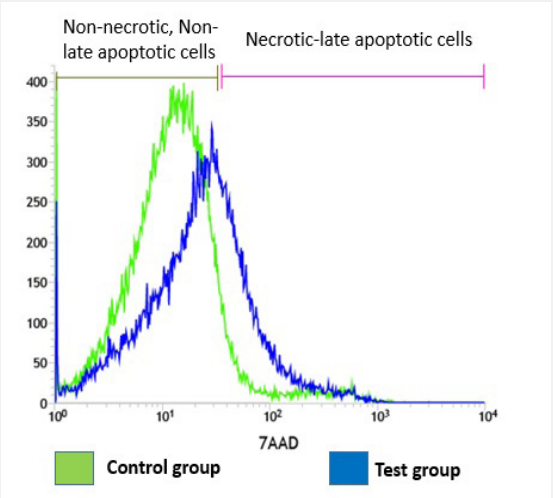

Fig. 12. The graphs of dead and live cells for the 7-AAD in the test and control groups.

Analysis of fluorometric caspase 3

Fluorometric caspase 3 analyses were performed in order to determine the apoptosis level of O. albiflora extract in the mechanism of death on HCT-116 cells. In fluorometric caspase 3 analyses, the value of caspase 3 expression of the negative control group was determined as 7.5x106 and the fluorescence value of the experimental group was determined as 15.3x106. In addition, the fluorescent value of the positive control group was recorded as 19.9x106 (Fig. 13).

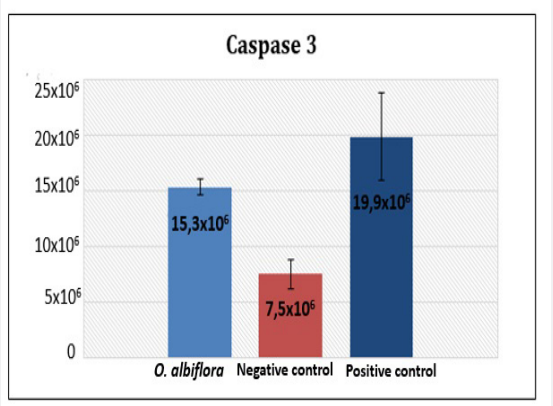

Fig. 13. The graphs of the value of the fluorometric caspase 3 in the O. albiflora and negative control, positive control.

4. Discussion

Anticancer drugs are expected to be selective against tumor cells and should not show any toxic effects to healthy cells but, it is observed that they can also kill healthy cells (Desai et al., 2008). Therefore, our study was continued with O. albiflora extract which has the least toxic effect against HEK-239 cells while, showed the most toxic

effect against HCT-116 cells, according to MTT analysis results. After 24 hours of incubation O. albiflora extract on HCT-116 cells IC50 value was determined as 1.4 mg/ml and 48 hours incubation value was 1.3 mg/ml. The results revealed that the cell viability and concentration of plant extract were inversely proportional during both of incubation periods. In terms of IC50 values, the 24 hours incubation period was not significantly different compared to the 48 hours incubation period. Considering the 24 hours incubation period would be advantageous in both in vivo and in vitro studies in terms of time, it was deduced to be appropriate to continue with 24 hours incubation period. In the flow cytometry analysis performed with Annexin V-FITC and PS, 80, 60 % of the cells in control group were alive and 8.56 % were apoptotic, while on research group which was subjugated to O. albiflora extract; 24.18 % of cells were alive and 25.95 % was apoptotic. These results showed O. albiflora extract induces HCT-116 cells to apoptosis significantly. To confirm that, O. albiflora induces apoptosis in HCT-116 cells, flow cytometry analysis with Apopxin green and 7-AAD was performed again. While the ratio of apoptotic cells was determined as 32.8 % in the control group, this rate increased to 35.33 % in the experimental group cells. In addition, the rate of necrotic/late apoptotic cells in the control group cells was 9.47 % and this ratio increased to 33.86 % in the experimental group cells. These results confirmed that cells exposed to a dose of 1.4 mg/ml of O. albiflora extract entered the early stage of apoptosis and were shown to be parallel to Annexin V-FITC early apoptosis/necrosis analysis.

