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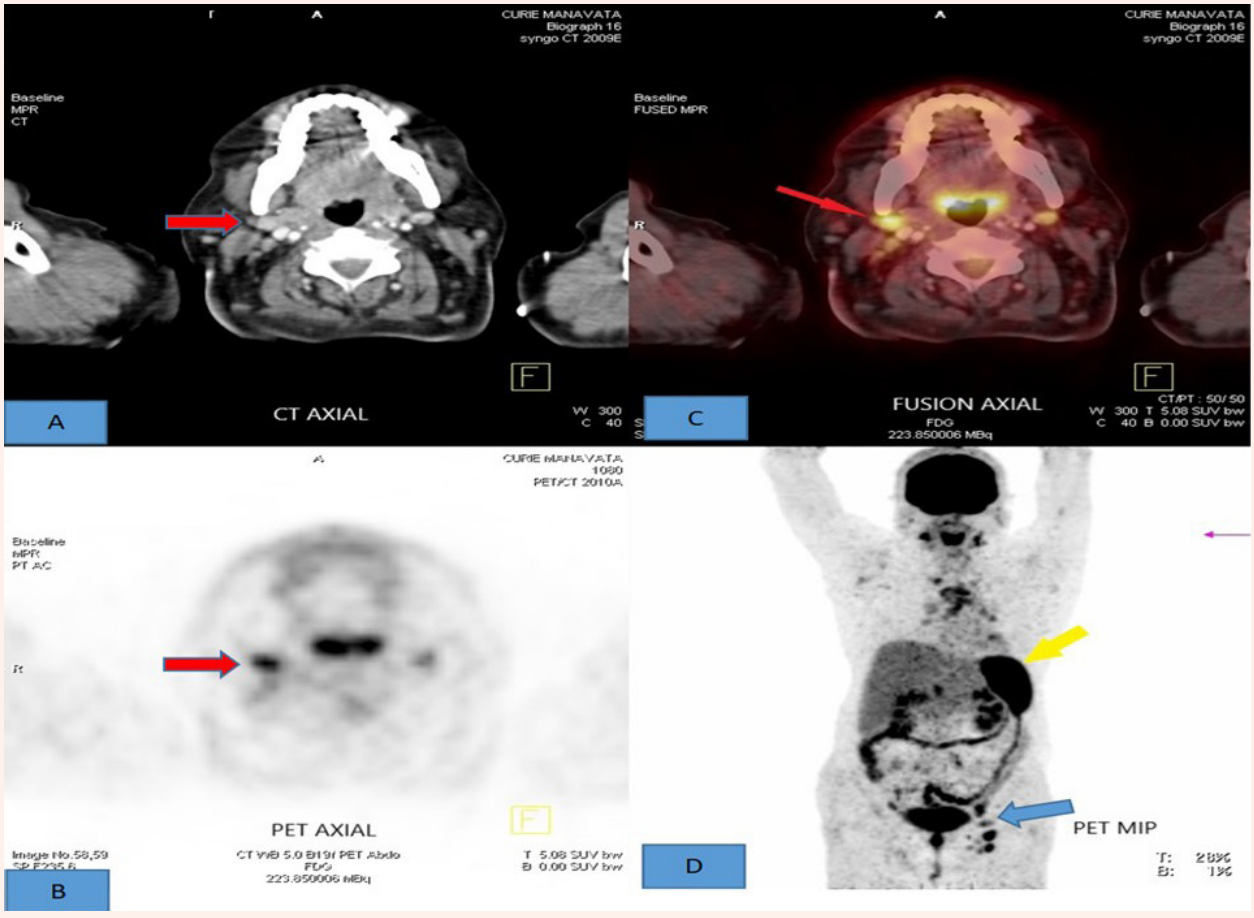

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Comparison of culture, Xpert MTB/RIF and EZN staining method to identify *Mycobacterium tuberculosis* from pulmonary and extra pulmonary specimens

