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1-World Medical Association.Declaration of Helsinki: ethical principles for medical research involving human subjects. http://www.wma.net/en/ 30publications/10policies/b3/index.html. Accessed October 14, 2010.

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Experimental Biomedical Research	Original article

The efficacy of active warming in preventing unplanned hypothermia during perioperative period in pediatric surgery patients in a tertiary care center

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ABSTRACT

Aim*:* To investigate the effects of using the active warming method on children with hypothermia in the perioperative period and examining the effects of hypothermia on awakening time, pain, shivering and hospital stay.

Methods: This study included patients 18 years of age and younger who underwent surgery in the pediatric surgery department of a tertiary hospital. The active warming group consisted of 28 patients and the control group consisted of 29 patients. The first group of patients was actively warmed during the surgery and the second group was treated as the control group and did not actively receive any warming therapy. Preoperative body temperatures of the cases were measured. In addition, body temperatures were recorded every 15 minutes in the intraoperative and postoperative periods.

Results: There was no statistically significant difference in preoperative body temperature between the control group and the active warming group. When body temperature were examined in the intraoperative period, there was no significant difference between the groups at the first 15 minutes of operation; however, the mean of the body temperature in active warming group was significantly higher than the control group at the 30th, 45th, and 60th minutes of operation. It was determined that patients in the control group had a longer stay in the hospital and the amount of time for waking at the end of the anesthesia was shorter in the active heating group than in the control group.

Conclusions: The results of our study suggest that active heating with a carbon fiber resistant system is an effective method to prevent unplanned hypothermia in operated children.

Keywords: Body temperature, surgical procedures, hypothermia, active warming, child.

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Introduction

General anesthesia affects the body's internal temperature in all children undergoing surgery. There is an increased risk of hypothermia, especially in prolonged and open body cavity operations [1]. Heat loss occurs more easily in children [2]. Hypothermia occurs when body temperature drops below 36°C in adults and below 36.5 °C in newborns. Cold stress in newborns is between 36-36.5°C, moderate hypothermia is considered 32-36°C and severe hypothermia is below 32°C [3,4]. During surgery, heat loss from the body occurs by radiation, convection, conduction and

evaporation, and radiation and convection make up 85% of this heat loss [5]. In addition, preterm, low birth weight and sick infants are much more susceptible to hypothermia due to the absence or scarcity of brown adipose tissue, and newborns also have a limited amount of movement, shaking and stretching [6,7]. Therefore, infants and newborns have a higher risk of developing intraoperative hypothermia during surgery. Since thermoregulation centers of babies are not developed, they should be supported in the perioperative period to maintain the hemodynamic state and maintain normal body temperature [8]. In addition, it has been reported that perioperative hypothermia causes wound infections, increases surgical site infections, increases oxygen demand, changes the pharmacokinetics of drugs, impairs coagulation, and causes cardiac arrhythmias and ventilation problems, especially in children [9–12]. Therefore, the quality of care and safety of children who have undergone surgery is very important in the perioperative period.

Passive and active warming methods are used to prevent unplanned hypothermia in the perioperative period. Passive warming methods consist of cotton or wool blankets, stockings, hats, and surgical drapes [13,14]. Active warming techniques include compressed air systems, resistive systems (electric, carbon fiber, jelly coats, etc.), radiant heaters, intravenous fluid, blood and blood products heaters, and heat and moisture exchanger filters [1-14]. There are few studies in the literature on active warming of children in the operating room. In addition, it is not known exactly whether an active warming method will have any effect on preventing unplanned hypothermia in the perioperative period. Therefore, the aim of this study is to investigate the effects of hypothermia on recovery time, pain, shivering, and duration of hospital stay in operated children, and the effects of perioperative active warming on preventing unplanned hypothermia in children.

Materials and Methods

The study was conducted on 57 cases, who underwent surgery in the pediatric clinic at the University Hospital between October 1, 2016, and December 30, 2016. Ethical approval was obtained from Clinical Trials Ethical Board at Bolu Abant Baysal University (Date and decision number: 2016/17-182). Necessary permissions were obtained from the hospital and families. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

We included all children under the age of 18 with normal weight and ranked ASA I-II-III and were operated upon. The exclusion criteria were those who needed intensive care, had infectious diseases, scored ASA IV and above, and had congenital anomalies.

The accidental sampling method was used by the investigator to divide the patients into groups and to prevent bias. The sampling size was determined by power analysis in a computer program. Two groups of 60 patients were placed randomly but patients who did not want to participate were excluded from the groups. For this reason, the control group consisted of 29 patients and the experimental group consisted of 28 patients. The operations included in the study were hernia, hernia + circumcision, neck mass excision, undescended testis + circumcision, cholecystectomy and pyeloplasty. The first group of patients were actively warmed during the perioperative period (Istanbul Medical- Medwarm resistive

system-W-500D + 80* 50 cm or 120*50). The system was set to 38^0C from 40^0C and disposable mattress covers were used for each patient in order to prevent surgical area infections. The second group was assigned as a control group. As a result of hospital procedures, all children were dressed in surgical gowns until they were taken to surgery. They were then wrapped in green covers. Apart from this, no application was made to the control group.

Body temperatures of all patients were measured with non-contact thermometer from temporal artery at 15 minute intervals during the preoperative, intraoperative and postoperative period. Body temperature measurement of children continued for 3 hours in the recovery room and pediatric clinic after surgery. The hospital staff measuring the patient's body temperature did not know to which group the patients were assigned. Blood pressure, pulse, respiration, O_2 saturation and pain scores were evaluated synchronously. Additionally, surgery room ambient temperature (ºC), surgery time, IV (intravenous) solution quantity (ml), type of anesthesia and ASA score were recorded. Visual pain scale (VAS) scale was used for pain measurement. Those whose body temperature was measured below 36 degrees were considered hypothermic.

Statistical analyses

Continuous data were coded in SPSS (Statistical Package for the Social Sciences) 22.0 program and statistical evaluation was performed with number, percentage, t test, Mann- Whitney U tests and Analysis of Variance tests (ANOVA). The results were assessed within *95% confidence interval* and a *p value of <0.05* was considered as statistically significant.

Results

If we look at the identifying characteristics of patients, 57 patients, that consisted of 28 patients in active warming group and 29 patients in control group, were included in the study. The anesthesia technique used was similar in all children. General anesthesia was applied to all patients.

All patients were normothermic before induction. It was determined that age average of all patients was approximately 3.5 years old, the operating room temperature mean was 25.9±1.3 ºC, awakening time from anesthesia was 8.4±3.0 minute, and the length of stay in hospital mean was 1.5±2.5 day in active warming group. Shivering was seen less frequently in patients who were warmed (Table 1).

Hypothermia was observed in 27.6% (8:29) of children in the control group during the intraoperative period. In the postoperative period, 13.8% (4:29) of the children were hypothermic in the control group. Hypothermia was not observed in children in the active heating group who were given intraoperative heating.

When body temperature was examined in the preoperative period, there was no statistically significant difference between the body temperatures in the control group (X: 36, 88±0.43) and the active warming group (X: 36, 80±0.35), indicating that the groups were homogeneous *(t: .817; p≥.05)*. The primary outcomes when body temperature were examined in the intraoperative period was that there was no significant difference between the groups at the first 15 minutes of operation *(p ≥ 0.05)*, but the active warming group was significantly higher than the control group in 30th, 45th, 60th minutes in intraoperative period *(p ≤ 0.05)*, (Figure 1).

Table 1. Identifying characteristics of patients (n: 57).

Groups*		A (n:28)		C (n:29)		Statistical Analysis
		n	%	n	%	
Gender	Female	7	12.3	10	17.5	X^2:.612 p: .43
	Male	21	36.8	19	33.3	
Age (year)	0-1	9	15.8	13	22.8	X^2:.967 p: .23
	2-18	19	33.3	16	28.1	
ASA	I	24	42.1	25	43.9	X^2:.536 p: .76
	II	3	5.3	2	3.5	
	III	1	1.8	2	3.5	
Operation time (minute)	0-60	21	36.8	25	43.9	X^2:1.149 p: .23
	Over 60	7	12.3	4	7.0	
Duration of fasting (hour)	0-4	1	1.8	2	3.6	X^2:2.987 p: .22
	5-8	19	33.9	23	41.1	
	Over 8	8	14.3	3	5.4	
Shivering	Yes	1	1.8	13	22.8	X^2:13.086 p: .001**
	No	27	47.4	16	28.1	
Sum		n	%	n	%	n	%
		28	49.1	29	50.9	57	100
		Mean± SD		Mean± SD		Statistical Analysis
Operating room temperature		25.9±2.0		26.7±1.3		t: 1.853 p:.06**
Awakening time from anesthesia (minute)		8.4±3.0		10.7±3.0		t: 2.832 p:.006**
Length of stay in hospital (day)		1.5±2.5		1.8±1.5		U: 281.0 p:.01**

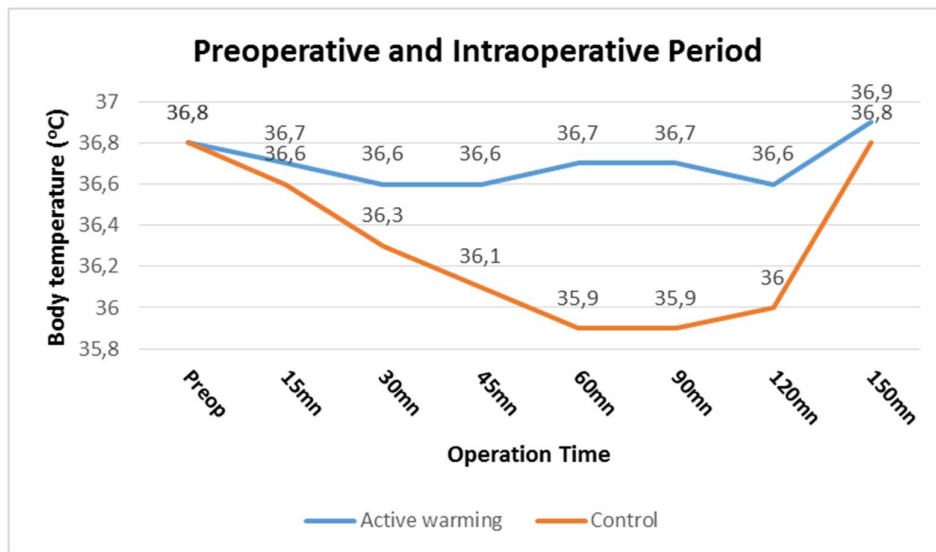*A: Active Warming Group; C: Control Group **p≤0.05

Figure 1. Change of body temperature during preoperative and intraoperative period. *Preop: Body temperature at the time of admissıın in operating room.
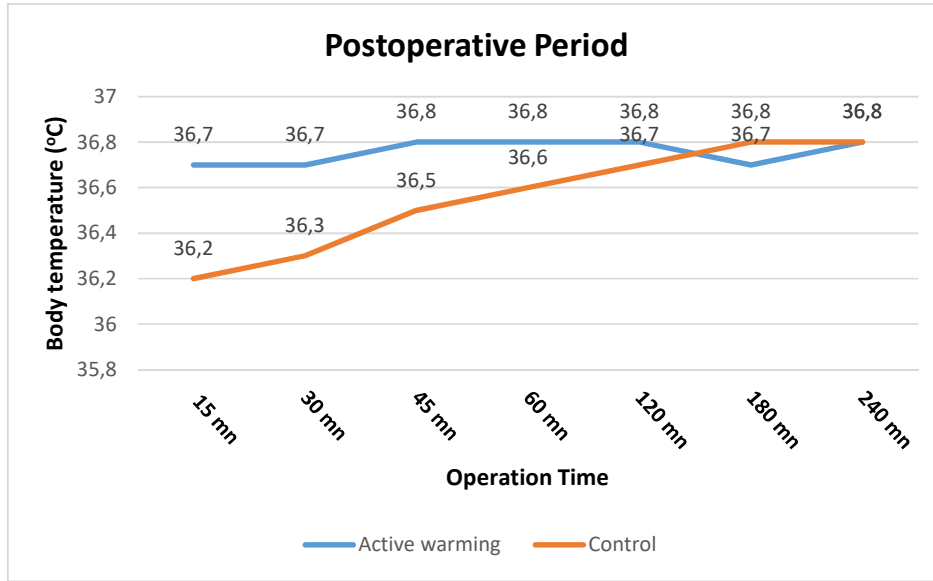
Figure 2. Change of body temperature during postoperative period.

In the postoperative period, there was no statistically significant difference in the body temperatures between the control group and the active warming group *(p≥.05),* (Figure 2).

When pain was examined in the postoperative period, it was observed that the pain mean score of control group was significantly higher than the pain mean score of the active warming group only in the 15th minute *(p ≤0.05)* (Table 2).

Table 2. Pain in the postoperative period.

Groups		A	C	*p***
Postoperative Pain	**15 mn**	6.0±3.4	3.5±3.2	*.006*
	30 mn	3.2±3.1	2.5±3.2	*.20*
	45mn	1.5±1.9	0.8±1.8	*.11*
	1 hour	0.7±1.3	0.2±0.8	*.10*
	2 hour	0.4±0.9	0.5±1.2	*.94*
	3 hour	0.2±0.8	0.4±0.9	*.22*
	4 hour	0.1±0.5	0.07±0.3	*.57*

Values: Mean ±SD, ******A: Active Warming Group; C: Control Group **p≤0.05*

Shivering were observed in 44.8% of patients in the control group and 3.6% of patients in the active warming group; the difference between the groups was significant *(X^2:13.086; p≤ 001).* It was determined that patients in the control group had a longer stay in the hospital *(Mann-Whitney U: 281000; p ≤0.05)* and the amount of time waking at the end of the anesthesia was shorter in the active warming group than the control group *(t: 2,832; p≤ 05).*

Discussion

Hypothermia is an important complication during the perioperative period [15,16]. Body temperature should be followed very carefully in both the intraoperative and postoperative period [17]. Healthcare professionals should know the underlying causes of hypothermia and the associated risk factors and also know the strategies to prevent or treat hypothermia. Nowadays, there are many ways that clinicians may help reduce hypothermia in children. It is reported that the use of an active patient warming for children under anesthesia is an effective method to protect patients from

unwanted perioperative hypothermia [18]. Tander states that children's body temperature drops within ten minutes after induction of anesthesia [9]. Continuous monitoring of core temperature is necessary to prevent hyperthermia.

Ying Pu et al. [19] reported that they warmed patients intraoperatively with warming blanket in gastrointestinal surgery; after the operation, heated patients were found to have lower pain than patients in the control group. Pain and delayed recovery are defined in relation to perioperative hypothermia [16]. Studies have reported that prevention from hypothermia reduced pain after surgery after the operation, especially in children, and this is confirmed by our study because the control group had higher pain scores in our study.

Gharavifard et al. [20] reported that awakening time of children from general anesthesia was 15.30±5.27 minutes after surgery and it was observed that the body temperatures of the children decreased during the surgery in their study. In our study, awakening time of children from general anesthesia was 10.7±3.0 minutes in control group. This value was found to be significantly higher than the awakening time of the warming group (8.4±3.0).

It was reported that normal thermoregulation should be provided in children, otherwise the hospitalization will be prolonged. In our study, it was seen that the control group stayed in the hospital longer than the warming patients.

Radiant heaters are not suitable for use in operating rooms. It also has its difficulties in when used for breastfeeding babies. The blanket or shield can be used for children [21]. The resistive heating system that was used in this study had a shield. Children can be transferred from one place to another using this system.

Studies have clearly shown the benefits of using the active warming systems in infants and children [22]. Sultan et al. [23] reported that the active warming after birth lowered the perioperative temperature and the incidence of hypothermia and shivering was decreased. In this study, postoperative shivering after intraoperative active warming was significantly lower than other patients.

Shen et al. reported that they found the incidence of postoperative hypothermia 5% in their study [24]. In this study, the postoperative hypothermia rate in the control group was 13.8%. No hypothermia was observed in children with the active warming group.

In the literature, force-air warming devices, radiant warmers, and circulating water mattresses are reported as a main method for perioperative hypothermia management [24]. Resistive heating method can be added to these active warming methods. It is also important that such materials can be cleaned due to the recent COVID 19 pandemic. In developing countries, the use of disposable covers cannot be applied due to cost. The resistive system with carbon fiber used in this study is recommended because it is a cost-effective method since it can be disinfected.

In addition to all these, if hypothermia develops, the children must be dressed and covered with a heated blanket and they should be heated by appropriate methods, such as active warmimg medhods. Anesthesia team, who provide perioperative care, are responsible for ensuring and maintaining temperature control during the surgical process [25]. Children' body temperature should be measured every 2 hours until it rises to more than 36.5 °C [26].

The limitations of this study are the fact that the body surface areas of the patients could not be

measured and the operating room temperature could not be fixed.

Conclusions

Consequently, active warming with a carbon fiber resistive system was found to be an effective method for the prevention of unplanned hypothermia in children. It was also determined that there was less shivering, the amount of time for waking the end of the anesthesia were faster and discharge times were shorter in active warming group.

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Investigation of a healthcare-associated candida infections in a Turkish intensive care unit: risk factors, therapy and clinical outcome

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ABSTRACT

Aim: Candida infections develop especially in intensive care unit (ICU) patients and increase the mortality rates. So, early and accurate diagnosis of Candida infections and determination of risk factors are very important. We aimed to retrospectively investigate Candida infections in terms of species and risk factors for candidemia caused by fungi.

Methods: Candida infections in critically ill patients hospitalized in the Intensive Care Unit of Bolu Abant Izzet Baysal University Training and Research Hospital between January 2014 and December 2018 were retrospectively analyzed. The isolated Candida species were evaluated according to the sample types. The cases were investigated in terms of mortality due to candidiasis, previously used antibiotics and isolated Candida species.

Results: 34 Candida species isolated from fungal cultures in ICUs were included in the study. *Candida albicans* (73.5%) was the most prevalent species isolated (NAC 26.5%). Patients with Candida isolated in their urine samples (76%) had a higher mortality rate than patients with Candida from other regions.

Conclusion: The results of our study suggest that the highest rate of candidiasis (88%) is in patients who received beta-lactam antibiotic treatment. In addition, we think that when Candida species are detected in the urine samples of critically ill patients in the ICU, care should be taken in terms of candidiasis.

Keywords: Candida, health care, intensive care unit, candidiasis, mortality.

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Introduction

Healthcare-associated infections (HAIs) are the infections that occur while receiving healthcare, that are developed in a hospital or another health center. These infections are seen within the first 48 hours or in longer period after hospitalization or that occur within 30 days after receiving healthcare. Center for Disease Control and Prevention (CDC) reported that 1.7 million hospitalized patients acquire HAIs every year while receiving a treatment for other health problems and more than 98.000 patients (one out of 17) die annually due to HAIs [1]. Candida species are opportunistic pathogenic microorganisms that are normally seen in human flora and may be the cause of an infection when the host's immune system weakens [2]. As a result of improving

healthcare services, many diseases are no longer a threat to people and their life expectancy is prolonged. However, various opportunistic infections occur due to prolonged hospital stays and antibiotics used. With the increase of awareness, the number of reported opportunistic fungal infections started to increase. In HAIs, besides patient-related factors, procedures applied to patients, long-term antibiotic use and candida colonization cause invasive candida infections with high mortality [3]. Candida species are the fourth most common pathogens causing nosocomial bloodstream infections [4]. The incidence of both *Candida albicans (C. albicans)* and *non-albicans Candida* (NAC) species is increasing [5]. Determination of risk factors in patients with Candida infections will be useful for the prevention of mortality and morbidity in the future. Hence, this study aimed to determine risk factors for candida infections due to *C. albicans* and NAC species and compare the patients infected by these two species.