Apoptosis, which is defined as programmed cell death, is composed of two basic pathways as extrinsic and intrinsic. Both pathways converge in a final pathway that involves activation of a number of proteases called caspases which break down regulatory and structural molecules and result in cell death. Therefore, caspase-3 expression in HCT-116 cells was measured. In the fluorometric caspase 3 analyses, the value of caspase 3 expression of the negative control group was determined as 7.5x106, while the fluorescence value of the experimental group was almost doubled to 15.3x106. In addition, the fluorescence value of the positive control group showed a significant parallelism with 19.9x106 (Fig. 13).

It has been scientifically determined that the risk of developing many chronic diseases such as cancer, diabetes, cardiovascular diseases, chronic inflammation, cataract, atherosclerosis, Alzheimer's disease is reduced by having fed with food rich of phenolic compounds known to have antioxidant effects. Phenolic compounds are one of the largest classes of secondary metabolites and can be grouped under many classes such as flavonoids, phenolic acids, coumarins, tannins, lignans, stilbenes (Choi et al., 2004; Goncalves et al., 2005; Conforti et al., 2008; Guha et al., 2011). Phenolic compounds, due to their potent antioxidant effects, provide the integrity of the biological system by protecting structures such as carbohydrates, proteins, lipids and DNA from the harmful effects of oxidation. In addition, many studies show that phenolic compounds prevent cancer through different mechanisms. Therefore, their effects are not only dependent on their antioxidant properties but also on their interactions with cellular mechanisms. Preclinical studies show that phenolic compounds exhibit effects such as inhibition, deceleration of initiation and progression of different types of cancer such as prostate, liver, colon and also inhibiting cell proliferation, inhibiting tumor growth and development (Basli et al., 2017). It is supported by studies that phenolic compounds, especially flavonoids reduce the incidence of cancer, and have an important role in cancer treatments especially in complementary therapy (Nicodemus et al., 2001). In many similar studies, when cell viability analyzes was taken as reference, results are significantly parallel to our conducted experiments in our study.

The prepared extract was prepared with a mixture of methanol: Water (80:20). When applying, these extracts were used by dissolving in water. This method is also supported by the reference of another study in which the effects of Nettle extract on colon cancer cells have been investigated (Korkmaz, 2010).

Interest and use of phytotherapy has been increasing recently. Traditionally, there are different methods of use in the form of extracts prepared by maceration or infusion or decoction of various parts of the plants. This way of use is widespread, but not sufficiently reliable. Because use of the dosages in traditional methods are not determined by scientific approaches. This may cause unexpected acute or chronic problems. For example, D-Pinitol, the main source of the Fabaceae family, has been shown to have an insulin-like lowering effect as well as anticancer properties (Bates et al., 2000). Without dose information, it can be thought that plants with D-Pinitol which are used by conventional methods can cause serious problems by damaging sugar metabolism in case of overdose.

Although the number of drugs and investigations made with the active ingredients isolated from the extracts of various plants is increasing day by day, it is not possible for people to isolate and use the chemicals used in traditional plants. In this aspect, the effect is expected from the whole plant. In many current studies, the individual medical effects of the compounds in plant contents are investigated. However, this method does not anticipate the consequences of interactions of individual chemical compounds with each other in the case of usage of all chemicals in the content. Our study is original and important in this aspect. Considering the data obtained in our study, and in case of continuation of analysis, it will light the way for examination of many other endemic species for their potential use for medical purposes.

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Pericardial tamponade as the initial sign of systemic lupus erythematosus within Myasthenia Gravis patient

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ABSTRACT

Systemic lupus erythematosus (SLE) is an autoimmune connective tissue disorder, mediated by numerous auto antibodies that has many different clinical manifestations and frequently affects the cardiac system. Pericarditis and pericardial effusion are well recognised cardiac complications. SLE is rarely reported with Myasthenia Gravis (MG). Myasthenia gravis (MG) is characterized by the dysfunction of neuromuscular junctions mediated the antibodies against the acetylcholine receptor and presents with weakness, fatigability of skeletal muscle. Thymectomy is a therapeutic option for patients with severe MG. Here we report a case, presented with dyspnea and revealed pericardial tamponade, pleural effusion who was diagnosed with SLE, three years after thymectomy performed for MG. Pericardial tamponade and pleural effusion are the first sign of SLE very rare and thymectomy facilitated the development of SLE.