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ABSTRACT

Mycobacterium tuberculosis is a major health problem worldwide, especially in developing countries. The effective treatment of *M. tuberculosis* depends on early diagnosis. In this study, 1577 *M. tuberculosis* strains were evaluated retrospectively from clinical samples sent between March 2014 and June 2018. The rate of pulmonary samples was determined as 694 (44%). Positive sample rate was 74 (4.69%) in culture, 19 (1.20%) in EZN staining and 75 (4.75%) in Xpert MTB/RIF. Compared to standard culture, the sensitivity, specificity, positive and negative predictive values of the Xpert MTB / RIF system were 100%, 99.93%, 98.66% and 100%, respectively. The sensitivity, specificity, positive and negative predictive values of EZN were 57.36%, 100%, 100% and 96.46%, respectively. Early diagnosis and treatment of *M. tuberculosis* is of great importance. According to these results, it can be concluded that Xpert MTB / RIF is a fast and reliable system that can be used in the diagnosis of tuberculosis and when used together with conventional tests, it can make important contributions to the diagnosis of tuberculosis.

1. Introduction

Mycobacterium tuberculosis is still a significant health problem, particularly in developing countries (Forbes et al., 2007). Rapid and accurate diagnosis of tuberculosis (TB) is important to control the disease. Diagnosis of TB especially in developing countries continues to rely on smear microscopy, which detects 40-60% pulmonary TB and 0-60% extrapulmonary TB (EPTB) cases (Mehta et al., 2012a; Mehta et al., 2012b; Fuchs et al., 2014). In addition, in order to detect bacilli in stained preparations, there should be approximately 5.000-10.000 bacilli/mL. Therefore, negative microscopy does not exclude the presence tuberculosis (Çöplü, 2002).

The gold standard and mostly used test for diagnosis of TB is the culture method (Trent, 2005). Culture method is not straightforward because isolation and drug susceptibility testing for this bacterium on solid media can take at least four to eight weeks or even longer. Furthermore, the turnaround time of *M. tuberculosis* is high and the method is not always accessible (Soini and Musser, 2001).

Rapid, specific and sensitive methods for the detection and identification of tuberculosis is needed to avoid unnecessary delay in making appropriate decisions. Molecular methods can advantageously complement and accelerate this process by minimizing the need to wait for a culture report (Sevilla et al., 2015).

The Xpert MTB/RIF assay (Xpert) was developed to improve TB and rifampin resistance (RIF-R) detection. This was accomplished by automating most of the steps required to process clinical samples and by improving the sensitive detection of both *M. tuberculosis* and RIF-R (Helb et al., 2010). This system is based on a fully automated nucleic acid amplification and detection, in which extraction, amplification and detection takes place inside a single-use cartridge, which is inserted into the GeneXpert Instrument System (Cepheid) (Vergara Gómez et al., 2017). The technique detects a fragment of the rpoB gene, which encodes for the subunit of RNA polymerase. Five molecular beacon type genetic probes are used, each labeled with a different fluorophore. They completely cover an area of 81 base pairs, RIF resistance determining region (RDRR), between the codons 507 and 533 (Vergara Gómez et al., 2017). The technique's lower detection limit, with 95% confidence, is 5 copies of DNA or 131 CFU/mL (World Health Organization, 2011). In comparison, the microscopic examination requires at least 10.000 CFU/mL while the culture between 100 and 500 CFU/mL (Lawn and Nicol, 2011).

The assay can be performed in approximately 2 h. Pooled data have shown Xpert MTB/RIF to have an overall sensitivity and specificity of approximately 89% and 98%, respectively (Steingart et al., 2014). Xpert MTB/RIF system has many advantages but it is not cost-effective as culture method or staining.

In this study we aimed to compare the results of the direct microscopic method of EZN, culture (LJ and MGIT 960), the Xpert MTB/RIF device, retrospectively.