Materials and Methods
The study protocol was approved by the Bolu Abant İzzet Baysal University Research Ethics Committee (Date and decision number: 2019/237). After getting approval from the local ethics committee, we retrospectively investigated candida infections in critically ill (A patient who needs monitoring to live because of organ/system failures or dysfunction) intensive care unit (ICU) patients in our institution between January 2014 and December 2018. Candida isolates were obtained from different clinical specimens such as urine, blood, sputum, wound, pleural fluid, and peritoneal fluid. Diagnosis of HAI candidiasis infection was made according to CDC criteria [6]. Only the first episode of candidiasis was considered in each patient,

recurrent episodes were excluded. Patients were followed up by an infectious diseases specialist and infection control nurse of our hospital on daily basis and required antibiotic and antifungal treatments were managed according to the guidelines.

Microbiologic sample identification
Samples sent to our microbiology laboratory were cultivated into Sabouraud Dextrose Agar (SDA) (Oxoid, England) medium and incubated at 37°C for 24-48 hours. After incubation, yeast isolates in which growth was found were identified at the level of species by using germ tube test and VITEK 2 Compact (BioMérieux, France) fully automated identification system.

Isolated candida species were evaluated according to the sample types. Cases were statistically compared in terms of mortality, previously used antibiotics and isolated candida species. Results were shown as the mean ± standard deviation for continuous variables, and the number of cases and percentage (%) for nominal variables. Categorical variables were evaluated using Pearson's Chi-square test and Fisher's exact test. Differences were considered statistically significant if the P value was less than *0.05*.

Results
A total of 34 candida species were isolated from fungal cultures of 34 patients (19 female and 15 male). Cultures were obtained from different clinical specimens. Among the isolates, 25 were *C. albicans* and 9 were NAC species. 17 isolates were from blood samples, 13 from urine samples and 4 from other types of samples (pleural fluid, peritoneal fluid, wound and sputum). When isolated candida strains were distinguished as *C. albicans* and NAC, the highest rate of mortality was seen in patients in

whom *C. albicans* was isolated. When the clinical course of the patients was evaluated in terms of discharge and mortality, the highest mortality rate was found in patients in whom *C. albicans* were isolated from their urine samples. Mortality rates according to sample types of *C. albicans* and NAC were also given in Table 1.

When the patients were classified according to the antibiotics (Beta-lactam, Carbapenem, Quinolone, anti-anaerobic antibiotics, etc.) they used before *C. albicans* and NAC were isolated, the highest rate of candidiasis (88%) was seen in patients who received beta-lactam antibiotic treatment (Table 2).

Table 1. Previously used antibiotics of the cases.

Antibiotics	Male (n/%)	Female (n/%)	Total (n/%)	*P*
Beta Lactam	17 / 89.50	13 / 86.70	30 / 88.20	*0.801*
Carbapenem	3 / 15.80	4 / 26.70	7 / 20.60	*0.436*
Quinolone	5 / 26.30	3 / 20	8 / 23.50	*0.666*
Anti-anaerobic	3 / 15.80	2 / 13.30	5 / 14.70	*0.841*
Anti-staphylococcal	3/ 15.80	5 / 33.30	8 / 23.50	*0.231*
Other	6 / 31.60	3 / 20	9 / 26.50	*0.447*

Table 2. Discharge and mortality rates of the cases according to culture type.

Culture type	Pathogen				Total (n/%)	
	Candida albicans (n/%)		*Non-albicans Candida* (n/%)			
	Discharge	Exitus	Discharge	Exitus	Discharge	Exitus
Urine	3 / 30	7 / 70	0 / 0	3 / 100	3/ 23.10	10 / 76.90
Blood	8 / 61.50	5 / 38.5	1 / 25	3 / 75	9 / 52.90	8 / 47.10
Sputum	0 / 0	0 / 0	1 / 100	0 / 0	1 / 100	0 / 0
Wound	1 / 100	0 / 0	0 / 0	0 / 0	1 / 100	0 / 0
Pleural Fluid	0 / 0	0 / 0	0 / 0	1 / 100	0 / 0	1 / 100
Peritoneal Fluid	1 / 100	0 / 0	0 / 0	0 / 0	1 / 100	0 / 0
Total	13 / 52	12 / 48	2 / 22.20	7 / 77.80	15 / 44.10	19 / 55.90
p	*0.235*		*0.198*			

Discussion

Candida infections are important health problems in critically ill patients in ICUs. These infections occur as a result of complicated surgical procedures, use of invasive medical devices and long term and broad-spectrum antibiotic treatment [5]. Thus, determining the risk factors for Candida infections will contribute to the decrease in occurrence of these types of infections. Recently, distribution of Candida species has shown an increase in favour of NAC rather than *C. albicans* [7]. Less response to antifungal treatment in NAC infections contribute to the increase in mortality rates [8-10]. In studies conducted in Turkey, *C. albicans* (CA) was generally isolated more often and the incidence rates of CA differs between 45.8% and 75.6% (Table 3) [11-16].

In this study, the rate of CA among isolated Candida infections that were found in critically ill ICU patients was 73.5%. In our study, we also found the incidence of CA infections to be more than NAC infections. Compared with other studies, it was thought that the limited number of patient groups included in this study (critically ill) may lead to a higher incidence of CA.

Candida infections are more commonly seen in patients hospitalized in ICUs due to many different reasons and also because of their weak immune system. In these kinds of patients, interventional procedures such as urinary catheter and intravenous catheter applications increase the susceptibility of patients against these infections. Candida infections are

Table 3. Previous studies of health care associated candidiasis infections from Turkey.

Studies	Institution	Subjects	Duration	Result
Çagan et al.[11]	Marmara University Faculty of Medicine	29 pediatric patients	2011-2013	55.1% *C. albicans*
Karalti[12]	Yeditepe University Hospital	249 urine specimen	2006-2016	54.2% *C. albicans*
Kilincel et al.[13]	Duzce University, Research and Training Hospital	81 blood cultures	2012-2018	55% *C. albicans*
Savci et al.[14]	Bozok University Research and Application Hospital	42 clinical specimens	2014-2016	66.7% *C. albicans*
Yapar et al.[15]	Tertiary-care educational hospitals in Turkey	83 Blood cultures	2008-2009	45.8% *C. albicans*
Sav et al.[16]	Erciyes University Hospital	3905 clinical specimens	2011-2012	75.6% *C. albicans*
Present study	Bolu Abant İzzet Baysal University Hospital	34 critically ill patients	2014-2018	73.5% *C. albicans*

characterized with high rate of mortality [17]. The most common types of infections seen in ICUs are bloodstream infections, catheter-associated infections and urinary tract infections [18]. In this study, the highest rate of Candida was isolated from blood (50%) and urine (38%) samples. Mortality rate was higher in patients in whom candida was isolated from urine samples (76%) compared to the patients in whom candida was isolated from other sites. It should be considered that Candida isolated especially from urine samples of critically ill patients in ICUs may increase mortality.

Multiple risk factors were attributed to Candida infections. One of the most important predisposing factors is long-term and broad-spectrum antibiotic treatment [19]. In this study, when the patients were classified according to the types of antibiotics used before the detection of Candida infection, beta lactam antibiotics (88.2%) was found to be the most commonly used antibiotic group. Studies on the predisposing factors causing Candida infections are not sufficient in Turkey. However, Yapar et al. [15] and Büyüktuna et al. [19], revealed a relationship between long term antibiotic use and Candida infection.

Our study has some limitations. Patients in ICUs who were included in the study were only analyzed according to the types of antibiotics used in terms of predisposing factors. Predisposing conditions such as hematopoietic stem sell or solid organ transplantation, diabetes mellitus, malignancy, trauma and renal failure were not questioned in patients. Moreover, the presence of urinary catheter and intravenous catheter was not investigated. In addition, several factors other than candida infection might have contributed to mortality. Therefore, we cannot definitely say that Candida infections were the only reason of mortality rates.

In conclusion, it was concluded in this study that the highest rate of Candida infections in ICU patients belonged to *C. albicans* species. It should be considered that Candida species isolated from candida infections may affect the prognosis of patients. Early diagnosis and treatment in Candida infections will help to decrease mortality rates. In this study, the highest rate of mortality was seen in *C. albicans* infections strains isolated from urine samples. Therefore, we think that when Candida species are detected in the urine samples of critically ill patients in the ICU, care should be taken in terms of candidiasis.

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Changes in levothyroxine pharmacokinetics following bariatric surgery in obese hypothyroid patients

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ABSTRACT

Aim: Levothyroxine (L-T4) requirements in obese hypothyroid patients receiving L-T4 therapy decrease following bariatric surgery. L-T4 absorption is also thought to decrease after surgery. The purpose of this study was to evaluate L-T4 requirements in hypothyroidism cases before and after weight loss through bariatric surgery.
Methods: Seventy-six cases of hypothyroidism receiving L-T4 replacement therapy and with a body mass index over 40 kg/m2 were included in the study. Patients losing at least 10% of basal body weight following bariatric surgery were assessed in terms of thyroid hormone levels and L-T4 requirements over follow-up of at least one year. The L-T4 requirements of patients in whom euthyroidism was achieved were compared in terms of bariatric surgery procedures and hypothyroidism etiology.
Results: Seventy-six patients (56 women, 20 men) with a mean age of 38 years (18-51) were included in the study. Mean weight before bariatric surgery was 121.6 ± 6.8 kg, and mean body mass index was 49.5 ± 1.6 kg/m^2. Euthyroidism was confirmed at pre- and post-bariatric surgery evaluation. No statistically significant postoperative changes were determined in thyroid-stimulating hormone or free thyroxine-3 and -4 ($p > 0.05$). A statistically insignificant decrease was observed in L-T4 dosages after surgery in cases of Hashimoto's thyroiditis ($p = 0.064$). A statistically significant decrease was determined in L-T4 dosages in cases of non-Hashimoto hypothyroidism ($p = 0.001$). L-T4 requirements decreased in both surgical procedures ($p = 0.001$)
Conclusion: Postoperative L-T4 requirements decrease with weight loss. In addition, no decrease appeared to occur in L-T4 absorption following the surgical procedures in this study.

Keywords: Obesity, hypothyroidism, bariatric surgery, levothyroxine.

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Introduction

Obesity is one of the most important present-day health risks. Hypothyroidism is commonly observed in obese populations [1]. Clinical and subclinical hypothyroidism leads to weight gain due to a decrease in basal metabolism rates. According to common perception, hypothyroidism is responsible for obesity. However, establishing a causal link between the two is controversial. Recent observations indicate that changes in thyroid-stimulating hormone (TSH) may well be secondary to obesity. At the same time, the replacement dose of Levothyroxine (L-T4) can be modified [2].

Drug absorption may be deficient in association with the type of bariatric surgery performed. Drug absorption deficiency can lead to an increased L-T4 requirement in hypothyroid patients. However, the data concerning this are inconsistent [3]. Research has shown that obese hypothyroid patients require higher L-T4 dosages than normal weight individuals [4]. L-T4 dosages decrease following weight loss [5]. However, it may be necessary to increase the dosages due to the possibility of resection-related reduced drug absorption.

Decreased L-T4 requirements have recently been shown in the majority of hypothyroid patients in line with weight loss achieved with modern bariatric surgery. The purpose of the present study was to investigate the effect of weight loss following bariatric surgery performed using mini-gastric bypass and sleeve gastrectomy procedures on L-T4 requirements in hypothyroid patients.

Materials and Methods

This study was performed in compliance with the Declaration of Helsinki and was approved by the Ethics Committee of the Medicana International Samsun Hospital, Turkey (12.08.2020-9/7109).

The research concerned 76 patients. The study group occurred of adult (over 18 years) hypothyroid patients (Hashimoto's thyroiditis, thyroidectomy, radioactive iodine ablation therapy, congenital hypothyroidism, central hypothyroidism, post-subacute thyroiditis, or infiltrative causes) receiving L-T4 replacement therapy under follow-up in our endocrinology and obesity surgery department, with body mass index exceeding 40 kg/m2 (class 3 obesity), and undergoing bariatric surgery. Patients were assessed using thyroid gland ultrasonography in order to avoid diagnostic confusion.

Patients with known thyrotoxicosis during screening, with primary endocrine/systemic disease, any inflammatory disease other than Hashimoto's thyroiditis, celiac disease and malabsorption, inflammatory gastrointestinal diseases, cancer, history of use of other medications reducing drug absorption, or receiving radiotherapy to the neck and head region were excluded. In the light of the study aims, patients achieving weight loss of at least 10% from baseline following bariatric surgery were evaluated in terms of thyroid hormone levels and LT-4 requirements initially and over a 12-month follow-up period at intervals of 2-3 months.

Patients were given detailed instruction concerning L-T4 use and asked to take the drug as described one hour before breakfast. The importance of the timing of L-T4 use was emphasized, particularly in cases using multivitamins and proton pump inhibitors in the postoperative period. Cases using the drug irregularly or frequently forgetting to take L-T4 were placed under close monitoring, and thyroid hormones were evaluated during periods when L-T4 was used regularly. If TSH values were below 2.5 mIU/L in cases losing more than 10% of body weight following bariatric surgery, the L-T4 dosage was reduced by 25%. The dosage was decreased by 30% in cases with TSH below 1 mIU/L at postoperative follow-up.

Hormonal data for TSH (normal range 0.35-4.94 mIU/L), free thyroxine-4 (fT4; normal range: 9.01-19.05 pmol/L), free triiodothyronine (fT3; normal range: 3.5-8.0 pmol/L), albumin (normal range: 3.5-5.5 g/dL), thyroglobulin antibody (TgAb; normal range: 0-4.11 lU/mL), and thyroid peroxidase antibody (TPOAb; normal range: 0-5.61 IU/mL) levels were investigated using automated immune chemiluminometric assay

(ICMA) kits (Monmouth, UK). Thyroid ultrasonography was applied using a high resolution device (Philips Medical system, USA) equipped with a 5–12 MHz broadband linear array probe. All procedures were performed by an experienced operative.

Statistical analysis

Data analysis was performed on SPSS version 21.0 software (SPSS Inc., Chicago, USA). Quantitative parametric data were expressed as mean plus standard deviation (SD), and quantitative non-parametric data as median values with minimum and maximum. The Kolmogorov Smirnov and Shapiro-Wilk test was used to analyze the distribution of variables. For non-parametric data, comparisons between different groups were performed using the Mann-Whitney U test, while the independent-t test was used to compare parametric data between the groups.

Results

Seventy-six patients (56 women, 20 men) with a median age of 38 years (18-51) were included in the research. Mini-gastric bypass was performed in 52 cases, and sleeve gastrectomy in 24. Postoperative follow-up time was 2.4 (1.7-3.6) years. Hypothyroidism associated with the surgical procedure was present in 28 cases, Hashimoto's thyroiditis in 32, and hypothyroidism associated with other causes (radioactive iodine [RAI] intake, congenital hypothyroidism, post-subacute thyroiditis, or infiltrative causes) in 16. Patients' mean weight before bariatric surgery was 121.6 ± 6.8 kg, and mean body mass index was 49.5 ± 1.6 kg/m^2. Mean weight after bariatric surgery decreased to 94.6 ± 5.6 kg and mean body mass index to 33.3 ± 1.4 kg/m^2. Cases' pre- and postoperative characteristics are shown in Table 1.

All bariatric surgery procedures were performed using standard techniques by an experienced bariatric surgeon. Euthyroidism was confirmed at pre- and post-bariatric surgery checks. No statistical postoperative changes were determined in levels of the thyroid hormones TSH, fT4 or fT3 ($p>0.05$). We found a statistically significant decrease in L-T4 dosages in parallel with weight loss after bariatric surgery in cases diagnosed with hypothyroidism developing after bariatric surgery and other hypothyroidism cases (RAI intake, congenital hypothyroidism, post-subacute thyroiditis, and infiltrative causes) ($p=0.001$). We also determined a non-significant decrease in L-T4 dosages in cases of Hashimoto's thyroiditis ($p=0.064$). The decrease in L-T4 dosages was highly significant in both surgical procedures ($p=0.001$). The findings are shown in Table 2. Albumin levels also decreased, particularly in the mini-gastric bypass cases ($p=0.047$).

Table 1. Preoperative characteristics of the patients.

Parameters	n=76
Age (years), median(min-max)	38 (18-51)
Sex	
Female, n (%)	56 (73)
Male, n (%)	20 (27)
Weight (kg), mean±SD	121.6±6.8
BMI, kg/m^2	49.5±1.6
Etiology of hypothyroidism	
Post-thyroidectomy	28
Hashimoto's thyroiditis	32
Other[1]	16
Type of bariatric surgery	
Mini-gastric bypass	52
Sleeve gastrectomy	24

BMI: Body Mass Index, SD: Standard Deviation
[1]RAI intake, congenital hypothyroidism, post-subacute thyroiditis, infiltrative causes.

Table 2. Changes in thyroid function and L-thyroxine dosages following bariatric surgery.

Parameters	Before bariatric surgery	After bariatric surgery	*p*
Weight (kg) (mean±SD)	121.6±6.8	94.6±5.6	*0.001*
BMI (kg/m^2)	49.5±1.6	33.3±1.4	*0.001*
TSH (mIU/L)	2.1±0.5	1.9±0.4	*0.652*
fT4 (pmol/L)	13.1 ± 0.8	14.9 ± 0.6	*0.860*
fT3 (pmol/L)	4.6 ± 0.4	4.5 ± 0.8	*0.972*

L-thyroxine, mcg/day, dose
    Surgical hypothyroidism
    Hashimoto's thyroiditis
    Other hypothyroidism[1]
	
	149.3±46.7
	152.5±52.3
	140.5±42.5
		
		112.5±32.4
		145.9±36.8
		115.6±31.9
			
			0.001
			0.064
			0.001

Albumin, g/dL	4.7±0.6	4.2±0.8	*0.043*

Type of bariatric surgery,
L-thyroxine, mcg/day, dose
    Mini-gastric bypass
    Sleeve gastrectomy
	
	148.4±49.2
	150.6±51.5
		
		125.2±35.7
		118.9±38.3
			
			0.001
			0.001

TSH, thyroid-stimulating hormone; fT4, free-T4; fT3, free-T3; RAI, radioactive iodine; SD: Standard Deviation. [1]RAI intake, congenital hypothyroidism, post-subacute thyroiditis, infiltrative causes.

Discussion

L-T4 dosage requirements decrease in parallel with weight loss in obese cases diagnosed with hypothyroidism. Loss of body mass eliminates anxiety concerning a decrease in L-T4 absorption resulting from the operation. Newly evidence [5] and our own study data show that thyroid hormone requirements may decrease with weight loss in obesity.