1. Introduction

Systemic lupus erythematosus (SLE) (non-tissue specific) and Myasthenia gravis (MG) (tissue-specific) are autoimmune diseases that genetic, environmental, and immunological factors have been implicated (Omar et al., 2010; Miskovic et al., 2015). The literature describes cases of SLE after thymectomy for myasthenia gravis, but pathophysiological mechanisms that lead to this phenomenon are not clear (Park et al., 2004). SLE is a connective tissue disorder, mediated by numerous autoantibodies that has many different clinical presentations and often affects the cardiovascular system (Park et al., 2004; Miskovic et al, 2015). Pericarditis and pericardial effusion are well described cardiac complications and pericardial effusions seen in up to 50% of patients with SLE (Omar et al., 2010; Castrejón et al., 2011; Kumar et al., 2012). But, cardiac tamponade has rarely described as an initial presenting feature of SLE (Topaloglu et al., 2006; Kumar et al., 2012). MG is decribed as dysfunction of neuromuscular junctions mediated the antibodies against the acetylcholine receptor. It presents with weakness and fatigability of skeletal muscle. Thymectomy is a therapeutic option for patients with severe MG (Park et al., 2004; Omar et al., 2010; Castrejón et al., 2011).

Here we report a case, presented with dispnea and revealed pericardial tamponade, pleural effusion who

was diagnosed with SLE, three years after thymectomy performed for MG. We found coexisting the pericardial tamponade and pleural effusion as the first clinical sign of SLE.

2. Case

A 35-year-old female patient presented with fatigue, fever, rashes, increased dyspnea symptoms. On physical examination showed a systolic blood pressure of 90 mmHg, a diastolic blood pressure of 60 mmHg, and a heart rate of 115/bpm. Heart sounds were deep and respiratory voices couldn't be obtained on the lung bases. Electrocardiography showed tachycardia, low voltage and electrical alternation (Fig. 1a). Bilateral pleural effusion and large pericardial effusion were observed in thorax tomography (Fig. 1b). Echocardiography revealed pericardial tamponade findings (Fig. 1c). Pericardial effusion was evacuated with pericardiocentesis. Pericardial fluid examination described as transudate and ibuprofen and colchicine therapy started. Patient's diuresis increased and dyspnea improved on follow-up. We learned from patient's history that the patient had a thymectomy operation three years ago with the diagnosis of MG and followed asymptomatic without medication. Laboratory analysis showed high rheumatoid factor, antinuclear antibody (ANA) 1/100 dilution +++ (1/1000), anti-ds-DNA 74.1 IU/ml (>22 IU/ml), anti-cardiolipin IgG 36.5 (> 10 U/ml), sedimentation 84 mm/h, CRP 22 mg/dl, anti-beta 2 glycoprotein-1 screening (IgA,G,M) 18.5 U/ml (>10 U/ml), p-ANCA/c-ANCA (qualitative) 1/10 dilution +++ (1/100) positive. The patient was consulted the rheumatology department. Treatment of methyl prednisolone, azathioprine, hydroxychloroquine were started with diagnosis of SLE. The patient defined hoarseness, so the pyridostigmine therapy was initiated with a diagnosis of myasthenia gravis by neurology. There was no pericardial fluid in control echocardiography. The patient's symptoms regressed and directed to the neurology and rheumatology departments. Pericardial effusion didn't repeat under one year follow-up.

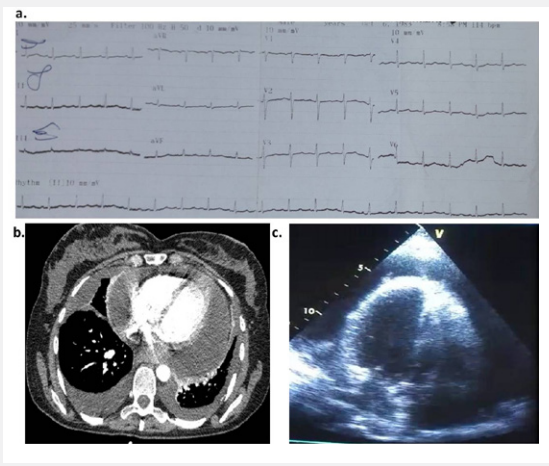

Fig. 1. Pericardial tamponade findings in different imaging modalities Fig. 1a: Electrocardiographic imaging of electrical alternation and voltage drop Fig. 1b: Thoracic tomographic imaging of pericardial tamponade and pleural effusion Fig. 1c: Echocardiographic imaging of pericardial tamponade.

3. Discussion

Pericardial tamponade as the first initial presentation of SLE is very rare and only limited to case reports (Topaloglu et al., 2006; Kumar et al., 2012). We describe a case of coexisting the pericardial tamponade and pleural effusion as the first presentation of SLE. SLE and MG are autoimmune diseases that share certain similarities (Omar et al., 2010). They happen more often in young women, have relapsing remitting clinical progression and usually positive anti-nuclear antibodies (Omar et al., 2010; Castrejón et al., 2011). But they are two different clinical syndromes and one autoimmune disease are increased risk of developing the second one. The entity of second autoimmune disorder is 13–22 % in patients with MG (Omar et al., 2010; Miskovic et al., 2015). Thymectomy is a therapeutic option for patients with severe MG. It can achieve complete clinical remission in 80% of patients (Castrejón et al., 2011; Miskovic et al., 2015). But many SLE cases described in the literature after thymectomy (Omar et al., 2010; Castrejón et al., 2011; Miskovic et al., 2015). So thymectomy operation predisposes patients to SLE and pathophysiological mechanisms are not clear. Thymectomy operation can cause to disproportion of auto-reactive and regulatory T cells and to induction of auto-immune processes due to the loss of central tolerance and over production of auto-antibodies (Omar et al., 2010; Castrejón et al., 2011; Miskovic et al., 2015). So after thymectomy, these patients should follow up intermittent. In the literature, polyarthritis and polyarthralgia are the most common clinical presentations of SLE after thymectomy operation (Park et al., 2004; Miskovic et al., 2015). In our patient, the first sign of SLE was pericardial tamponade and pleural effusion. Although thymectomy is an effective treatment modality in MG patients, clinical cases and literature datas support that this surgical operation may be a precipitating factor for other autoimmune diseases, especially SLE (Park et al., 2004; Omar et al., 2010; Castrejón et al., 2011). Some MG patients who undergo thymectomy, SLE can thrive over the years. The probability of secondary autoimmune pathologies should be highly noted after thymectomy operation (Park et al., 2004; Omar et al., 2010; Castrejón et al., 2011). In our case report, SLE was described as three years after thymectomy operation that was performed for MG and by which long-term improvement of MG was achieved. Also, coexisting of pericardial tamponade and pleural effusion as initial clinical SLE signs is very rare in the literature.

Conclusion

MG patients should be careful about the development of different autoimmune diseases especially SLE after thymectomy operation. So, these patients should follow up intermittent. Coexisting the pericardial tamponade and pleural effusion may rarely be the first sign of SLE, as seen in our case.

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Transient ST-segment elevation due to intracranial hemorrhage in a patient presenting with acute anterior myocardial infarction

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ABSTRACT

Neurogenic stunned myocardium (NSM) is defined as sudden onset, reversible or irreversible myocardial injury, often mimicking acute myocardial infarction. It is arised from an imbalance in the autonomic nervous system in various acute brain injury situations, most commonly in the subarachnoid hemorrhage. Different electrocardiographic abnormalities and arrhythmias are very common even in the absence of structural heart disease. It should be carefully distinguished from acute coronary events. We presented a case of temporary ST-segment elevation after intracranial hemorrhage mimicking re-infarction that developed after thrombolytic therapy which reperfused acute anterior myocardial infarction.

1. Introduction

The electrocardiographic and cardiac changes are not uncommon after central nervous system injuries such as subarachnoid, intracranial hemorrhage, subdural hematoma and ischemic stroke (Woon et al., 2012; Mierzewska et al., 2015). Neurogenic stunned myocardium (NSM) is defined as sudden onset, reversible or irreversible myocardial injury, often mimicking acute myocardial infarction. It is arised from an imbalance in the autonomic nervous system in various acute brain injury situations, most commonly in the subarachnoid hemorrhage (Mierzewska et al., 2015). This condition may have many different clinical manifestations as well as different electrocardiographic findings (Katsanos et al., 2013; Mierzewska et al., 2015). It should be carefully distinguished from acute coronary events. We presented a case of temporary ST-segment elevation after intracranial hemorrhage mimicking re-infarction that developed after thrombolytic therapy which reperfused acute anterior myocardial infarction.