A few studies have determined significant decreases in TSH and fT3 levels with weight loss after obesity surgery. Weight loss also reduces thyroid hormone requirements [5]. Similarly in the present study, we found a statistically significant decrease in L-T4 requirements in association with weight loss. L-T4 dosage requirements decrease in parallel with weight loss to yield similar TSH levels in both types of surgery. In our previous study we observed that TSH levels decreased in correlation with insulin resistance following bariatric surgery [6]. Juiz-Valiña et al. [7] also reported similar findings. We therefore think that L-T4 requirements decreased through a similar pathway to the decrease in insulin resistance occurring with weight loss in association with thyroid hormone resistance in obese patients. It possibly concluded that weight loss restores thyroid hormone homeostasis. Research has shown that severely obese individuals may require higher L-T4-suppressing or replacement therapy than

normal weight subjects in association with impairment of the L-T4 pharmacokinetic parameters [8].

Decreased thyroid gene expression (particularly TSH-receptors) has been reported in visceral adipose tissue and subcutaneous in obese patients [9]. The decrease in adipose tissue in parallel with weight loss following obesity surgery results in corresponding changes in serum TSH and thyroid hormone levels. These changes show that adipocytes are involved in the regulation of thyroid hormones. Our research revealed that L-T4 dose requirements decreased in parallel with weight loss in cases of surgical hypothyroidism and other hypothyroidism cases. This finding suggested that the level of adverse effects on drug absorption caused the decrease in gastric acid in the sleeve gastrectomy group can be overlooked. In the mini gastric bypass cases, however, approximately 1/3 of the length of the intestine was bypassed. This method may have permitted sufficient drug absorption through the non-bypass intestine.

Research has shown that L-T4 requirements decrease in the postoperative period. The principal correlation shown in research is between weight loss and dosage [1]. The principal L-T4 fluid formulation has been shown to be capable of preventing the problem of malabsorption after bariatric surgery in malabsorptive operations (Roux-en-Y gastric bypasses and biliary pancreatic diversions) [10]. Our findings showed no L-T4 malabsorption in sleeve gastrectomy and mini gastric bypasses. Loss of both adipose and non-adipose mass following these operations may result in a decrease in postoperative L-T4 requirements by preventing absorptive effects resulting from surgery [11]. These studies have shown no decrease in L-T4 absorption in Roux-en-Y bypasses. Small but significantly delayed L-T4 absorption has been observed after surgery in these cases [12].

Research involving sleeve gastrectomy and mini gastric bypasses has shown no significant L-T4 absorption in the gut, duodenum, or upper part of the jejunum [13]. These are bypassed or removed in bariatric surgery. These studies offer powerful support for our own findings. L-T4 dosage requirements also decrease in parallel with weight loss. In contrast, although we observed a decrease in drug requirements in both types of operation in hypothyroidism cases associated with Hashimoto's thyroiditis, this was not statistically significant. This variation may be due to an increase dose requirement deriving from the chronic progressive course of Hashimoto's thyroiditis. Alternatively, these operations may have resulted in additional absorption insufficiency due to Hashimoto's thyroiditis being frequently accompanied by celiac disease, inflammatory bowel disease, and autoimmune gastric diseases. We think that these factors may account for the decreased L-T4 dosage requirements determined in other cases not being observed in Hashimoto's thyroiditis cases.

Conclusion
Both adipose and non-adipose body mass decrease with significant weight loss achieved with bariatric surgery. Marked weight loss results in a decrease in postoperative L-T4 requirements. This decrease is more powerful than the effect of resection on absorption. This study shows that sleeve gastrectomy and mini gastric bypasses do not result in L-T4 malabsorption. This study adds new findings to the actual evidence regarding changes caused by weight loss in thyroid morphology.

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Optimization and screening of solid lipid nanoparticle production for gene delivery by factorial design and response surface methodology

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ABSTRACT

Aim: A successful gene therapy requires a delivery system for overcoming various biological barriers. For this, we adapted the factorial design and response surface methodology to the cationic solid lipid nanoparticle production process.

Methods: Screening and optimization of formulations were carried out with factorial design with 3 factors and 3 levels using Box-Behnken Design. Then, solid lipid nanoparticles were physicochemically characterized. Furthermore, optimal SLN formulation is examined in terms of complex formation with plasmid DNA, its protection potential against nucleases, cytotoxicity profile, and storage stability.

Results: Response-surface analyses demonstrated that the selected quadratic model holds significance for particle size and zeta potential. The interaction of independent variables was statistically determined. Optimization and prediction were performed using obtained second-order polynomial equations. Optimal formulation and complexes were found to be nanosized, positively charged and their polydispersity-index values below 0.3 as an indicator of being monodispersed. Cytotoxicity of the optimal formulation is compatible for further studies and no significant increase was observed in particle size until day 21 and until day 60 for polydispersity-index.

Conclusion: Optimal formulation provides a good basis as a gene delivery system was produced with developed systematic. Briefly, this methodology could be used to obtain SLNs with desired conditions.

Keywords: Solid lipid nanoparticle, factorial design, response surface method, gene delivery.

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Introduction

According to the U.S. Food and Drug Administration (FDA), gene therapy purpose to change or control the expression of a gene or to make adjustments to the biological features of living cells for therapeutic use [1].

Successful gene therapy requires a gene delivery system for overcoming various biological barriers and the effects of the environment. The gene delivery system must protect the genetic material from lysosomal degradation when taken into cell cytoplasm with endocytosis and then be able to transcend the nuclear membrane as a biological barrier. Genetic material also should be protected against harsh environmental conditions such as pH and temperature change, redox reactions [2–5].

Various gene transfer techniques are applied. They are divided into viral and non-viral techniques. As viral techniques; adenoviruses, adeno-associated viruses, lentiviruses, and retroviruses are often used for gene delivery. Non-viral techniques contain; inorganic particles (calcium phosphate, silica, gold, magnetic), synthetic or natural biodegradable particles (polymeric, cationic lipid, peptide-based vectors) and physical methods (needle injection, ballistic DNA injection, electroporation, sonoporation, photoporation, magnetofection, hydroporation) [6–8].

Although viral techniques are mostly used in clinical trials, some of their difficulties caused alternative techniques to be considered. These difficulties are biosafety problems (high immunogenicity and potential insertional mutagenesis) and limited gene package capacity [9–11].

One of the non-viral methods, solid lipid nanoparticles (SLN); are non-toxic carrier systems stabilized by surfactants and made from solid lipids for controlled and targeted delivery. Their advantageous features are, biocompatibility, assembling of hydrophilic and hydrophobic drugs, maintain the release of the drug and avoid photochemical, chemical, or oxidative degradation [12]. These systems have several advantages such as improved transfection, reduced immunogenicity, large gene package size, ease of manufacture, protection from degradation, prolong circulation of the nanoparticles (NPs), reduce side effects, realize controlled release of the contents, and enhanced targeted delivery [13]. SLNs are preferred as gene delivery systems due to their large surface areas, small dimensions, ability to transfect genetic material, biocompatibility, and suitability for large-scale production [9,14]. SLNs could be produced by various methods; high shear homogenization and ultrasound, high-pressure homogenization (hot and cold), solvent emulsification/evaporation, microemulsion based SLN preparations [15–19].

For the production of SLNs, the selection of components, their proportions, and the differences depending on the preparation method lead to the presence of a wide alternative sample space. Therefore, a method that allows a systematic analysis should be applied prior to experimental investigation. In a factorial design, the appropriate number of samples for various factors and levels are determined for the analysis of independent variables and dependent variables. The main goal is to achieve the highest level of output with the least number of data in order to reduce the labor force and cost while increasing efficiency. Box-Behnken designs are used to generate higher-order response surfaces using fewer required runs with factorial design technique. Box-Behnken design can also perform statistical analysis of the model, response optimization, and prediction of the formulations that meet the desired conditions [20,21]. In this study, we used a factorial design for scanning microemulsion components and optimizing SLN formulation. We examined the Design of Experiments (DOE), which we propose to be used in the development of formulations that are potential as gene delivery systems using Response Surface Methodology (RSM). Moreover, we characterized the complex formed by the optimal SLN formulation with plasmid DNA and examined it in terms of its protection potential against nucleases, cytotoxicity profile, and storage stability.

Materials and Methods

This study has been projected by the Scientific and Technological Research Council of Turkey

(TÜBİTAK) under code TÜBİTAK-SBAG-218S682. EGFP encoding plasmid pcDNA3-EGFP was a gift from Doug Golenbock (Addgene plasmid # 13031; http://n2t.net/addgene:13031; RRID: Addgene_13031). Behenoyl polyoxyl-8 glycérides (Compritol® HD5 ATO) (C-HD5) was kindly donated by Gattefosse, France. Macrogolglycerol ricinoleate (Kolliphor® ELP) (K-ELP) was kindly donated by BASF, Germany. Mouse fibroblast cell line (L929) was purchased from ATCC, USA. Ethanol (EtOH), Sodium dodecyl sulfate (SDS) were provided from Merck-Co., Germany. Dimethyldioctadecylammonium bromide (DDAB) was purchased from Sigma-Aldrich Co., USA. Alamar Blue cell viability assay kit was purchased from Thermo Fisher Scientific, USA. All other chemicals were of analytical grade and used as received. Ultrapure water was used in all stages needed.

Nanoparticle preparation
Cationic SLNs were produced with slight modifications to the previously described melt-emulsification technique [22,23]. As formulation components, C-HD5 and DDAB were used as lipid phase; K-ELP was used surfactant (S); EtOH was used as co-surfactant (CoS), and UPH$_2$O was used as an aqueous phase. In the first step of production, microemulsion contents were weighed in a sealed cap glass vial as determined by factorial design based on preliminary laboratory studies. Then, the vial and its content were kept in a water bath at 10 degrees above the lipid melting point (75°C) until transparent microemulsion was self-established. Obtained oil in water (o/w) microemulsion was dispersed in cold ultra-pure distilled water (0-4 °C) at a ratio of 1:10 (w/v) under vigorous stirring. SLNs were obtained by sudden freezing of microemulsion droplets in cold water.

Experimental design
Screening and optimization of SLN formulations were carried out with factorial design with 3 factors and 3 levels. The amount of lipid (3%, 4%, and 5%) and the total amount of surfactant and co-surfactant (18%, 24%, and 30%) were selected as two variables. These variables are important since they form two main corners of the pseudo-ternary phase

Table 1. Variables and their levels in Box-Behnken Design.

Variables		Level of variables		
Independent variables		Low Level (-1)	Mid-Level (0)	High Level (1)
A	Lipid (%)	3%	4 %	5%
B	Surfactant + Co-Surfactant (1:2, w/w) (%)	18 %	24%	30%
C	Lipid to cationic lipid ratio (w/w)	2:1	1:1	1:2
Dependent variables		**Goals**		
Y1	Particle Size (nm)	Minimize		
Y2	Polydispersity Index	Minimize		
Y3	Zeta Potential (mV)	Target 35 mV		

Table 2. Designed formulation parameters and measured responses using Box-Behnken Desing for SLN production.

No	Formulation Code	A	B	C	Y1	Y2	Y3
		Lipid (%)	S + CoS (%)	L/Cat.L (w/w)	Particle Size (nm) ±S.D.	PDI ± S.D.	Zeta Potential (mV) ±S.D.
1	C-HD5-SLN 1	3	24	2: 1	3155 ±116	0.756 ±0.060	33.8 ±7.87
2	C-HD5-SLN 2	3	24	1: 2	200.4 ±29.37	0.341 ±0.070	34.6 ±2.67
3	C-HD5-SLN 3	5	24	2: 1	3513 ±117.1	0.983 ±0.046	64.6 ±4.09
4	C-HD5-SLN 4	5	24	1: 2	146.1 ±15.6	0.239 ±0.042	41.4 ±8.25
5	C-HD5-SLN 5	4	18	2: 1	4024 ±470.2	0.858 ±0.202	59.8 ±0.96
6	C-HD5-SLN 6	4	18	1: 2	65.31 ±2.578	0.628 ±0.038	40.0 ±3.12
7	C-HD5-SLN 7	4	30	2: 1	690.2 ±172.4	0.740 ±0.150	35.3 ±14.4
8	C-HD5-SLN 8	4	30	1: 2	45.86 ±16.63	0.468 ±0.224	40.6 ±3.96
9	C-HD5-SLN 9	3	18	1: 1	514.3 ±62.17	0.531 ±0.082	61.5 ±2.40
10	C-HD5-SLN 10	5	18	1: 1	708.4 ±118.2	0.561 ±0.108	60.8 ±1.68
11	C-HD5-SLN 11	3	30	1: 1	592.7 ±92.47	0.596 ±0.076	36.3 ±2,85
12	C-HD5-SLN 12	5	30	1: 1	222.1 ±11.95	0.459 ±0.122	39.9 ±3.13
13	C-HD5-SLN 13	4	24	1: 1	399.3 ±19.44	0.516 ±0.291	34.9 ±2.73
14	C-HD5-SLN 14	4	24	1: 1	353.1 ±62.41	0.573 ±0.081	33.5 ±3.48
15	C-HD5-SLN 15	4	24	1: 1	424.5 ±152.4	0.573 ±0.006	32.8 ±1.66

diagram, which is commonly used in the production of the microemulsion [24,25]. The presence and amount of cationic lipids, which are essential for cationic SLN production, were aimed to be investigated as a third variable. For this, the lipid to cationic lipid ratio (2:1, 1:1, 1:2, w/w) was determined as the third variable. Dependent on the preliminary studies and according to the literature, the factorial design matrix was created using the Minitab 19 software (Minitab LLC, USA) using the variables represented in Table 1. Totally 15 different compositions were determined as 12 alternative microemulsion contents and 3 midpoint replicates. Dynamic light scattering (DLS) analysis is widely used for the

characterization of SLNs. Particle size, PDI, and zeta potential measurements obtained by DLS analysis were selected as dependent variables. Response optimization was determined to aim the smallest particle size and the most homogeneous particle size distribution in the manner of the lowest PDI value. Zeta potential was set as a target response value and determined as 35 mV. The designed variables with codes and actual responses in terms of DLS measurement results are given in Table 2. In order to provide screening in the sample space and according to the analysis of variance (ANOVA), the quadratic model was chosen for a Box-Behnken design. With the RSM analysis, P-Value, F-Value, coefficient determinant (R^2),

and adjusted coefficient determinant (R^2 adj) data were evaluated statistically. Actual data of the formulation determined by response optimization were measured and the statistically produced predicted values were calculated. The fit of the model and second-order polynomial equations were determined. Then, the 3D-surface and contour plots were analyzed.

Agarose gel retardation assay, SDS-induced release, and DNase I protection studies
Agarose gel retardation assay was performed to determine the optimal complex formation ratio of C-HD5-SLN and pcDNA3-EGFP. Complexes with various ratios were formed by mixing the constant amount of pcDNA3-EGFP (100 ng/µL) with the increasing amount of C-HD5-SLN under gentle shaking for 30 min at room temperature, which allows the formation of electrostatic interactions between the positive charges of SLNs and the negative charges of pcDNA3-EGFP. The resultant complexes were characterized by agarose gel retardation assay [26–28].
In order to show the release of pcDNA3-EGFP, SDS induced release study was performed. To evaluate the capacity of the C-HD5-SLN to protect pcDNA3-EGFP, complexes were incubated with DNase I (0.4 IU DNase I/1 µg pDNA) at 37°C for 30 minutes, then decomplexed in presence of SDS 1% (w/v) and further subjected agarose gel retardation assay [29]. The bands were observed by a UV transilluminator with a digital imaging system (Vilber Lourmat, France).

Physicochemical characterization for freshly prepared SLNs and stability studies
The particle size, polydispersity index, and zeta potential values of C-HD5-SLN and C-HD5-SLN:pcDNA3-EGFP complex were measured by DLS (Zeta sizer Nano ZS, Malvern Instruments Ltd., UK) method using non-invasive backscattering mode with the detector positioned at 173°. DLS measurements were reported as averaged intensity weighted distribution for particle size measurements. The refractive index of the Compritol (1.456) as a main ingredient of the lipid matrix was used for DLS calculations [30]. The triplicate measurements were performed using disposable polystyrene microcuvettes.
The morphology of C-HD5-SLN and C-HD5-SLN:pcDNA3-EGFP complex were visualized by using Scanning Electron Microscope (SEM, Carl Zeiss 300VP, Germany). Sample preparation was done by drying the nanoparticles on metal plates and then coating with 100 A° thick gold in the brand coating device (Quorum Q150 Res, UK).
The physicochemical stability of the formulation was followed up to 2 months. SLNs were stored at 4 °C and measured at days 0, 7, 14, 30, and 60 to evaluate the stability.

Cytotoxicity analysis
The *in vitro* cytotoxicity profile of the C-HD5-SLN and C-HD5-SLN:pcDNA3-EGFP complex was evaluated on the fibroblast cell line (L929). One day before the experiment, L929 cells were plated in 96-well plates at a density of 5×10^3 cells per well in 100 µL. After overnight incubation, cells were treated with C-HD5-SLN and C-HD5-SLN:pcDNA3-EGFP complex at increasing formulation volumes (3, 6, 9, 12, 15 µL/well), with respect to SLN volume for 24 h. The percentage of living cells was investigated by the Alamar Blue proliferation kit (Thermo Fisher Scientific, USA) according to the kit's manual.

Statistical analysis
Except for the experimental design and RSM, the data analysis was performed with Prism 6

(GraphPad Software, Inc., USA) software. The statistical analysis between different groups has been assessed by a non-paired t-test and Two-way ANOVA followed by multiple comparison tests. Differences were considered statistically significant if the P value was less than 0.05. The results of all experiments were reported as mean ± S.D.

Results and Discussion

Microemulsion content is the main factor determining the physicochemical properties of SLNs obtained by the microemulsion dilution method. With this method, the first step in the production of SLN is to obtain clear transparent microemulsions. One of the most common methods to obtain clear microemulsions is to

is required to decide which point to choose. In order to make this statistical evaluation, dependent and independent variables and related responses should be determined. Particle size is the most widely used variable for SLN evaluation. While encapsulation efficiency is an eligible parameter for SLNs loaded with drugs, the zeta potential value is an important parameter for cationic SLNs that will interact with nucleic acids via electrostatic forces on the surface.

On the pseudo-ternary phase diagrams used in the conventional determination of the content ratios of the microemulsion, each corner has one of the main components of the formulation. In most cases, these are the lipid phase, the aqueous phase, and the total of surfactant and

Table 3. The fit of the model and second order polynomial equations of the responses

Response	Regression analysis of variance				Regression equation in coded units
	R^2	R^2 adj	F-Value	P-Value	
Particle Size	94.19%	83.73%	376.83	0.003	$Y1 = 1824 - 4268\ C - 2077\ A + 303\ C + 1029\ C*C + 332\ A*A - 5.98\ B*B - 103\ C*A + 138.1\ C*B - 23.5\ A*B$
PDI	88.70%	68.37%	6.16	0.143	$Y2 = -0.39 + 0.251\ C + 0.567\ A - 0.0061\ B + 0.1015\ C*C - 0.0377\ A*A + 0.00086\ B*B - 0.0822\ C*A - 0.00592\ C*B - 0.01154\ A*B$
Zeta Potential	88.62%	78.14%	64.12	0.015	$Y3 = 319.9 - 5.7\ C - 61.5\ A - 12.99\ B + 2.08\ C*C + 7.78\ A*A + 0.2252\ B*B - 6.00\ C*A + 1.046\ C*B + 0.179\ A*B$

obtain pseudo-ternary phase diagrams and determine the microemulsion formation region on this diagram [24,25,31]. Then, SLNs are produced by selecting an initial microemulsion formulation based on the central point or several different points of this area. However, there can be an infinite number of alternative microemulsion formulation, regardless of the size of the area. A reliable statistical evaluation

co-surfactant amounts in percentages. Among these factors, the lipid phase and total of surfactant and co-surfactant were selected as formulation independent variables (Table 1). The aqueous phase was not chosen as a variable because determining the percentage of lipid and the percentage of total surfactant and co-surfactant ensures that the percentage of water is also determined since the total of the system

will be equal to one hundred percent. Therefore, the solid lipid/cationic lipid ratio in the lipid phase was chosen as the 3rd variable. The cationic lipid plays an important role in the charge of the formulation, hence its stability and its electrostatic interaction with negatively charged nucleic acids.