2. Case

A 5 4-year-old male patient admitted to emergency service on the second hour of chest pain. Electrocardiography (ECG) showed a 3 mm ST-segment elevation in leads V1-V4. The patient given

antithrombotic therapy at appropriate doses with an acute anterior myocardial infarction. Thrombolytic therapy was planned to be given as it is not in percutaneous coronary intervention center. Intravenous 10 IU of two doses of reteplase (r-PA) for 30 min administered to the patient who has no contraindication for treatment. Then, the patient transferred to our center. After thrombolytic therapy, the patient was clinically reperfused and hemodynamically stabilized. ECG taken again and the QS pattern and biphasic T wave were detected in leads V1-V3. Echocardiography revealed hypokinetic septum and anterior wall with 40% ejection fraction. The patient transferred to the coronary angiography laboratory with percutaneous intervention plan. Coronary angiography showed diffuse atherosclerosis in the left anterior descending artery and medical follow-up planned (Fig. 1a/Video-1). Following intensive care, the patient's blurring of consciousness and apneic breathing suddenly developed. Subsequently, the patient suffered hemodynamic instability and intubated. ECG showed widespread ST-segment elevation (Fig. 1b). An acute cerebrovascular event diagnosed by cranial tomography with a 63*65 mm size intracerebral hematoma on the left frontotemporal region, which resulted in a shift of to the right and a subfalcial herniation (Fig. 1c). Echocardiography revealed no new segmental wall motion abnormality. The ST-segment elevation improved one hour after the acute phase. Cardiac markers didn't increase in follow up. Coronary angiography wasn't considered again. ECG findings were evaluated as a complication of intracranial haemorrhage. Patient transferred to intensive care unit of neurology department. Antithrombotic therapy stopped. New ECG changes or new echocardiographic wall motion abnormality didn't detect. The patient died on the second day of follow-up due to intracerebral hematoma.

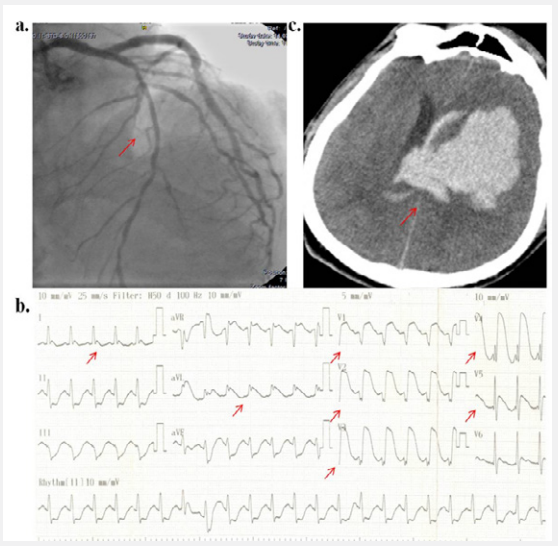

Fig. 1a. Electrocardiographic imaging of diffuse ST-segment elevation after intracranial bleeding. 1b. Angiographic imaging of left anterior descending coronary artery with diffuse 1c. Computed tomographic imaging of intracranial bleeding

3. Discussion

Neurogenic stunned myocardium may be mixed with acute coronary syndromes especially in emergency departments (Mierzewska et al., 2015). NSM is a difficult clinical condition due to diagnostic difficulties and may result in ECG changes, elevation in cardiac biomarkers, LV dysfunction or cardiogenic shock (Mierzewska et al., 2015). Common ECG changes; prolonged QT interval, ST-segment changes, T-wave inversion, new Q waves or U wave (Katsanos et al., 2013; Mierzewska et al., 2015; Murthy et al., 2015). NSM may also cause echocardiographic bazal, mid-ventricular segmental wall motion abnormalities. Although NSM is thought to be primarily a consequence of myocardial injury induced by local massive catecholamine release from the nerve endings in myocardium, the precise pathogenetic process is still unclear and further investigations are needed. Coronary vasospasm and hypoperfusion, hypotension and hypoxia, direct cardiotoxic effect of catecholamines are thought to be another mechanisms (Yusuf et al., 2007; Wybraniec et al., 2014; Köklü et al., 2015; Murthy et al., 2015).