With this information, as indicated in Table 1, lipid percentage, total surfactant and co-surfactant percentage, and solid lipid cationic lipid ratio were determined as three independent variables. For the analysis of the response related to these variables, particle size, PDI, and zeta potential values were examined. Box-Behnken Design allows these variables to be evaluated with fewer samples. While it is necessary to test $3^3 = 27$ different microemulsions in 3-factor 3-level factorial design, with Box-Behnken Design optimization, this factorial design can be completed with only 15 experiments with 3 replicate center points. The contents of the microemulsion presented accordingly are given in Table 2 together with the response characterization values.

Response surface regression analysis was performed using the values in Table 2. The second order polynomial equations and statistical evaluations obtained accordingly are summarized in Table 3. According to the RSM analysis, the F value for the particle size was determined as 376.83 (p = 0.003 <0.05). The F value demonstrated that the selected quadratic model holds significance [20,21].

The coefficient determinant (R^2) value was found 94.19% value indicates the reliability of the model together with adjusted R^2 value. Overall, it can be predicted that particle size is affected by all variables. The same interference is valid for zeta potential depending on the statistical data presented in Table 3. However, PDI value does not fit the model while F value

was found to be 6.16 and the P-value is 0.145 >0.05.

When we investigate the surface and contour plots of response surface regression analysis in terms of particle size, PDI, and zeta potential, we observed that the surface and contour plots lines are curved. If the model showed no interaction, the 3D surface plots would be planar [32,33]. Curved structures are the sign of interaction between variables. Likewise, in contour plots, this situation can be demonstrated by the presence of elliptical lines. These inclined plots are indicative of a second-order polynomial equation for the model (Figure 1,2,3). In the polynomial equation, the negative sign coefficient value indicates the antagonist effect and positive values indicate the agonist or synergist effect [20,34]. Regression Equation in Coded Units was given in Table 3. For particle size, A, C * C, A * A, and C * B values have positive coefficient values while B, C, B * B, C * A, and A * B values have negative coefficient values. In terms of PDI, A, C, C*C, and B*B values have positive coefficient values while B, A*A, C*A, C*B, and A*B values have negative coefficient values. And for Zeta potential, C*C, A*A, B*B, C*B, and A*B values have positive coefficient values while A, B, C, and C*A values have negative coefficient values.

RSM helps in the prediction of the theoretical optimum conditions for desired responses [20,21,32]. Mathematically offered formulation components were experimentally obtained. Optimal desired formulation component ratios and, predicted and actual experimental values were shown in Table 4. The obtained data successfully predicts the responses in parallel significance with P values. The particle size and zeta potential values of this formulation, coded as C-HD5-Opt-SLN, are very close to the predicted value. The PDI value was relatively
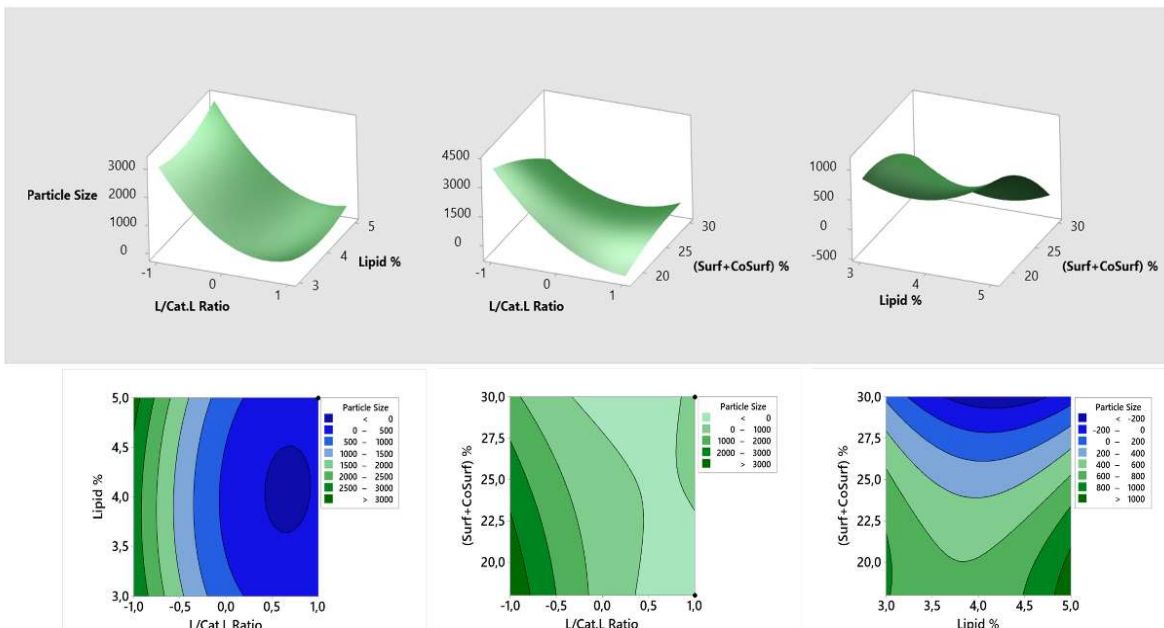
Figure 1. 3D surface response plots and contour plots showing the interference of the variables on particle size.
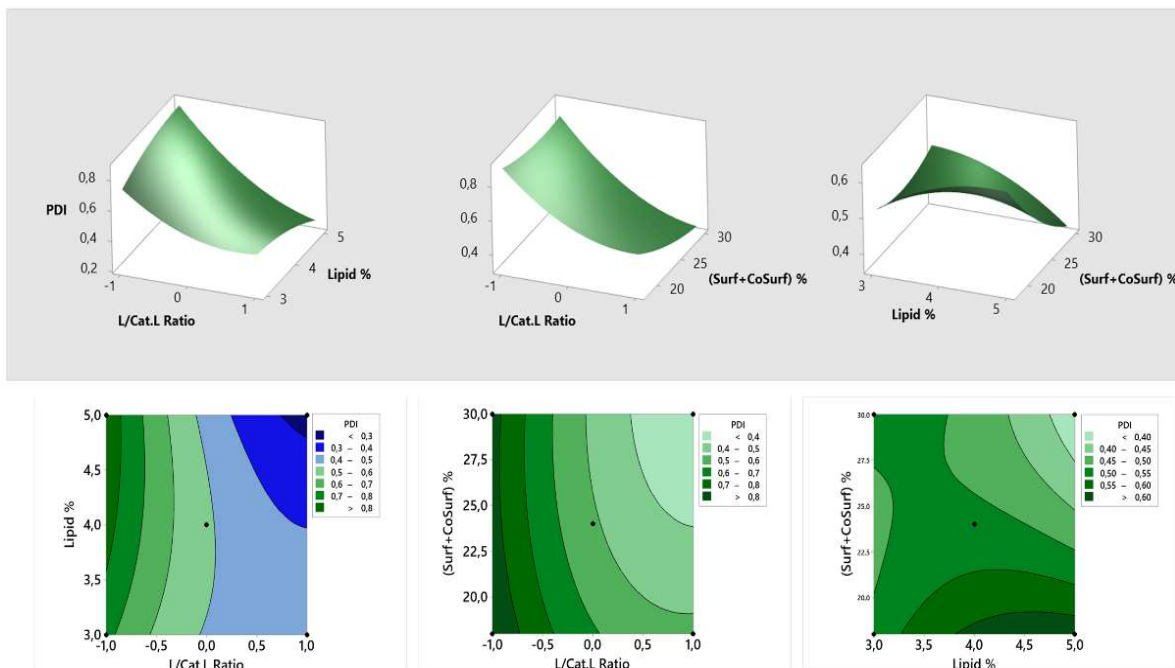

Figure 2. 3D surface response plots and contour plots showing the interference of the variables on PDI.

far from the predicted value, as it was shown in the regression analysis that the PDI value was not fit for the model (P>0.05). Following the determination of consistency in optimized formulation values with the predicted values, the potential of this optimal SLN formulation was investigated in terms of the gene delivery system requirements.

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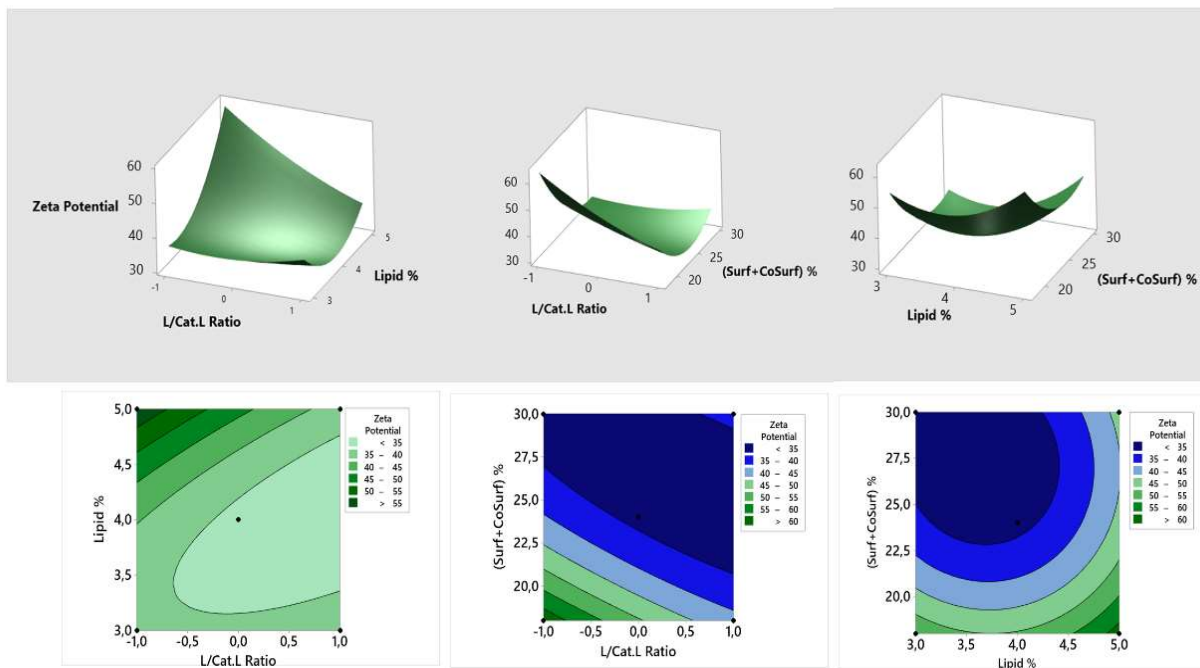

Figure 3. 3D surface response plots and contour plots showing the interference of the variables on zeta potential.

Table 4. Observed and predicted responses of the optimal formulation.

C-HD5-SLN-Opt	Optimized variables			Actual and predicted responses		
	A	B	C	Y1	Y2	Y3
Predicted Values	% 4.45	% 28.3	0.88 (1:1.83 w/w)	48.13	0.279	35.01
Actual Values				51.37 ±0,2843	0.209 ±0.005	35.1 ±5.09

significance with P values. The particle size and zeta potential values of this formulation, coded as C-HD5-Opt-SLN, are very close to the predicted value. The PDI value was relatively far from the predicted value, as it was shown in the regression analysis that the PDI value was not fit for the model (P>0.05). Following the determination of consistency in optimized

completely stopped at the ratio of 1:1 (v/v) as seen in the fourth well. (Figure 4a). The same ratios investigated for the SDS-induced release study and at all ratios, SLNs were released the pcDNA3-EGFP for the total concentration of 1% SDS (w/v) solution (Figure 4b). In Figure 4c, all complex ratios performed in electrophoretic mobility assay were prepared
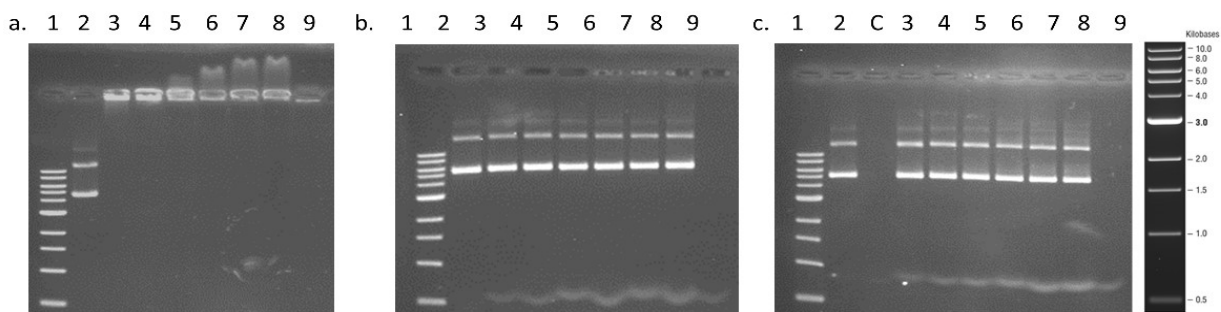

Figure 4 (a-c). Gel retardation, SDS-induced release and DNase I protection study agarose gel images. a) Complex formation ratios of C-HD5-SLN-Opt: pcDNA3-EGFP, b) SDS-induced release of C-HD5-SLN-Opt: pcDNA3-EGFP complexes, c) Enzymatic degradation of C-HD5-SLN-Opt: pcDNA3-EGFP. Lanes from left 1:1kb DNA ladder as molecular weight marker, 2: naked plasmid DNA (100ng/well), 3-7: C-HD5-SLN-Opt: pcDNA3-EGFP complexes (respectively 0.2:1, 0,5:1, 1:1, 2:1, 3:1, v/v). C: Naked pDNA degraded with DNase I enzyme as a control.

formulation values with the predicted values, the potential of this optimal SLN formulation was investigated in terms of the gene delivery system requirements.

Cationic SLNs developed as gene delivery systems form complexes with plasmid DNA *via* electrostatic interactions. However, the ratio of this complex should be determined to find optimal C-HD5-SLN-Opt: pcDNA3-EGFP ratio. For this, electrophoretic mobility assays were performed for the pcDNA3-EGFP plasmid. Samples were prepared by forming complexes with increasing amounts of C-HD5-SLN-Opt, against a fixed amount of pcDNA3-EGFP (100 ng) with vigorous shaking at room temperature. When the agarose gel electrophoresis images were examined, it was observed that the electrophoretic mobility

freshly and then treated with DNase I (0.4 IU DNase I/1 μg pDNA) enzyme to determine the protection capacity of the formulation [27,35]. After incubation at 37°C for 30 minutes, SDS-induced release of pcDNA3-EGFP was performed on both samples and further subjected by agarose gel electrophoresis to compare the condition of the released plasmid. Naked pcDNA3-EGFP was treated with the same amount of DNase I as control of enzyme activity (Lane C). As shown in Figure 2c, the best protection was provided in starting 1:1 (v/v) ratio, which is seen in lane 4 and determined as optimal according to the electrophoretic mobility assay.

Next, the physicochemical properties of the complex (C-HD5-SLN-Opt: pcDNA3-EGFP, 1:1, v/v) were characterized. Particle size was

again found nanosized and below 80 nm. This size range is adequate for traverse into the cells [36]. Moreover, the charge of the system remained cationic. This is a factor that increases the entry of particles and genetic material into the cell by electrostatic interaction with the negatively charged cell membrane (Table 5). One of the precursors of an optimal and compact complex formation ratio is the PDI value. The PDI values below 0.4 indicate that the nanoparticle system is considered as monodispersed for drug delivery and PDI values over 0.7 indicate that the nanoparticle system has a broad size distribution for being a drug delivery system [23,37]. Another important parameter for the characterization of nanoparticles is the particle size distribution plot. For both C-HD5-SLN-Opt and C-HD5-SLN-Opt: pcDNA3-EGFP (1:1, v/v) complex, a single peak was observed (Figure 5). If the structure of the complex was not adequately

compact, more than one peaks would have been observed representing the excessive amount of pcDNA3-EGFP or bare C-HD5-SLN-Opt. Increasing the amount of C-HD5-SLN-Opt and C-HD5-SLN-Opt: pcDNA3-EGFP (1:1, v/v) complex was evaluated for cytotoxicity test (Figure 6). In cytotoxicity studies, C-HD5-SLN-Opt and C-HD5-SLN-Opt: pcDNA3-EGFP (1:1, v/v) complex containing an equivalent amount of nanoparticle were tested in L929 cells. Cytotoxicity profiles are parallel to each other for the nanoparticle and the complex. Although there is dose-dependent cytotoxicity in both cell lines, the cell viability for the required amounts for transfection studies has not fallen below 70%. Delgado et al. used 2.5 µg / mL plasmid DNA for transfection. This corresponds to the use of the C-HD5-SLN-Opt: pcDNA3-EGFP complex of less than 3 µL sample volume used in the cytotoxicity study [38].

Table 5. Physicochemical characterization of optimal complex.

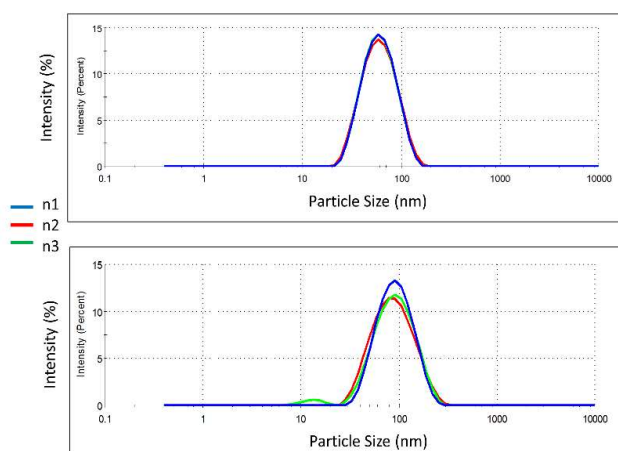| Characterization of Complex	Particle Size (nm) ±S.D.	PDI ± S.D.	Zeta Potential (mV) ±S.D.
C-HD5-SLN-Opt: pcDNA3-EGFP (1:1, v:v)	73.76 ± 0.093	0.253 ±0.007	30.1 ± 1.48

Figure 5. Size distribution plot of C-HD5-SLN-Opt and C-HD5-SLN-Opt: pcDNA3-EGFP, (1:1, v/v) complex. n=3.
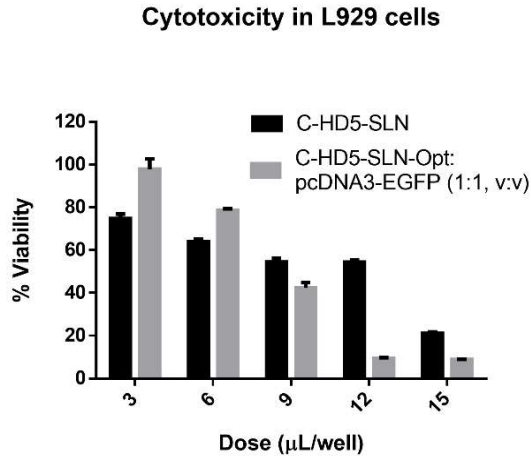
Cytotoxicity in L929 cells

Figure 6. Viability percentages of L929 cells treated with increasing doses of C-HD5-SLN-Opt and equivalent SLN doses of C-HD5-SLN-Opt: pcDNA3-EGFP, (1:1, v/v) complex.

The physicochemical stability of the developed C-HD5-SLN-Opt vector system was also evaluated. For this purpose, C-HD5-SLN-Opt were stored at 4 °C and particle size was measured at days 0, 7, 14, 30, and 60 (Figure 7). No significant increase was observed in particle size until day 21 (p>0.05). PDI value did not change significantly during the duration of stability monitoring.

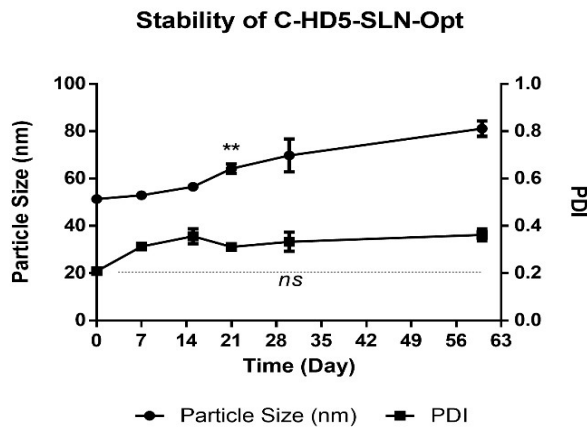**Stability of C-HD5-SLN-Opt**

Figure 7. The storage stability of the developed C-HD5-SLN-Opt delivery system over 60 days at 4 °C. Left Y axis represents the particle size and right Y axis represents the PDI of C-HD5-SLN-Opt. ** represent P<0.01, ns: not significant, n=3.

Representative SEM photographs shows that of C-HD5-SLN-Opt and C-HD5-SLN-Opt: pcDNA3-EGFP, (1:1, v/v) complex are in the global structure and their size is correlated with the DLS measurement (Figure 8). For the advanced characterization of C-HD5-SLN-Opt and C-HD5-SLN-Opt: pcDNA3-EGFP, (1:1, v/v) complex statistical evaluations can be enriched with various methods such as differential scanning calorimetry (DSC) analysis, permeability studies, and pharmacokinetic studies [19].
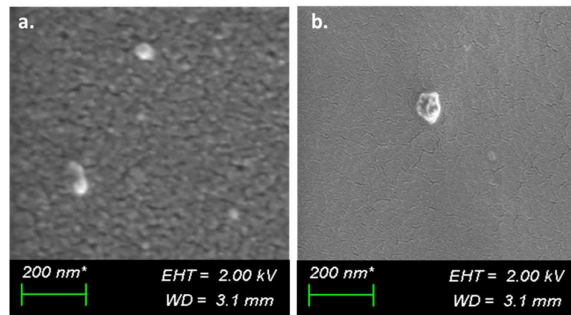

Figure 8. SEM images of (a) C-HD5-SLN-Opt and (b) C-HD5-SLN-Opt: pcDNA3-EGFP, (1:1, v/v) complex.

Conclusion

The cationic solid lipid nanoparticles were successfully prepared using factorial design. For this purpose, Box Behnken design was used to optimize and analyze the formulation parameters. Response optimization was performed via software to find out the optimum conditions for desired SLN formulation. Optimum solid lipid nanoparticle formulation was investigated in terms of its protection potential against nucleases, cytotoxicity profile, and storage stability. In this regard, the obtained C-HD5-SLN-Opt coded solid lipid nanoparticle formulation provides a good basis as a gene delivery system. We showed that Box-Behnken design is a useful model to reduce labor force and cost especially for compounds with low quantities and high prices. With this method, it

is possible to produce solid lipid nanoparticles with the desired feature by differing the variables and responses. Moreover, transfection efficiency can also be analyzed and incorporated into the Box-Behnken Design as a dependent variable and its efficiency can be optimized in the further studies.

Conflict of Interest: None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.
No Competing interests are at stake and there is No Conflict of Interest'' with other people or organizations that could inappropriately influence or bias the content of the paper.

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Experimental Biomedical Research	Original article

Evaluation of risk factors associated with pancreatic adenocarcinoma in Black Sea region, Turkey

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ABSTRACT

Aim: To evaluate the risk factors in patients with pancreatic adenocarcinoma in Turkey's Black Sea region and to determine groups at high risk for pancreatic cancer.

Methods: 106 newly diagnosed pancreatic adenocarcinoma patients living in Black Sea region of Turkey who applied to our clinic between January 2015 and December 2016 were included in this study. In the same period a control group was formed with 92 patients of similar age. Both groups were asked to fill out a form that questioned the pancreatic cancer risk factors. Data were analyzed.

Results: Pancreatic cancer risk was 3.5 times higher in people over 65 years of age. Patients with deficient level of serum vitamin D level (<20 ng/ml) had 10.2 times more risk of pancreatic cancer than patients with normal level of Vitamin D (≥30 ng/ml). While newly diagnosed type 2 diabetics had 19.5 times higher risk of pancreatic cancer, long term type 2 diabetics had 1.2 times higher risk than normal individuals. Those with pancreatic cancer in family had 4.3 times higher risk than those who did not. It was observed that people with rhesus (Rh) antigen negative blood group has 70% less risk of pancreatic cancer.

Conclusion: Determination of pancreatic cancer risk factors, organization of imaging and screening programs for high-risk people can provide early diagnosis of the disease and prolong survival.

Keywords: Pancreatic adenocarcinoma, risk factors, pancreatic neoplasms, etiology, epidemiology.

Introduction

Although pancreatic cancer is the 11th most common cancer type, it ranks as the third leading cause of cancer-related death in the USA [2]. Pancreatic cancer is predicted to be the second most common cause of cancer-related deaths in coming 20 years [3]. In a study conducted by Parkin et al. in 2002, pancreatic cancer accounts for 3% of all cancer types worldwide, however, it is among the top in cancer-related deaths due to its poor diagnosis [1]. In many patients, when pancreatic cancer is detected, the disease is advanced and the tumor is not operable. Median survival rate is less than a year, and mortality rate is approximately 99%. Only less than 10% of pancreatic tumors can be detected at an early phase and resected. Usually the disease starts to develop two years before its diagnosis [4]. Most patients initially have a non-specific pain in epigastrium. As the disease develops in time, the pain spreads over to the back, upper thoracic and lumbar region. Upon progress of the disease, most patients consult doctors with complaints of pain, jaundice, and

loss of weight. It is often the case that when patient seeks medical advice with these complaints, the disease has already reached the advanced stage [1-5].

There are numerous studies conducted to identify causes of pancreatic cancer. In etiology, environmental and genetic causes are considered to play a joint role. Risk factors are divided in two as changeable and unchangeable risk factors. Unchangeable risk factors are age, gender, race, genetic factors, family history, atopy/allergy history, blood groups, surgery history, diabetes and chronic pancreatitis. Changeable risk factors, on the other hand, can be listed as smoking, obesity, use of alcohol, dietary, socio-economic conditions, infections, levels of vitamin D and folic acid. Analysis of risk factors and identification of factors that significantly increases the risk are of high importance [1-10]. Identifying populations of high-risk, establishing imaging and screening programs for these groups can support early diagnosis of the disease and increase survival period. Therefore, in this study, we aimed to identify high-risk groups together with risk factors in patients with pancreatic adenocarcinoma in Turkey's Black Sea region.

Materials and Methods

Ethical approval for this study was obtained from the local ethics committee (IRB approval number 144/2015). Necessary permissions were obtained from the hospital and families. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

106 newly diagnosed pancreatic adenocarcinoma patients living in Black Sea Region of Turkey who applied to our clinic

between January 2015 and December 2016 were included in this study. In the same period, a control group was also formed with 92 patients of similar age group who visited our clinic for another disease and had not been diagnosed with cancer. Both groups were asked to fill out a form that questioned the risk factors. The factors to be investigated in this study, conducted as a case-controlled and cross-sectional study, are defined as follows: age (≤50, 51-65, >65); gender (female, male); family history of pancreatic cancer (yes, no); atopy/allergy (yes, no); blood type (A,B,O,AB; rhesus (Rh) antigen negative, Rh antigen positive); cholecystectomy (yes, no); gastrectomy (yes, no); type 2 diabetes (yes, no); duration of diabetes - newly diagnosed type 2 diabetes (<2 years), long term type 2 diabetes (≥2 years); smoking (never smoked, still smoking or stopped smoking); body mass index (BMI) normal weight (18.5-24.99), pre-obesity (25-29.99), obesity (≥30); alcohol consumption (yes, no); place of residence (rural, urban); helicobacter pylori history (yes, no); level of vitamin D normal (>30, 80 ng/ml), insufficient (20, ≤30 ng/ml), deficient (<20 ng/ml); level of folic acid low (<4.6 ng/ml), normal (4.6-18.7 ng/ml), high (>18.7 ng/ml).

Statistical analysis

The collected data were analyzed with IBM SPSS V23. In the evaluation of the study data, besides the descriptive statistical methods (mean, standard deviation), in the comparison of qualitative data; Pearson's chi-squared test were used. The p-value <0.05 was significantly considered. Independent Sample T-test was applied in the analysis of the significance of the difference between the averages obtained.

Results

There was no significant difference across groups concerning age and gender *(p>0.05)*

(see Table 1). Pancreatic cancer risk was found 3.5 times higher in patients over 65 years of age compared to the control group *(p<0.05)*. Gender was not associated with increased pancreatic cancer risk *(p>0.05)*. The risk of pancreatic cancer was observed 3.4 times higher in people with insufficient serum vitamin D level. For people with deficient level of serum vitamin D, the risk increased 10.2 times more *(p<0.05)*. When non-smokers were taken as reference, there was no increase in the risk of pancreatic cancer in individuals who quit smoking or who are smoking *(p>0.05)*. The risk of pancreatic cancer was detected to increase 1.2-fold in diabetic patients *(p<0.05)*. When diabetic patients were classified as newly diagnosed type 2 diabetes (<2 years) and long-term type 2 diabetes (≥2 years), the risk of pancreatic cancer increased by 19.5-fold in newly diagnosed type 2 diabetic patients *(p<0.05)*. There was no association between BMI, alcohol use, chronic pancreatitis and pancreatic cancer risk *(p>0.05)*. Compared to the control group, the risk of pancreatic cancer was observed to increase 4.3-fold in patients with pancreatic cancer history in the family *(p<0.05)*.

There was no significant difference in terms of pancreatic cancer risk between those living in urban areas and rural areas *(p>0.05)*. Similarly, we observed no relation between the level of serum folic acid, distribution of ABO blood types, helicobacter pylori, atopy/allergy, gastrectomy, cholecystectomy, and the risk of pancreatic cancer *(p>0.05)*. However, the risk of pancreatic cancer was found less patients with Rh antigen negative blood group *(p<0.05)* (See table 2).

There was no significant difference across groups concerning age and gender *(p>0.05)* (See Table 1). Pancreatic cancer risk was found 3.5 times higher in patients over 65 years of age compared to the control group *(p<0.05)*. Gender was not associated with increased pancreatic cancer risk *(p>0.05)*. The risk of pancreatic cancer was observed 3.4 times higher in people with insufficient serum vitamin D level. For people with deficient level of serum vitamin D, the risk increased 10.2 times more *(p<0.05)*. When non-smokers were taken as reference, there was no increase in the risk of pancreatic cancer in individuals who quit smoking or who are smoking *(p>0.05)*. The risk of pancreatic cancer was detected to increase

Table 1. Age and gender distribution.

Parameters		Pancreatic Cancer	Control Group	*P value*
Average age (Years)		65 ±11	62 ±13	*0.207*
Age groups	**≤50**	11 (10.4%)	19 (20.7%)	*0.052*
	51-65	33 (31.1%)	33 (35.9%)	
	>65	62 (58.5%)	40 (43.5%)	
Gender	**Male**	63 (59.4%)	52 (56.5%)	*0.679*
	Female	43 (40.6%)	40 (43.5%)	

Table 2. Comparison of pancreatic cancer risk factors between groups.

Parameters	Pancreatic Cancer Group	Control Group	*OR*	*P*
Age groups				
≤50	11 (10.4%)	19 (20.7%)	*Reference*	
51-65	33 (31.1%)	33 (35.9%)	*1.3 (0.4 – 4.1)*	***0.014***
>65	62 (58.5%)	40 (43.5%)	*3.5 (1.2 – 10.2)*	
Gender				
Male	63 (59.4%)	52 (56.5%)	*Reference*	0.616
Female	43 (40.6%)	40 (43.5%)	*0.9 (0.4 – 2.2)*	
Serum vitamin D level (ng/ml)				
Normal (>30, 80)	3 (2.8%)	10 (10.9%)	*Reference*	
Insufficient (20, ≤30)	6 (5.7%)	15 (16.3%)	*3.4 (0.5 – 25.4)*	***0.001***
Deficient (<20)	97 (91.5%)	67 (72.8%)	*10.2 (2 – 52.6)*	
Smoking				
Never smoked	51 (48.1%)	52 (56.5%)	*Reference*	0.242
Still smoking or smoked before/quit smoking	55 (51.9%)	40 (43.5%)	*1.6 (0.7 – 3.8)*	
Body mass index (kg/m^2)				
Normal (18.5-24.99)	22 (20.8%)	28 (30.4%)	*Reference*	
Pre-obesity (25-29.99)	44 (41.5%)	32 (34.8%)	*1.7 (0.7 – 4.3)*	0.757
Obesity (≥30)	40 (37.7%)	32 (34.8%)	*1.3 (0.5 – 3.6)*	
Type 2 diabetes				
No	65 (61.3%)	73 (79.3%)	*Reference*	***0.001***
Yes	41 (38.7%)	19 (20.7%)	*1.2 (0.4 – 3.1)*	
Duration of type 2 diabetes (years)				
No	65 (61.3%)	73 (79.3%)	*Reference*	
Newly diagnosed type 2 diabetes (<2 years)	23 (21.7%)	2 (2.2%)	*19.5(3 – 127.3)*	***0.003***
Long term type 2 diabetes (≥2 years)	18 (17.0%)	17 (18.5%)	*1.2 (0.6 – 2.5)*	
Family history of pancreatic cancer				
No	96 (90.6%)	89 (96.7%)	*Reference*	***0.041***
Yes	10 (9.4%)	3 (3.3%)	*4.3 (0.8 – 22.3)*	
Alcohol consumption				
No	88 (83.0%)	77 (83.7%)	*Reference*	0.796
Yes	18 (17.0%)	15 (16.3%)	*0.9 (0.3 – 2.6)*	
Place of residence				
Rural	50 (47.2%)	27 (29.3%)	*2 (0.9 – 4.3)*	0.054
Urban	56 (52.8%)	65 (70.7%)	*Reference*	
Chronic pancreatitis history				
No	104 (98.1%)	91 (98.9%)	*Reference*	0.814
Yes	2 (1.9%)	1 (1.1%)	*0.7 (0.1 – 32.4)*	
Helicobacter pylori history				
No	93 (87.7%)	85 (92.4%)	*Reference*	0.310
Yes	13 (12.3%)	7 (7.6%)	*1.5 (0.5 – 5.3)*	
Serum folic acid level (ng/ml)				
Low (0, <4.6)	9 (8.5%)	18 (19.6%)	*Reference*	
Normal (4.6-18.7)	91 (85.8%)	69 (75.0%)	*3.7 (1.2 – 11.5)*	0.099
High (>18.7)	6 (5.7%)	5 (5.4%)	*2.8 (0.5 – 15.1)*	
Blood groups				
O	30 (28.3%)	32 (34.8%)	*Reference*	
A	56 (52.8%)	36 (39.1%)	*1.5 (0.7 – 3.4)*	
B	14 (13.2%)	19 (20.7%)	*0.6 (0.2 – 1.8)*	0.835
AB	6 (5.7%)	5 (5.4%)	*1.1 (0.2 – 5.4)*	
Rh status				
Rh antigen negative	91 (85.8%)	69 (75.0%)	*Reference*	***0.006***
Rh antigen positive	15 (14.2%)	23 (25.0%)	*0.3 (0.1 – 0.8)*	
Atopy/allergy				
No	99 (93.4%)	82 (89.1%)	*Reference*	0.453
Yes	7 (6.6%)	10 (10.9%)	*0.6 (0.1 – 2.3)*	
Cholecystectomy				
No	91 (85.8%)	83 (90.2%)	*Reference*	0.466
Yes	15 (14.2%)	9 (9.8%)	*1.6 (0.5 – 5.3)*	
Gastrectomy				
No	102 (96.2%)	90 (97.8%)	*Reference*	0.926
Yes	4 (3.8%)	2 (2.2%)	*1.1 (0.2 – 8.2)*	

1.2-fold in diabetic patients *(p<0.05)*. When diabetic patients were classified as newly diagnosed type 2 diabetes (<2 years) and long-term type 2 diabetes (≥2 years), the risk of pancreatic cancer increased by 19.5-fold in newly diagnosed type 2 diabetic patients *(p<0.05)*. There was no association between BMI, alcohol use, chronic pancreatitis and pancreatic cancer risk *(p>0.05)*. Compared to the control group, the risk of pancreatic cancer was observed to increase 4.3-fold in patients with pancreatic cancer history in the family *(p<0.05)*. There was no significant difference in terms of pancreatic cancer risk between those living in urban areas and rural areas *(p>0.05)*. Similarly, we observed no relation between the level of serum folic acid, distribution of ABO blood types, helicobacter pylori, atopy/allergy, gastrectomy, cholecystectomy, and the risk of pancreatic cancer *(p>0.05)*. However, the risk of pancreatic cancer was found less patients with Rh antigen negative blood group *(p<0.05)* (see Table 2).

Discussion

In the United States, 10% of pancreatic cancers are under the age of 50. It has been reported that the risk of cancer increases with age [5-7]. When people aged 50 and below are referenced, the risk of pancreatic cancer has increased 3.5-fold for those who are older than 65 *(p<0.05)*. Many studies on pancreatic cancer have not revealed a significant difference between genders [8, 9]. In our study, gender was not correlated to pancreatic cancer risk *(p>0.05)*. The best indicator of vitamin D level in serum is 25-hydroxyvitamin D3. This level is determined by dietary vitamin D and sun exposure. It is stated that serum vitamin D level may vary depending on age, race, BMI and diseases. Experimental studies suggest that the risk of pancreatic cancer may be reduced if the

optimal level of serum vitamin D is achieved, depending on the diet or sun rays. However, clinical studies are inadequate; there are some conflicting data available, too. In one of these, it has been suggested that there may be an increased risk of pancreatic cancer with increased serum 25-hydroxyvitamin D3 levels. However, at the end of the study, the difference was considered to stem from the difference in patient-control group analysis of the studied population or the difference in the method used to measure the level of 25-hydroxyvitamin D3 [10]. In an experimental study conducted by Kasiappan et al. [11] a group of mice with tumors were given a high-fat diet for 30 days; another group with a tumor was given a high-fat diet and the vitamin D analogue seacalcitol (EB1089). When the two groups were compared, tumor growth was more than 50% in mice that did not receive the vitamin D analogue seacalcitol. In our study, the risk of pancreatic cancer increased by 10.2-fold in people with deficient serum D level compared to individuals with normal vitamin D level *(p<0.05)*. Pre-clinical studies contain data which indicates that cancer risk will decrease with vitamin D support. Clinical studies cannot adequately express this. This relationship is maybe really weak; perhaps it may be present in certain cancers.