Both acute coronary syndromes (ACS) and intracranial hemorrhage (ICH) are vital clinical conditions and require different treatment approaches (Katsanos et al., 2013). Despite the fact that antithrombotic, anticoagulan therapies are mandatory in ACS, these medications are contraindicated in ICH (Yusuf et al., 2007; Woon et al., 2012; Katsanos et al., 2013; Wybraniec et al., 2014; Köklü et al., 2015). Even if the patient is presenting with ACS as in our case, it should be kept in our mind that ECG changes may occur when consciousness and neurological findings develop as a result of ICH. Cardiac abnormalities secondary to intracranial events are usually transient, as in our case. In such a case, diagnosis should be clarified in a short time with cranial imaging methods and focused on the treatment of the intracranial event.

Conclusion

Different electrocardiographic and cardiac abnormalities are developed after various acute brain injuries and defined as neurogenic stunned myocardium. Acute coronary syndromes and intracranial hemorrhage are vital clinical conditions, require different treatment approaches and should be carefully distinguished.

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Video Legends

Video 1: Coronary angiographic imaging of vessels

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A case of scalp myiasis from a non-endemic region

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Prevention
Treatment
Unsanitary environment

ABSTRACT

Myiasis is an infestation of maggot flies, usually found in tropical and non-tropical regions such as Africa and South America. Although cases are reported after trips to the endemic regions, it should not be overlooked, as in our case, that they may appear especially in poor socioeconomic conditions and the individuals who have to live in the unsanitary environment. Here, we present a 7-year-old myiasis case in our region because it is rare but it can be easily treated when it is diagnosed and it causes unwanted complications if it is not treated.

1. Introduction

Myiasis is defined as an infestation of the mammalian tissue by developing larva of a variety of fly species (Burkhart et al., 2018). Classification of human myiasis by localization includes cutanous cavity and enteric myiasis. Cutanous myiasis is divided into wound myiasis and furuncular myiasis (Duro et al., 2007). In wound myiasis an open wound or orifice is infested by fly larvaes such as dermatobia hominis which is the leading cause. A carrier fly lays its eggs on an open wound or flesh that hatch in response to elevated temperature followed by larvae development and rapid skin penetration (Cetınkaya et al., 2008). The diagnosis is simple when larvae are visible on wound surface and challenging if they are burrowed deeply. Myiasis is vastly a self-limiting disease with minimal morbidity. The indications for treatment are pain reduction, cosmetic concerns, and psychological relief. The incidence of misdiagnosis at the beginning of myiasis cases and accordingly the use of ineffective antibiotic therapy is high (Johnston and Dickinson, 1996). Secondary infections such as cellulitis, lymphangitis, and lymphadenitis are thought to develop due to the faeces of larvae (Schembre et al., 1990).

Treatment options include surgical debridement under local anestesia which is usually curative but remaining larva pieces can induce a foreign body reaction. Occlusion/suffocation method involves placement of occluding material like liquid parafin, petroleum jelly, beeswax over the wound. Aerobic larvae is forced to rise to surface for air over the course of several hours then they are captured by forceps. Alternatively, lidocain injection into tissues inhabited by larvae forces them to the surface (McGraw and Turiansky, 2008). Oral ivermectin is used successfully for oral and orbital myiasis.

2. Case

A 7-year-old female patient admitted to our clinic with the complaint of a wound on scalp and seeing worms on it time to time. Wound occured one month earlier after minimal travma and grew since. Worms were noticed for one week. Detailed history revealed poor socio-economic status. She was living with her old and careless grandparents. Inspection of the scalp showed a supurated, 1.5x1.5 cm size ulcer on erythematous, scaly base in occipital area (Fig. 1). The tip of moving larvae heads was visible to naked eye by close examination.
First, we cut patient's hair then disinfected the scalp

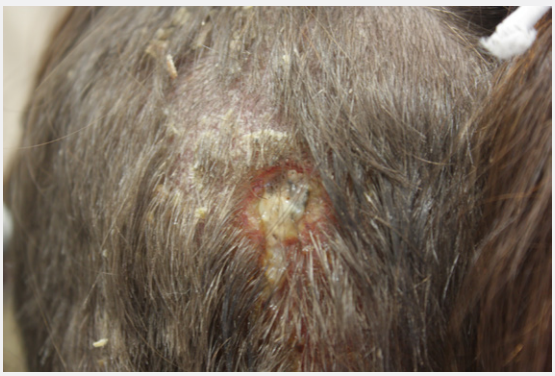

Fig. 1. Inspection of the scalp showed a supurated, 1.5x1.5 cm size ulcer on erythematous, scaly base in occipital area.