Smoking is the most important known risk factor associated with pancreatic cancer. In a study, the risk of pancreatic cancer was found to be two times higher in smokers than in nonsmokers. It was stated that this risk would increase in direct proportion with the amount of cigarettes used and the duration of time smoked [12]. According to our data, there was no correlation between smoking and the risk of pancreatic cancer *(p>0.05)*. Based on American Cancer Society studies, those with BMI ≥30 had a 2.08 times greater risk of pancreatic

cancer than those with BMI < 25 [13]. In the data obtained, there was no correlation BMI and pancreatic cancer risk *(p>0.05)*. Diabetes is a risk factor for pancreatic cancer; it can also be a sign of the emergence of pancreatic cancer or a condition that may occur after surgery. In a meta-analysis of 20 studies conducted by Everhart and Wright in 1995, the risk of pancreatic cancer in diabetic individuals was found to be 2 times more than in non-diabetic [1]. In the data obtained, the risk of pancreatic cancer in diabetic patients was found 1.2 times higher than in non-diabetic patients *(p<0.05)*. In a study conducted with 1428 pancreatic cancer and 1528 healthy control groups, the rate of diabetes was 27.6% in people with pancreatic cancer, while the rate of diabetes was 9.9% in people in the control group. Of the patients who have both pancreatic cancer and diabetes, 76.2% had newly diagnosed diabetes, which was significantly higher than the control group [14]. When non-diabetic patients were referenced, the risk of pancreatic cancer increased 19.5-fold in the presence of newly diagnosed type 2 diabetes (<2 years); those with long-term type 2 diabetes (≥2 years) had a 1.2-fold increased risk of pancreatic cancer *(p<0.05)*.

Familial pancreatic cancer accounts for 10% of all patients with pancreatic cancer. In a study, the risk of pancreatic cancer for people in whose immediate family (mother, father, and siblings) there are at least two with pancreatic cancer increases the risk ten times compared those with no family history [15]. According to our data, the risk of cancer increased 4.3 times in patients with a family history of pancreatic cancer *(p<0.05)*. 1530 pancreatic cancer patients and 1530 healthy individuals from the Pancreatic Cancer Cohort Consortium were evaluated in a case-control study. There was no significant statistical difference between those

who had >0 or <5 grams of alcohol daily and those who had ≥60 grams of alcohol daily [16]. In another study, it was reported that consuming 40 grams of alcohol daily increased the risk of pancreatic cancer 1.45 times, and that it could increase up to 1.62 times [17]. The results from these studies are contradictory. In our study, there was no correlation between alcohol use and the risk of pancreatic cancer *(p>0.05)*.

Research has shown that pancreatic cancer is more common in people living in urban areas with low socioeconomic condition. According to our data, no relationship was found between the place of residence and the risk of pancreatic cancer *(p>0.05)*. Four percent of patients with chronic pancreatitis are believed to develop pancreatic cancer [18]. If a person has a history of familial pancreatitis, the risk increases 50-60 times [19]. In our study, there was no correlation between the risk of chronic pancreatitis and pancreatic cancer *(p>0.05)*. The International Agency for Research on Cancer classifies the helicobacter pylori as a proven carcinogen. In four contemporary studies conducted, it was iterated that there is a possible relationship between pancreatic cancer and helicobacter pylori, however further studies are needed [20]. According to our data, there was no correlation between helicobacter pylori and pancreatic cancer risk *(p>0.05)*. The International Agency for Research on Cancer has proposed that foods containing folate may be protective against pancreatic cancer [21]. In a study evaluating 14 prospective cohort studies that involves 319716 men and 542948 women, the relationship between folate use and pancreatic cancer was investigated. Over 7-20 years after the study, 2195 pancreatic cancers were identified. There was no correlation between folate intake and pancreatic cancer risk when the 15% quartile of the highest folate intake diet and 15% quartile of the lowest folate

intake diet were compared [22]. In the current literature, inconsistent findings have been observed between the amount of folate in the diet and the level of serum folate and the risk of pancreatic cancer. In the data obtained, no correlation between folic acid level and pancreatic cancer risk *(p>0.05)* was found.

In a study conducted on blood types with 224 patients who have pancreatic cancer, the risk was 1.5 times higher in individuals with blood type B than in individuals with other blood types; the risk was lower in individuals with 0 type blood [23]. In our study, there was no correlation between ABO blood types and pancreatic cancer risk *(p>0.05)*. It has been investigated whether there is any difference in pancreatic cancer risk based on Rh status or not. According to the statistical analysis, the risk of pancreatic cancer in Rh antigen negative people was 70% less *(p<0.05)*. Given the differences in distribution of ABO and Rh blood types of people living all over the world and current studies concerning blood types, further studies are needed to determine whether there is a link between blood types and pancreatic cancer risk. When 13 community-based studies were examined, the risk of pancreatic cancer in atopic/allergic individuals was 30% less [24]. Studies examining the relationship between pancreatic cancer and atopy/allergy suggest that there is need for further studies. According to our data, there was no significant association between atopy/allergy and pancreatic cancer risk *(p>0.05)*. A study shows that cholecystectomy increased the risk of pancreatic cancer by 23% and gastrectomy by 50% [25, 26]. In our study, there was no relation between the risk of pancreatic cancer and cholecystectomy and/or gastrectomy *(p>0.05)*. In the current literature, the relationship between cholecystectomy or gastrectomy and the risk of pancreatic cancer is not clear enough.

The limitation of this study is that it is case-controlled and cross-sectional. Therefore, the results may suggest that vitamin D deficiency is more common in patients with pancreatic cancer, but we cannot clearly predict the increased risk of pancreatic cancer in cases of vitamin D deficiency. Smoking is a proven risk factor for pancreatic cancer, but this was not demonstrated in our study. On the other hand, a similar situation applies to the relationship between Rh status and pancreatic cancer risk. We cannot expressly conclude that the risk of pancreatic cancer is reduced in people with Rh antigen negative. A population-based follow-up study is required for more reliable results. This article may contribute to the following population-based studies.

The main problem with pancreatic cancer is that the disease cannot be detected early; when diagnosed, the disease is often at an advanced stage. Because the survival rate is low and that diagnosis cannot be made in the early stages of pancreatic cancer, primary prevention is important. Therefore, identification of risk factors and better elucidation of etiology is necessary.

Conclusion
Determination of pancreatic cancer risk factors, organization of imaging and screening programs for high-risk people can provide early diagnosis of the disease and prolong survival. Population-based studies are needed to reveal pancreatic cancer risk factors.

University Ethics Committee (Date and decision number: 144/2015).

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Effect of gallic acid on liver injury during obstructive cholestasis after bile duct ligation in rat

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ABSTRACT

Aim: To investigate the hepatoprotective, anti-inflammatory and antioxidant effects of gallic acid (GA) against obstructive cholestasis (OC) -induced liver damage in rats.

Methods: Thirty female Sprague-Dawley rats were divided into three groups. Group 1 (n=10) was the sham-operated group. In group 2 and group 3, hepatoduodenal ligament dissection was performed after laparotomy. Once the common bile duct was made apparent, it was ligated with 4/0 silk surgical suture and cut between both sutures. Group 2 (n=10) was the control group. Group 3 (n=10) was the GA group. GA (50 mg/kg) was administered by oral gavage daily for 10 days. At the end of the experiment on day 10, the rats were anesthetized. Fibrosis, inflammation, ductal proliferation and necrosis were evaluated histopathologically. Serum levels of AST, ALT, TBIL, DBIL, LDH and GGT levels were determined. In the serum and liver, TAS, TOS, MDA, TNF-α, IL-1, IL-6, and IL 10 levels were evaluated.

Results: When group 2 and group 3 were compared histopathologically, fibrosis and inflammation were significantly lower in group 3. In group 3, all LFTs (except DBIL), liver and serum IL-6, IL-1, TOS, MDA, and TNF-α levels were significantly lower than group 2, whereas IL-10 and TAS values were increased.

Conclusion: Findings of this research indicate that GA may be effective against OC-induced liver damage in a rat model. We presume that the beneficial effects of GA are closely associated with its antioxidant and anti-inflammatory activities. Therefore, we think that using GA can save us time before resorting to the surgical method.

Keywords: Obstructive cholestasis, bile ducts ligation, rats, gallic acid, liver, oxidative stress, fibrosis.

Introduction

Obstructive cholestasis (OC) is a common condition that requires surgery. Choledocholithiasis is the most common cause of OC; other causes include biliary cyst stenosis, pancreatitis, parasitic infections, congenital bile duct anomalies, infectious cholangitis, cholelithiasis, biliary atresia, primary sclerosing cholangitis, abdominal traumas, and cholangitis associated with Langerhans cell histiocytosis [1,2].

The liver is the primary organ affected by OC. Retention of hydrophobic bile salts via membranes causes it to accumulate in hepatocytes, thereby disrupting membrane fluidity and function and causing cell damage [2,3]. Patients with OC experience many pathophysiological alterations that influence

several organs and physiological processes. Bilirubin accumulates at high levels in patients with OC due to dysfunctional bile secretion. Thus, bile acid in hepatocytes causes acute liver damage as a result of oxidative stress [4]. Subsequently, hemostatic abnormalities, cardiac disorders, and multiple system disorders (e.g., renal failure) occur, which cause changes in patient immunity [5]. It remains unknown why OC causes multiple organ dysfunction; however, bilirubin breakdown and impaired bile acid homeostasis have been suggested [6].

Although the most common treatment for OC is surgery, percutaneous transhepatic biliary drainage and medical treatment are occasionally needed to stabilize the patient's condition and restore biliary flow before surgery [7]. Therefore, drugs are primarily used to reduce hepatic damage caused by inflammatory cells and elevated bilirubin levels, as well as to reduce elevated biliary pressure in ducts and portal veins. For prevention of intrahepatic cholestasis, novel drugs are needed to provide an alternative to surgical treatment of OC [8].

In numerous scientific reports, polyphenolic compounds were demonstrated to have different pharmacological activities that may be associated with the antioxidant system [9]. In *in vitro* studies [10], the ethyl acetate fraction (EF) was shown to have an antifibrotic effect. Based on mass spectrometry, EF has two main components: gallic acid (GA) and ellagic acid [11]. Gallic acid is a natural phenolic antioxidant; it is found in green tea leaves, sumac, gallnuts, witch hazel, mulberries, blackberries, oak bark, raspberries, strawberries, and dragon fruit [12]. The antioxidant effects of GA are presumably due to the hepatoprotective activity of hydroxyl groups [13]. GA has attracted considerable

attention due to its antioxidant effects and the cleaning properties of reactive oxygen species (ROS) [14,15].

In this study, the hepatoprotective effects of gallic acid (GA) against liver damage in obstructive cholestasis (OC) model caused by bile duct ligation in rats were investigated.

Materials and Methods
Animals
Thirty female Sprague-Dawley rats (230–270 g) were obtained from Dicle University Animal Experimental Care Unit (Diyarbakir, Turkey). All experiments were approved by the Dicle University Animal Experiments Local Ethics Committee (DUHADEK) (Decision No: 14-TF-149) and were performed in compliance with the DUHADEK guidelines. Rats were housed in cages and fed with rat diet and tap water ad libitum at a room temperature of $26\pm3\circ C$, a relative humidity of %55-70, and a time period of 12 h light and 12 h dark.

Groups and surgical method
Animals were divided into three groups. Each group was consisted of ten animals. After an overnight fasting, rats were anesthetized before the experimental procedure. The rats' abdomens were shaved and cleaned with 10% povidone-iodine solution. Under sterile conditions, an incision was made at the midline of the abdomen (i.e., laparotomy was performed); the liver, diaphragm, and adjacent organs with apparent hepatoduodenal ligament were carefully dissected.

Group 1 (n=10) was the sham group. After laparotomy, the hepatoduodenal ligament was dissected; the common bile duct was exposed, but no binding or cutting was conducted. Then, the animals were given standard feed and water for 10 days.

Group 2 (n=10) was the control group. After laparotomy, hepatoduodenal ligament

dissection was performed. Once the common bile duct was made apparent, then it was ligated with 4/0 silk surgical suture and cut between both sutures [16]. Animals were given standard feed and water for 10 days.

Group 3 (n=10) was the GA group, which underwent the same procedures as in group 2. In addition, 50 mg/kg GA dissolved in 1 mL saline solution (Gallic acid®, 97.5–102.5% (titration), Sigma-Aldrich, Shanghai, China) was administered by oral gavage daily for 10 days [17].

After surgical procedures had been completed, the abdominal incisions were sutured en bloc with 3/0 silk sutures. Postoperative feeding was allowed. The rats were kept in the preoperative room and fed as described above. On the 10th day, the rats were anesthetized. Then, an abdominal incision was made, liver samples were collected, and blood was drawn. Finally, the animals were sacrificed by exsanguination. Blood samples were centrifuged at 4°C and 4,000 rpm for 15 min. Plasma samples (200 μL) were obtained and stored at -80°C for biochemical parameter analysis.

Two rats (one in Group 2 and one in Group 3) died during the course of the experiment, prior to sample collection, and that replaced with new ones.

Histopathological examination
Dissected liver tissues were processed in 10% formalin solution, then embedded in paraffin wax in accordance with a routine embedding protocol. Next, 4-μm-thick sections were cut from paraffin blocks. In addition to hematoxylin and eosin staining, Masson's trichrome (Bioptica, Milan, Italy) staining was performed on liver slides to facilitate the evaluation of periportal fibrotic bands. Sections were evaluated using a Zeiss imager A2 microscope. Fibrosis, inflammation, and necrosis were evaluated histopathologically in accordance with the method described by Ishak et al. [18], ductal proliferation was evaluated in accordance with the method described by Ara et al. [19]

Liver function tests (LFTs)
For LFTs, sera were obtained by centrifuging blood samples at 4,000 g for 5 min. Subsequently, an automated biochemical analyzer (Abbott Laboratories, Chicago, IL, USA) was used, in accordance with the manufacturer's protocol, to determine the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), direct bilirubin (DB), total bilirubin (TB), lactate dehydrogenase (LDH) and gamma-glutamyl transferase (GGT).

Assessment of oxidative stress and inflammatory cytokines in liver and serum
The total oxidant status (TOS) and total antioxidant status (TAS) in serum samples were determined using commercial kits (Rel Assay Diagnostics, Gaziantep, Turkey) and an autoanalyzer (Abbott Architect C16000, Abbott Diagnostics Laboratories), based on automatic colorimetric methods described by Erel et al. [20] TAS results are offered as micromolar Trolox equivalent per liter (μmol Trolox equiv/L). TOS results are shown as micromolar hydrogen peroxide per liter (μmol H_2O_2 equiv/L). Malondialdehyde (MDA) levels were detected by spectrophotometric measurement of the color produced between the reaction of thiobarbituric acid and MDA [21].

Serum levels of interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 10 (IL-10) and tumor necrosis factor-α (TNF-α) were measured using the ELISA method, in accordance with the manufacturer's instructions. Biochemical parameter results were recorded. Tissues weighing 0.13–0.25 g were washed with saline water, then dried using blotting paper. Next, the tissues were placed in Eppendorf tubes and

stored at -80°C until use. Before biochemical analysis, the samples were brought to -20°C, warmed to +4°C, and gradually thawed. Tissues were homogenized in phosphate-buffered saline (pH 7.4) on ice using a WiseTis Homogenizer. Tissues were centrifuged at 3,000 rpm for 10 min in a Nüve™ centrifuge and the supernatant was collected. Tissues were prepared for ELISA and washed in a BioTek ELX50 microplate washer; ELISA results were recorded at 450 nm (Triturus GRI FOLS™). TAS, TOS, MDA, TNF-α, IL-1, IL-6 and IL-10 levels were evaluated in liver tissues. The optical density values and standard (std) concentration obtained from the standard solution were used to plot the distributions of each inflammatory cytokine in Excel software. The formula calculated based on the curve was used to calculate the concentrations of the samples by means of their optical densities.

Statistical analysis

Statistical analyses were computed by SPSS Statistics, version 24.0; scores were shown as mean ± standard deviation (SD). Kruskal-Wallis and Mann-Whitney U tests were used for differences between group comparisons. When a *p* value was less than 0.05, the differences were considered as statistically significant.

Results

Histopathological findings

Ductal proliferation, inflammation, necrosis, and fibrosis were found to be significantly increased in the liver tissues of all groups with biliary obstruction compared to the Group 1. Although necrosis and ductal proliferation were improved in Group 3, no statistically significant difference was observed when compared with Group 2. However, when Groups 2 (Figures 1A, B and C) and 3 (Figure 2A, B and C) were compared, fibrosis and inflammation levels were statistically significantly lower in group 3 (p < 0.05; Table 1).

Liver function tests (LFTs)

All LFTs values studied in groups with biliary duct obstruction *(Group 2 and Group 3)* were significantly higher (p<0.05) compared to group 1 values. When group 2 and group 3 were compared; all LFTs except DBIL were significantly lower (p<0.05) in group 3 (Table 2) than group 2.

Liver interleukin levels and oxidative stress parameters

The liver IL-6, TOS, MDA, and TNF-α levels were significantly (p<0.05) increased in the biliary obstruction groups *(Group 2 and group 3)* compared to the Group 1. When groups 2 and 3 were compared, IL-6, IL-1, TOS, MDA, and TNF-α levels were significantly lower (p < 0.05) in the group 2, whereas IL-10 and TAS values were significantly higher in group 3. In addition, mean TAS values were significantly higher in group 3 than in group 1 (Table 3).

Blood interleukin and oxidative stress parameters

Significant increases in TNF-α levels, MDA, IL-6, IL-1, and TOS, as well as a significant decrease in IL-10 levels, were followed in

Table 1. Comparison of liver histopathological evaluation of the groups.

Groups	Ductal proliferation	Fibrosis	Inflammation	Necrosis
G 1 (n=10)	0,01±0,01	0,01±0,01	0,42±0,20	0,42±0,29
G 2 (n=10)	2,28±0,28*	1,28±0,18*	1,71±0,18*	4,14±0,73*
G 3 (n=10)	1,57±0,29*	0,42±0,20*▲	0,85±0,14*▲	2,57±0,36*

*Values are expressed as mean ± SD. G1: Sham-operated group. G2: Control group. G3: GA group. * Group 1 vs Group 2 and Group 3 p<0.05; ▲ Group 2 vs Group 3 p<0.05.*

group 2, compared with group 1 (p < 0.05). However, in group 3, the IL-6, IL-1, TOS, MDA, and TNF-α levels significantly decreased, while IL-10 and TAS levels significantly increased (p < 0.05), compared with group 2. In addition, no significant differences were followed between groups 1 and 3 for all parameters, except MDA and TNF-α; however, MDA and TNF-α values were higher in group 3 than in group 1 (p < 0.05; Table 4).