with povidone iodine. She was given intravenous ampicillin-sulbactam therapy 1 gr four times daily to treat secondary infections. We applied occlusion/suffocation method with petrolatum jelly and captured 22 larvaes with forceps aid (Fig. 2). To rule out brain invasion, we performed computed tomography scan of the brain that showed no intracranial lesion with minimal defects in cutaneous and subcutaneous tissue of right occipital area. After 7 days of systemic antibiotic therapy regression of the lesion was achieved and patient was discharged with topical fucidic acide. Two weeks later, during control examination, only a minimal alopecic area with scarring was noticable (Fig. 3).

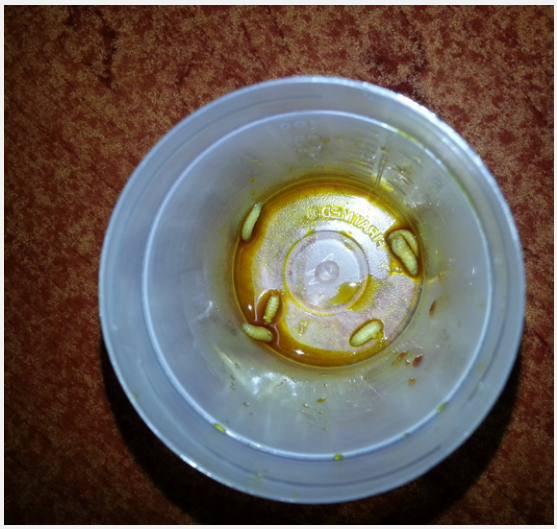

Fig. 2. Captured larvaes with forceps aid.

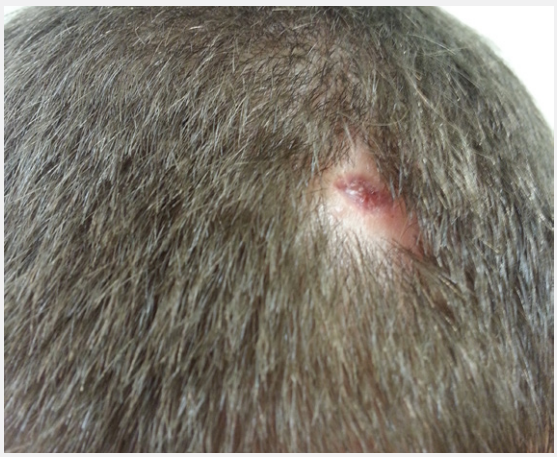

Fig. 3. Two weeks later, during control examination, only a minimal alopecic area with scarring was noticable.

3. Discussion

Myiasis is almost always seen in people from poorer, unsanitory environment with debilitating conditions (Obasa and Sowunmi, 2012). Although it mostly involves exposed intact or damaged skin, eyes, nose, ears, urogenital tract, scalp and even brain can also be involved (Amitay et al., 1998). Despite myiasis is thought to be a disease of tropical and subtropical climates, individuals from other regions with poor socioeconomic status can also be affected and it is important to keep myiasis in differantial diagnosis of cutaneous suppurative ulcerated lesions. In addition, people traveling to endemic areas such as South America and Africa should be advised to wear insect repellent and protective clothing from mosquito bites to prevent the transmission.

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ORGANIZATION OF THE ARTICLE

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Title page: The title page should contain the following items: (1) complete title; (2) full names of all authors; (3) complete affiliations of all authors; (4) the number of text pages of the whole manuscript and the number of figures and tables; (5) the name and complete address of the corresponding Author that includes telephone number, facsimile number and E-mail address to whom correspondence and proofs should be sent.

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Keywords: Provide at least **4-6 keywords** and avoiding general and plural terms and multiple concepts. These keywords will be used for indexing purposes.

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Research Reports should be divided into numbered sections headed by a caption (e.g. **Abstract**, **1. Introduction; 2. Experimental Procedure; 3. Results; 4. Discussion; Acknowledgements, References**).

Introduction: The objectives of the research should be clearly stated in this section. Relevant background information and recent published studies should be described concisely, and be cited appropriately.

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