Discussion

Cholestasis, known as disruption of biliary flow, leads to the collection of bile acids and other toxins in the liver; this causes hepatotoxicity, thus damaging intracellular compartments and cell membranes. The main hypothesis regarding the mechanism of liver damage due to liver cholestasis is that hepatocellular apoptosis increases due to bile acid accumulation in hepatocytes and blood [22]. An alternative hypothesis is that excess
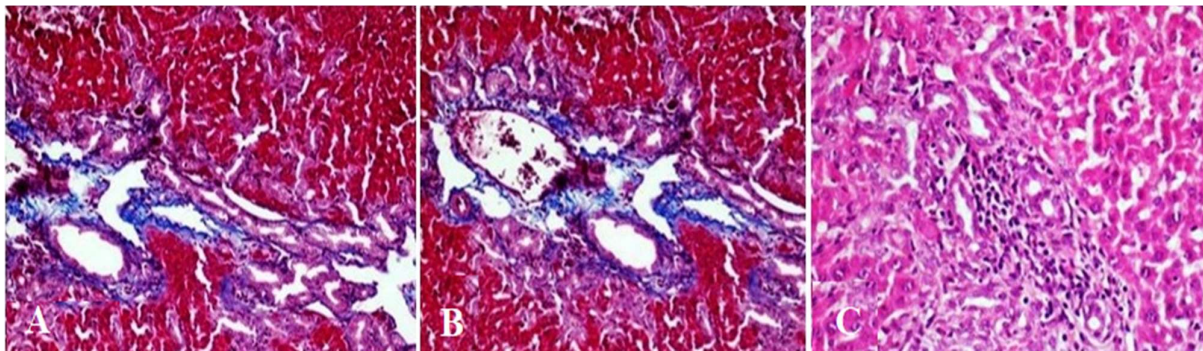

Figure 1. In control group (Group 2), fibrous expansion of some portal areas **(A, B)** (Masson's trichrome, x20); moderate portal inflammation **(C)** (Masson's trichrome, x20).
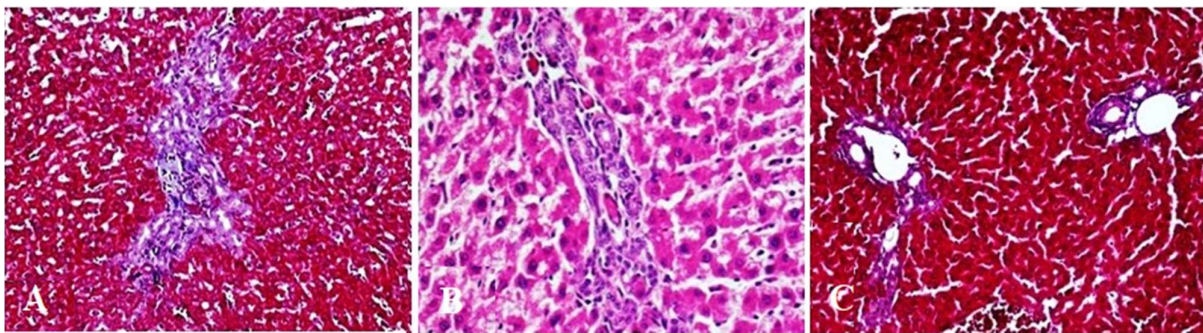

Figure 2. In gallic acid treated group (Group 3), mild portal fibrosis **(A)** (Masson's trichrome, x20); no portal inflammation **(B)** (Masson's trichrome, x20); no portal fibrosis **(C)** (Masson's trichrome, x20).

Table 2. Comparison of liver function tests of the groups.

Groups	LDH	GGT	AST	ALT	TBIL	DBIL
G 1 (n=10)	261±29,1	10,1±1,2	105±6,8	39±2,9	0,27±0,10	0,06±0,01
G 2 (n=10)	1426±82,3*	74,7±3,3*	883±104*	218±28*	11,9±1,20*	7,58±0,91*
G 3 (n=10)	768±50,4*▲	38±1*▲	407±40*▲	102±12*▲	7,30±0,54*▲	6,15±0,78*

*Values are expressed as mean ± SD. G1: Sham-operated group. G2: Control group. G3: GA group. * Group 1 vs Group 2 and Group 3 p<0.05; ▲ Group 2 vs Group 3 p<0.05. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; DBIL: Direct bilirubin; GGT: Gamma-glutamyl transferase; LDH: Lactate dehydrogenase; TBIL: Total bilirubin.*

Table 3. Comparison of liver interleukin and oxidative stress parameters.

Groups	IL- 6	IL-1	IL-10	TAS	TOS	MDA	TNF-α
G 1 (n=10)	65±6,5	1282±118	151±20	0,81±0,04	15,2±1,01	12,3±0,94	250±115
G 2 (n=10)	182±11,5*	2034±175*	105±15,1	0,87±0,04	34,8±2,1*	42,2±1,67*	350±120*
G 3 (n=10)	111±8,5*▲	1273±94▲	217±26▲	1,48±0,86▲¥	23,8±2,1*▲	22,8±2,38*▲	275±120*▲

*Values are expressed as mean ± SD. G1: Sham-operated group. G2: Control group. G3: GA group. * Group 1 vs Group 2 and Group 3 p<0.05; ▲ Group 2 vs Group 3 p<0.05; ¥ Group 1 vs Group 3 p<0.05. IL, interleukin; MDA: Malondialdehyde; TAS: Total antioxidant status; TNF-α: Tumor necrosis factor-α; TOS: Total oxidant status.*

Table 4. Comparison of serum interleukin and oxidative stress parameters.

Groups	IL- 6	IL-1	IL-10	TAS	TOS	MDA	TNF-α
G 1 (n=10)	83±22*	1348±173*	202,1±41*	1,1±0,2*	18,7±6*	14±3,8*¥	251±9*¥
G 2 (n=10)	170,5±50▲*	1783±497▲*	106±28▲*	0,8±0,1▲*	28±3,8▲*	37±9▲*	338±35▲*
G 3 (n=10)	107±42▲	1277±233▲	176±47▲	1,4±0,3▲	21±6▲	21,6±7▲¥	275±15▲¥

*Values are expressed as mean ± SD. G1: Sham-operated group. G2: Control group. G3: GA group. * G1, G2 vs G3 p<0.05; ▲ G2 vs G3 P<0.05; ¥ G1 vs G3 P<0.05. IL: Interleukin; MDA: Malondialdehyde; TAS: Total antioxidant status; TNF-α: Tumor necrosis factor-α; TOS: Total oxidant status.*

accumulation of bile acids occurs in the blood and liver (sterile inflammation); many studies have been performed regarding this issue. The toxic build-up of bile acids is an important mechanism for cell toxicity, including oxidative stress, apoptosis, and fibrosis [23]. Neutrophils are recruited to the area of injury approximately six hours after bile duct ligation; they peak at 48–72 hours, when liver damage is most commonly observed [24]. Neutrophil accumulation is directly associated with injury or inflammation. Neutrophils can be especially toxic to leukocytes when superoxides are generated by potent oxidant hypochlorous acid and nicotinamide adenine dinucleotide phosphate oxidase is generated by myeloperoxidase. This shows that hypochlorous acid can cause intracellular oxidant stress in hepatocytes and consequently cause cell damage [25].

Histopathological findings have been observed in the liver following bile duct ligation in various studies. In the study by Hongwei et al., livers with bile duct ligation showed elevated bile duct proliferation, parenchymal necrosis, and inflammatory cell infiltration. When Hongwei et al. treated the rats with retinoic acid and ursodeoxycholic acid, the pathological changes were reduced and liver tissues were restored to normal structure [26].

In another study by Zong et al., less collagen deposition was observed in the group subjected to both bile duct ligation and treatment with ethyl pyruvate. In addition, the liver fibrosis score was lower in rats treated with ethyl pyruvate. Thus, Zong et al. concluded that treatment with ethyl pyruvate delayed liver fibrosis [27].

In the present study, inflammation and cellular necrosis were observed by means of

histological analysis in both bile duct ligation groups (groups 2 and 3). This was an indicator of neutrophil collection after bile duct ligation. Furthermore, histological examination revealed a reduction in the parameters associated with liver fibrosis and inflammation in the group 3, which confirmed the above-mentioned morphological results.

Gamma-glutamyl transferase, bilirubin, and LFTs (e.g., AST, ALT, and lactate dehydrogenase) are particularly sensitive determinants of liver damage and dysfunction in membrane transport, as well as in cell and mitochondrial membrane properties [28].

Zong et al. reported that the bile duct ligation group had significantly higher serum AST and ALT levels, compared with the sham group. Ethyl pyruvate treatment significantly reduced ALT and AST levels, compared with the bile duct ligation group [27].

In the study by Xie et al., the serum ALT, AST, and total bilirubin levels were significantly lower in the bile duct ligation + dexmedetomidine group than in the bile duct ligation group [29].

In the present study, significant increases in LFTs were observed in the biliary obstruction groups (Groups 2 and 3). However, a decrease in LFTs (except direct bilirubin) in the group 3 compared to Group 2 indicated that GA inhibits or reduces OC-induced liver membrane damage due to its antioxidant properties; this findings is consistent with the results of other studies [30,31].

The anti-inflammatory and antioxidant properties of GA may be associated with elevated liver superoxide dismutase and liver catalase activities, liver TNF-α expression, liver MDA, and serum protein carbonyl reduction [31]. MDA is an indicator of lipid peroxidation and a commonly used marker of oxidative stress [14]. In the study by Cao et al.,

elevated MDA was observed in liver tissue after bile duct ligation; rats in the GA-treated group showed reduced MDA levels [32]. In the study by Demirbilek et al., the MDA level was significantly increased in rats with bile duct ligation. However, decreased MDA levels were observed in rats treated with sulfasalazine and pyrrolidine dithiocarbomate [33]. In the present study, due to bile duct ligation-induced liver injury, MDA levels in liver and serum were significantly higher in Group 2 than in Group 1. Conversely, treatment with GA led to lower MDA levels in liver tissue and serum, which is consistent with the previously reported findings [34]. These effects may be, at least in part, due to the antioxidant properties of GA.

TAS and TOS measurements are valuable to evaluate oxidative stress. The TOS is an important indicator of oxidative stress [35], while TAS functions to preserve tissue integrity from oxidative injury. The TAS is a precise indicator of antioxidant level [36]. In the study by Coban et al., the TOS level increased and the TAS level decreased in rats in the bile duct ligation model; conversely, the TOS level decreased and the TAS level increased in the treatment group [37]. Similar results were observed in other experimental injury models [38]. In the present study, the TOS level was significantly higher in the Group 2 than in Group 1. In the Group 3, a significant decrease in TOS and a significant increase in TAS levels were observed ($p < 0.05$), presumably due to the antioxidant properties of GA.

IL-1 and IL-6 play crucial roles in inflammatory events and immunity [39,40]. In the liver, TNF-α production induces apoptosis and necrosis of hepatocytes, leading to liver inflammation [41]. In a study by Xie et al., dexmedetomidine treatment decreased serum IL-6 and TNF-α proinflammatory cytokine levels, compared with the bile duct ligation

group [29]. In a study by Pan et al., following bile duct ligation, IL-1 levels were improved in the treated rats [42].

In the present study, significantly higher serum TNF-α, IL-1, and IL-6 levels were observed in Group 2 (p < 0.05). However, Group 3 showed significantly lower serum TNF-α, IL-1 and IL-6 levels, compared with Group 2 (p < 0.05). Thus, the observed reductions in TNF-α, IL-1, and IL-6 serum levels confirm that GA has hepatoprotective effects via its anti-inflammatory properties.

IL-10 is a Th2-type cytokine that exerts inflammatory effects by inhibiting the production of pro-inflammatory cytokines (e.g., TNF-alpha, IL-1, IL-6, and IL-8). In addition, IL-10 plays a role in regulating monocyte production of soluble TNF-α and IL-1 receptor antagonist [43]. In the present study, IL-10 levels were lower in the group 2 than in the group 1, which is consistent with previously reported results. However, IL-10 levels were increased in Group 3 compared to Group 2 and Group 1 (p < 0.05).

Conclusion

Findings of this research indicate that GA may be effective against OC-induced liver damage in a rat model. We presume that the beneficial effects of GA are closely associated with its antioxidant and anti-inflammatory activities. Therefore, we think that using GA can save us time before resorting to a surgical method. However, further studies are needed to investigate the optimal dosage and duration of application.

Abbreviations

GA: Gallic acid
OC: Obstructive cholestasis
LFTs: Liver function tests
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
TBIL: Total bilirubin
DBIL: Direct bilirubin
LDH: Lactate dehydrogenase
GGT: Gamma-glutamyl transferase
ROS: Reactive oxygen species
ELISA: Enzyme-linked immunosorbent assays
TAS: Total antioxidant status
TOS: Total oxidant status
MDA: Malondialdehyde
IL: Interleukin
TNF-α: Tumor necrosis factor-α
EF: Ethyl acetate fraction

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Experimental Biomedical Research   **Original article**

Evaluation of epicardial fat tissue thickness in patients with multiple sclerosis

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A B S T R A C T

Aim: Multiple sclerosis (MS), which is inflammatory in its pathogenesis, damages the myelin sheath in the central nervous system (CNS) and causes axonal loss. Epicardial fat tissue (EFT), located between the myocardium and the visceral layer of the pericardium, surrounds the heart and several inflammatory cytokines is secreted from this tissue. In this study, we aimed to investigate EFT thickness in MS patients and compared with that of volunteer non-MS subjects.

Methods: A total of 154 subjects comprising 61 MS patients and 93 volunteers matched for gender and age were included in our study. Epicardial fat tissue thickness was measured by echocardiography. All values were compared between groups.

Results: Echocardiographic parameters were similar in both groups. However, the mean EFT thickness was significantly higher in the MS group than in the control group *(p<0.001)*. Epicardial fat tissue thickness was also significantly correlated with the presence of MS *(r=0.33, p<0.001)*.

Conclusion: The results of our study suggest that the increase in epicardial adipose tissue thickness in MS patients may be a predictive factor for cardiovascular disease. However, the clinical significance of this finding and its relevance to MS pathogenesis should be investigated in further studies.

Keywords: Multiple sclerosis, epicardial fat tissue, cardiovascular diseases, inflammation, biomarkers.

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Introduction

Multiple sclerosis (MS), which is inflammatory in its pathogenesis, damages the myelin sheath in the central nervous system (CNS) and causes axonal loss [1,2]. Although its etiology is unknown, genetic and environmental factors are presumed to trigger an autoimmune response, resulting in damage to myelin and axons [3]. However, inflammation might also be neuroprotective or contribute to the repair of damaged tissue within the CNS [4].

Epicardial fat tissue (EFT), located between the myocardium and pericardial visceral layer, surrounds the heart. Embryologically, EFT and abdominal adipose tissue are of the same origin [5]. The levels of lipolysis and lipogenesis in EFT are quite high compared to those of other adipose tissues [6]. Moreover, EFT is in contact with coronary arteries and secretes many inflammatory cytokines [5,7]. Studies have shown that EFT may be an independent risk factor for the presence and severity of cardiovascular disease (CVD), probably through inflammation or vasoactive pathways [8].

Inflammatory cytokines play a crucial role in the pathogenesis of MS [9]. MS, which is an autoimmune disease, progresses with demyelination in the CNS [10]. EFT thickness is associated with various autoimmune diseases [11,12], and there are various studies showing the release of various cytokines from EFT [13]. Hence, EFT thickness may be considered a significant parameter in MS patients.

In this study, we aimed to investigate echocardiographic EFT thickness, which may be an indicator of coronary artery disease, in MS patients and compared it with that of normal subjects.

Materials and Methods

The patients who were admitted to a university hospital between February 2019 and March 2020 were included in the study. The study was approved by Bolu Abant Izzet Baysal local ethics committee (Date: 30/01/2019; Decision number: 2019/27). Written and verbal consent were obtained from all patients.

A total of 154 subjects comprising 61 MS patients and 93 volunteers matched for gender and age were included in our study. The demographic characteristics of the participants were recorded. The laboratory parameters of the patients at the time of admission were recorded from the laboratory database. Echocardiography (echo) was performed on all participants.

Exclusion criteria

Patients who had a history of coronary, carotid, or peripheral artery atherosclerosis, valvular heart disease, heart failure, arrhythmias or conduction disorders, prosthetic heart valves, pacemaker implantation, significant renal failure, severe liver dysfunction, thyroid disorders, electrolyte imbalances, systemic inflammatory or infectious disease, and poor ultrasound image quality were excluded.

Measurement of epicardial fat thickness

EFT thickness was measured with a 4-Mhz Vivid S6 transducer (GE Vingmed, N-3191, Horten, Norway). A blinded cardiologist performed the echo under continuous electrocardiographic monitoring in the left lateral position. Measurements were recorded as the average of the values obtained over three cardiac cycles. EFT thickness was noted as an echo-free or hyperechoic area between the epicardium and the visceral layer of the pericardium and reflects visceral adipose tissue [14]. The assessment of EFT thickness was performed from the parasternal long- and short-axis views of the free wall of the right ventricle. The measurement was made at the level of the aortic annulus perpendicular to the free wall of the right ventricle. The measurements of maximum values were made at the end of diastole. The intraobserver variation for EFT was <5%.

Statistical analysis

For all statistical analyses, the 18.0 version of SPSS (Statistical Package for the Social Sciences) for Windows, (Chicago, Illinois, USA) was used. The numerical data were expressed as mean ± standard deviation.

The difference between the study and the control groups was evaluated by Student t-test for data which were normally distributed. For the data which were not normally distributed Mann-Whitney's U-test was used. The correlation between EFT and MS was evaluated using Spearman's correlation analysis. A p-value < 0.05 was considered as significant.

Results

The clinical risk factors and baseline characteristics of the patients are shown in Table 1. No significant difference was found between the groups in terms of baseline characteristics (Table 1).

Likewise, echocardiographic findings were similar in both MS and non-MS groups. However, the mean EFT thickness value was found significantly higher in MS group than in non-MS group (p<0.001) (Table 2). No significant difference was found between the groups in terms of laboratory parameters such as creatinine, triglyceride, and cholesterol values. The Spearman's correlation test revealed that EFT thickness (r=0.33, p<0.001) was significantly correlated with the presence of MS (Table 2, Figure 1).

Table 1. Baseline characteristics of the study groups.

Baseline characteristics	Control Groups (n=93)	MS Groups (n=61)	*P Value*
Age (years)	33±12	36±12	*0.25*
Sex (Male/female), n	46/47	22/39	*0.08*
SBP (mmHg)	116±14	111±12	*0.05*
DBP (mmHg)	71±9	70±7	*0.64*
Body mass index (kg/m^2)	25±4	25±3	*0.17*
Smoking	19 (20%)	17 (28%)	*0.27*
Diabetes Mellitus	1 (1%)	3 (5%)	*0.14*
Hypertension	2 (2%)	4 (7%)	*0.16*
MS: Multiple sclerosis, DBP: Diastolic Blood Pressure, SD: Standard Deviation, SBP: Systolic Blood Pressure.			

Table 2. Echocardiographic and laboratory parameters of the study groups.

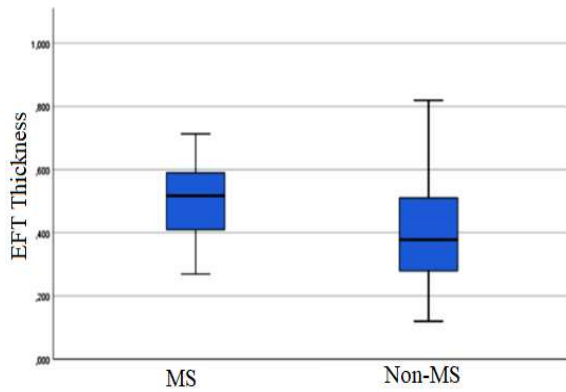| Variables	Control Group (n=93)	MS Group (n=61)	*P Value*
	Mean ± SD		
EF	66±3	65±3	*0.36*
LVDD(cm)	4.6±0.4	4.5 ±0.3	*0.28*
LVSD(cm)	2.8±0.4	2.8± 0.3	*0.26*
PW(cm)	0.86±12	0.88±0.12	*0.33*
IVST(cm)	0.88±0.14	0.93±0.14	*0.04*
Left atrium	2.9±0.5	3.0±0.4	*0.69*
Creatinine (mg/dl)	0.78±12	0.78±11	*0.98*
Triglyceride (mg/dl)	107±52	107±59	*0.97*
EFT(cm)	0.41±0.17	0.50±0.12	***<0.001***
	Median (IQR)		
HDL-cholesterol (mg/dl)	46 (8.8)	51(17.1)	*0.07*
LDL-cholesterol (mg/dl)	101(13.7)	102 (38.1)	*0.79*
Total cholesterol (mg/dl)	169 (21)	178 (34)	*0.08*
EF: Left ventricular ejection fraction, LVDD: Left ventricular end-diastolic diameter, LVSD: Left ventricular end-systolic diameter, PW: Posterior wall thickness, IVST: Interventriculer septum thickness, EFT: Epicardial fat thickness, SD: Standard Deviation, IQR: interquartile range.			

Figure 1. EFT thickness is significantly higher MS group compared to non-MS group.

Discussion

In the present study it is found that EFT thickness was significantly higher in the MS group than in the non-MS group, and EFT thickness was significantly correlated with MS. There is no consensus on whether MS patients are at greater risk of CVD when compared to the general population [15-18]. While Roshanisefat et al. found no difference between MS and non-MS patients in terms of CVD incidence, Moccia et al. reported that the Framingham General Cardiovascular Disease Risk Score (FR) was associated with the severity, disability, and clinical course of MS [19-20]. In some studies, it has been reported that indirect markers of CVD, such as high-sensitive C-reactive protein and carotid intima media thickness (CIMT), were related to MS [21]. Hence, the overall data suggest an increased CVD risk in patients with MS [22].

In the context of the reported studies, it is not clearly understood whether the increase in CVD is related to the presence of obesity, hypertension, hyperlipidemia, or diabetes mellitus in MS. Further studies are needed to solve this issue [22]. An association between EFT thickness, CVD, and metabolic syndrome has been observed in several studies [8,23]. Therefore, our finding of increased EFT

thickness in MS patients may provide indirect evidence and a pathway for the association of MS with CVD.

In the early phase of relapsing-remitting MS, demyelination by inflammation predominates, while in the progressive phase, neuroaxonal damage by neurodegeneration predominates [24]. Frischer et al. reported a significant association between inflammation and axonal injury in MS. In older MS patients with longer disease duration, inflammatory infiltrates declined to levels similar to those found in age-matched controls [25]. Therefore, although it is not known whether the role of inflammation is causative or protective, there is a strong association between neurodegeneration and inflammation in MS.

Epicardial fat tissue is a paracrine and endocrine tissue and is a source of adiponectin, adrenomedullin, anti-inflammatory adipokines, and several proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1, IL-6, and nerve growth factor (NGF) [13]. Mazurek et al. [7] examined epicardial and subcutaneous adipose tissues among elective coronary artery bypass grafting surgery patients. Significantly higher mRNA and protein levels of IL-1, IL-6, MCP-1, and TNF-α were detected in EFT when compared to subcutaneous adipose tissue. Coincidentally, IL-6, IL-1, and TNF-α levels are associated with MS, and these cytokines have been found to be related to axonal injury [26]. Additionally, Sharief et al. reported that TNF-α levels are correlated with MS disease progression and disability level [27].

The clinical significance of the inflammatory potential of EFT and its role in other diseases is unclear. Our finding of increased EFT thickness among MS patients may provide new insight into the association between inflammation and

MS. Nevertheless, future studies are needed to further explore this aspect.

There are some limitations not studied. This is a single-center study with a small sample size, which predisposes it to an inherent selection bias. The accuracy and reproducibility of EFT thickness measurements with MRI and CT are higher than that of echo. Therefore, the use of echo for the measurements of EFT thickness may reduce the reliability of our results. Nonetheless, echo was performed as it is simple and inexpensive. Lastly, since EFT has a three-dimensional distribution, echo, which produces a two-dimensional image, may not fully evaluate the total amount of epicardial adipose tissue.

Conclusions

The results of our study suggest that the increase in epicardial adipose tissue thickness in MS patients may be a predictive factor for cardiovascular disease. To our knowledge, this is the first report about epicardial adipose tissue in MS patients. The clinical significance of this finding and its relevance to MS pathogenesis should be explored in further studies.

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical statement: The study was approved by Bolu Abant Izzet Baysal University local ethics committee (Date: 30/01/2019; Decision number: 2019/27).

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Experimental Biomedical Research   **Original article**

Investigation of the presence of pregnancy rhinitis in the third trimester with rhinomanometry

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ABSTRACT

Aim: Pregnancy rhinitis (PR) is characterized with nasal symptoms during pregnancy without any signs of respiratory infection and it usually disappears within 2 weeks after delivery. We aimed to investigate the relationship between pregnancy rhinitis and findings derived from anterior rhinoscopy (AnR), anterior rhinomanometry (ARM) and subjective nasal obstruction score (SNOS).

Methods: This prospective, controlled study was performed in otorhinolaryngology and obstetrics and gynecology departments of our tertiary care center. A total of 30 pregnant women in the third trimester and 30 non-pregnant women were involved. All participants underwent otorhinolaryngology examination, as well as clinical evaluation for AnR, ARM and SNOS.

Results: Pregnancy rhinitis was detected in 66.7% of the pregnant women. The mean AnR was 3.60 ± 1.35 in pregnant women and 0.77 ± 0.73 in the control group. Total nasal inspiratory resistance (TNID) was 0.46 ± 0.23 in pregnant women and 0.27 ± 0.06 in the control group. The mean SNOS was 1.37 ± 0.72 in pregnant women and 0.57 ± 0.63 in the control group. AnR, ARM and SNOS findings were significantly higher in pregnant women $(p<0.05)$. There is a low positive and significant correlation between AnR, ARM, and SNOS values in pregnant women $(p<0.05)$.

Conclusion: Our data yielded that nasal obstruction and pregnancy rhinitis were common in pregnant women. Nasal symptoms and complaints must be carefully examined during pregnancy. Further prospective, controlled, randomized trials on larger series are warranted to elucidate the clinical and pathophysiological features of pregnancy rhinitis.

Keywords: Pregnancy rhinitis, subjective nasal obstruction score, anterior rhinoscopy, pregnancy rhinitis, anterior rhinomanometry.

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Introduction

Pregnancy rhinitis (PR) is defined as the presence of nasal symptoms occurring only during pregnancy for more than six weeks without any known allergic cause and no signs of respiratory infection. These symptoms disappear in the first two weeks after delivery. PR occurs in 20-30% of women between the 3-7 months of pregnancy [1, 2]. The pathophysiological mechanism causing pregnancy rhinitis is that vasomotor imbalance in the nasal mucosa due to changes in estrogen

and progesterone levels causes hyperemia and edema in the nasal mucosa. Also, placental growth hormone was found to be high in pregnancy rhinitis [3, 4].

Rhinitis does not directly affect pregnancy. However, uncontrolled rhinitis can increase stress by indirectly disturbing the nutritional and sleep status of the pregnant woman. Pregnancy hypertension, low Apgar score, preeclampsia and intrauterine growth retardation may be encountered due to rhinitis induced snoring [5, 6]. Control of rhinitis during pregnancy reduces the risk of additional medication by increasing the quality of life of pregnant women [7, 8].

We aimed to investigate the frequency of pregnancy rhinitis and seek whether there is a correlation between anterior rhinoscopy (AnR) findings, anterior rhinomanometry (ARM) measurements, and subjective nasal obstruction scores (SNOS).

Materials and Methods

This prospective study was carried out by the otorhinolaryngology and obstetrics & gynecology clinics of our institution. The study was approved by the local ethics committee and consent was obtained from all the participants (2017/73). A total of 30 pregnant women aged between 19-35 years who were in the third trimester of pregnancy were recruited. The control group included 30 non-pregnant women. The participant women had no history of allergic rhinitis (AR), septal deviation, and signs of acute or chronic upper respiratory tract infections, non-allergic rhinitis, nasal polyps, and sinonasal tumors.

Outcome parameters

A complete otorhinolaryngologic examination and decongestant-free active anterior rhinomanometry (ARM) were performed routinely for all participants.

All examinations and tests were performed between 27th and 40th weeks of pregnancy. In the history, pregnant women were asked to score the level of nasal congestion as for SNOS (none=0, mild=1, moderate=2, severe=3, fully blocked=4).

In the first examination, nulliparous women were asked whether they had PRs in the first and second trimesters. Multiparous women were asked whether they had PR in the previous pregnancies or during the current pregnancy. The gender of the baby was noted and pregnant women were asked to describe the nasal obstruction during pregnancy as either "increased", "decreased" or "unchanged".

In endoscopic examination, turbinate hypertrophy in a nasal cavity was scored by 1-point, the mild congestion of the mucosa was scored as 1 point and severe congestion was scored as 2-points. Therefore, the highest score a pregnant can get in anterior rhinoscopic examination was 6 points [9].

Decongestant-free ARM was performed on all pregnant women by using Rhinostream SRE 2000 (*Interacoustics A/S, Drejervaenget 8, DK-5610 Assens, and Denmark*) rhinomanometry device. The mask covered both the mouth and the nose. The pressure probe passed through the mask was placed in a nostril and the nasal flow probe in the other nostril. During placement of the probes, attention was paid not to deform the nostrils and to prevent the air leakage. Pregnant women were asked to keep their mouths closed and breathe through their noses. The values were read at 150 Pa. After expiration and inspiration, nasal resistance was recorded in Pa/cm^3 and the individual resistances of each nostril and the total inspiratory nasal resistance were calculated. Patients who had nose clearance before ARM had rested for 20 minutes in a room with a temperature of 20 ± 3 °C, the humidity was 50%, and the illumination

was moderate. The patients were instructed about the test. They had not exercised for 2 hours preceding the test and they were asked not to have tea, coffee, and cigarettes.

Results from the pregnant group were compared with 30 non-pregnant women in the control group who were non-smokers and did not have any signs of acute upper respiratory tract infections, or chronic sinonasal diseases. Thus, AnR, ARM and SNOS scores were recorded under same conditions.

Statistical analysis

Statistical analysis was performed by using SPSS 24.0 software (*Statistical Package for Social Sciences Inc., Chicago, IL, USA*). In terms of descriptive statistical methods, Mann Whitney U test for difference tests and Spearman Brown test for correlation were used since the data displayed non-parametric features. The results were evaluated with a confidence interval of 95% and a p-value less than *0.05* was considered as statistically significant.

Results

The mean ages in the study and control groups were 30.43±3.65 and 30.37±4.07, respectively. The gestational age during examination ranged between 27 and 39 weeks (mean: 34.2±3.71; median: 36). Of the 30 pregnant women, 9 (30%) were nulliparous and 21 (70%) were multiparous. A total of 16 newborns were male (53.3%) and 14 (46.7%) were female.

None of the pregnant women reported any relief of nasal congestion during pregnancy. The complaints did not change in 11 women (36.7%) and were increased in 19 women (63.3%). Fifteen of 21 multiparous women (71.4%) reported absence of PR, while 6 (28.6%) of them reported presence of PR in their previous pregnancies. In their current pregnancies, no PR were diagnosed in 10

pregnant women (33.3%), while PR was diagnosed in 20 pregnant women (66.7%) (Table 1).

Table 1. Gestational features and rhinitis findings in pregnant women.

Parameters	Study group (n=30)
Average pregnancy weeks	34.2±3.71
Median pregnancy weeks	36
Nulliparous	30% (n=9)
Multiparous	70% (n=21)
Nasal congestion decreased	0% (n=0)
Nasal congestion unchanged	36.7% (n=11)
Nasal congestion increased	63.3% (n=19)
Presence of rhinitis	66.7% (n=20)
Absence of rhinitis	33.3% (n=10)

The mean ANR values in the study and control groups were 3.60±1.35 and 0.77±0.73, respectively. Therefore, pregnant women exhibited higher AnR scores compared to non-pregnant women.

Regarding ARM findings, the mean total nasal inspiratory resistance (TNID) was 0.46±0.23 in the study group and 0.27±0.06 in the control group. The minimum and mode TNID scores were similar between 2 groups (0.19 and 0.18; 0.24 and 0.23). However, the maximum TNID was 0.39 in the control group and 1.07 in the study group. Therefore, the average TNID score was higher in pregnant women. Moreover, TNID values were grouped according to 0.12-0.33 Pa/ml/s, which was accepted as the normal value range in rhinomanometric measurements. With reference to this threshold, 9 (30%) of pregnant women in the study group and 25 (83.3%) of the non-pregnant women in the control group were within this range.

Table 2. AnR, ARM and SNOS results in the study and control groups.

Groups	Minimum	Maximum	Average	Median	Mode
Study group					
AnR	0	6	3.60±1.35	4	4
ARM	0.19	1.07	0.46±0.23	0.39	0.24
SNOS	0-none	3-severe	1.37±0.72	1-mild	1-mild
Control group					
AnR	0	2	0.77±0.73	1	1
ARM	0.18	0.39	0.27±0.06	0.25	0.23
SNOS	0-none	2-moderate	0.57±0.63	0.50-mild	0-none

[Abbreviations: *Anterior rhinoscopy (AnR), anterior rhinomanometry (ARM) and subjective nasal obstruction score (SNOS)].

Table 3. Comparison of AnR, ARM and SNOS results in the study and control groups.

	Groups (n=30)	$\bar{x}$	Ave. Row	Rows Total	U	Z	*p-value**
AnR	**Study**	3.60	44.20	1326.00	39.000	-6.200	*<0.00*
	Control	0.77	16.80	504.00			
ARM	**Study**	0.46	40	1200.00	165.000	-4.219	*<0.00*
	Control	0.27	21	630.00			
SNOS	**Study**	1.37	38.88	1166.50	198.500	-4.042	*<0.00*
	Control	0.57	22.12	663.50			

* Mann-Whitney U Test. [Abbreviations: **Anterior rhinoscopy (AnR), anterior rhinomanometry (ARM) and subjective nasal obstruction score (SNOS)].

On the other hand, the mean SNOS in the study and control groups were 1.37±0.72 and 0.57±0.63, respectively. We noted that SNOS averages of pregnant women were significantly higher than non-pregnant women (Table 2). The analysis of descriptive findings yielded that AnR, ARM and SNOS displayed significant differences between study and control groups *(p<0.05)*. AnR, ARM and SNOS findings of in pregnant women were significantly higher than the control group (Table 3).

In the study group, the correlation between AnR, ARM and SNOS was sought and the correlation between AnR and ARM was 0.176. The correlation between AnR and SNOS was 0.295 and the correlation between ARM and SNOS was 0.197. These correlations were found to be low and positive, but they were significant *(p<0.05)*.

As for these results, if any of AnR, ARM and SNOS values of the pregnant women increased, the others also tended to increase mildly. The

determination coefficient (explained variance=r^2) was calculated to determine the impact of changes of AnR, ARM and SNOS measurements on the other. As a result, r^2 value between AnR and ARM was found to be 3.1. This value was 8.7 between AnR and SNOS; and it was 3.8 between ARM and SNOS. Any changes in AnR or ARM constitutes an alteration of 3.1%. In the other parameters, these percentages were found to be 8.7% and 3.8%.

Furthermore, the regression equation between AnR and SNOS was y=0.6+0.21x. It was y=1.16+0.46x between ARM and SNOS, and y=0.39+0.02x between AnR and ARM.

In the control group, the correlation was 0.134 between AnR and ARM, 0.131 between AnR and SNOS and -0.36 between ARM and SNOS. Therefore, the correlations between AnR-ARM and AnR-SNOS were low and positive. However, the relationship between ARM and SNOS was very low and negative.

As for these results, r^2 between AnR and ARM was 1.8, r^2 between AnR and SNOS was 1.7, and r^2 between ARM and SNOS was 0.1. These findings may be interpreted as any change in AnR or ARM constitutes 1.8% change. In the other parameters, these percentages were 1.7% and 0.1%.

Discussion

In the present study, the prevalence of PR was 66.7% and it was reported as 40% in previous publications [10]. The nasal obstruction symptom evaluation (NOSE) scores in the study and control groups displayed no significant differences (p = 0.866). Minimal cross sectional areas at internal nasal valve and at the level of the head of inferior turbinate decreased significantly between the first and third trimesters (2). There was no difference between the trimesters as for total nasal resistance. The correlation analysis between the NOSE score, total volume and minimal cross sectional area at internal nasal valve demonstrated no significant differences [11].

In Sweden, the prevalence of PR was found to be 22% in a series of 599 patients [12]. Thus, it is possible to say that the global prevalence is considerably higher than the prevalence in Turkey. However, relatively larger samples should be studied for achievement of more objective outcomes.

We noted that nasal obstruction and PR were common during pregnancy. The previous PR was found in 28.6% of multiparous pregnancies. The presence of rhinitis in the current pregnancy was detected as 66.7%. The mean AnR score was 3.60±1.35. Our results were close to the value of 3.47 reported by Philpott et al. [13]. The utilities of AnR and ARM has been recommended for evaluation of rhinitis and nasal physiological changes during pregnancy [13]. Subjective nasal obstruction scale (SNOS) has been validated as a reliable tool of nasal congestion in previous studies [14].

Ülkümen et al. [15] reported that an incidence of 38.89% for PR. Nasal congestion was significantly associated with body-mass index and gestational weeks. Patients should be informed about adverse fetal and maternal outcomes of pregnancy related nasal congestion which may be aggravated by obesity and excessive weight gain during pregnancy.

In our study, the mean ARM was 0.46±0.23 and the mean SNOS was 1.37±0.72. The correlations between AnR-ARM, ARM-SNOS and AnR-SNOS were 0.176, 0.195 and 0.295, respectively.

The nasal obstruction associated with PR may lead to with diminution of the quality of life and sleep [16]. Gilbey et al. [17] suggested that

rhinosinusitis-specific quality of life was lower in the third trimester compared to that of the second trimester and that of patients without rhinosinusitis. Thus, increased awareness is important to improve the quality of life in pregnant women, prevent sleep apnea and related morbidity, and positively affect the gestational outcomes. There is controversy on the influence of atopy on the pregnancy outcomes since some authors postulated that atopy may be associated with more favorable results [17]; whereas some publications indicated that there was no link between atopy and recurrent abortions [18,19].

The main limitations of our study include relatively small sample size, subjectivity of detection of SNOS and data limited to the experience of a single center. On the other hand, prospective design and integrity of specific data constituted the strengths of our study.

Conclusions

To conclude, nasal obstruction and PR are common conditions in pregnant women. As reflected in higher scores of AnR, ARM and SNOS values, nasal symptoms and complaints must be questioned during follow-up in pregnancy. A low positive, but significant correlation was noted between AnR, ARM and SNOS measurements in pregnant women. The implementation of further multicentric trials on larger series may aid in accomplishment of more accurate results.

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