2. Material and methods

A total of 1577 samples (694 pulmonary samples from sputum, bronchoalveolar lavage, and tracheal aspirate and 883 extrapulmonary samples from urine, pleural fluid, aspirate, cerebrospinal fluid [CSF], etc.) sent to the Ondokuz Mayıs University Medical Faculty Tuberculosis Laboratory between March 2014 and June 2018 on suspicion of tuberculosis disease were included in the study.

Culture

The *M. tuberculosis* culture and first-line phenotypic DST were performed in automated BD MGIT 960 (BD, USA) system. All sputum samples were processed using 4% sodium hydroxide (NaOH) method, and then cultured on MGIT 960 medium. *M. tuberculosis* strains grown on MGIT medium were tested for drug susceptibility in MGIT 960.

Specimens were decontaminated using sodium hydroxide (NaOH). After concentration by centrifugation at 3000 g for 15 minutes, the sediment was resuspended in 1.5 ml of 0.5 M phosphate buffer (pH 6.8) and inoculated onto Lowenstein-Jensen (LJ) medium and MGIT-7H9 broth supplemented with oleic acid-albumin-dextrose-catalase (OADC) and PANTA (BD, USA). Inoculated MGIT test tube were incubated using MGIT 960 instrument (BD, USA) and Lowenstein-Jensen (LJ) medium at 37°C in the incubator. *M. tuberculosis* strains grown on MGIT medium were tested for drug susceptibility in MGIT 960.

Microscopy

Preparations were arranged from the same sample for EZN staining and examined under the light microscope at 1000x magnification.

Procedures for Xpert testing

Xpert MTB/RIF testing was performed on samples, using version 4 cartridges, according to the manufacturer's recommendations. The Xpert assay sample reagent (containing NaOH and isopropanol) was added in a 1:3 ratio to the tubes to kill the mycobacteria and liquefy the sample. The mixture was shaken vigorously and held for 15 minutes. It was left for another 5 minutes before shaking again. Finally, 2 ml was pipetted into the Xpert assay cartridge and inserted into the Xpert MTB/RIF instrument for PCR testing. The measurement and analysis were conducted automatically and reported by the GeneXpert Dx software (version 4.0).

The specificity, sensitivity, and positive and negative predictive values were used for the evaluation of the performance of Xpert MTB/RIF.

3. Results

A total of 1577 samples with suspected tuberculosis that had been tested by three methods were evaluated. Of the 694 (44%) pulmonary samples 308 (44.4%) were sputum, 255 (36.7%) were bronchoalveolar lavage fluid, 120 (17.3%) were pleural fluid and 11 (1.6%) were endo tracheal aspirate. And of the extrapulmonary samples 294 were gastric fluid, 215 of urine, 88 were exudate, 82 were cerebrospinal fluid and 79 were surgical material.

Culture, EZN, and Xpert MTB/RIF system positivity of the 1577 samples were 4.7% (n=74), 1.2% (n=19) and 4.75% (n=75), respectively. When compared with culture results, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the Xpert MTB/RIF system for pulmonary samples were 100%, 99.7%, 96%, and 100%, respectively. These values for extrapulmonary samples were 96.3%, 100%, 100%, and 96.9%, respectively. The comparison of EZN staining with culture results revealed sensitivity of 61.5%, specificity of 100%, PPV of 100%, and NPV of 95.5% for respiratory samples; these values for nonrespiratory samples were 50.9%, 100%, 100%, and 97.1%, respectively.

Three isolates were determined positive for rifampicin by both two methods Xpert MTB/RIF system and MGIT automated system.

Table 1. Sensitivity, specificity, PPV, and NPV in comparison of Xpert MTB/RIF system and EZN with culture.

			Sensitivity %	Specifity%	PPV%	NPV%
Pulmonary Samples (694)	Sputum (308)	Xpert MTB/RIF	100	98.3	78.26	100
		EZN	66.66	100	100	96.98
	BAL* (255)	Xpert MTB/RIF	93.33	100	100	99.12
		EZN	59.57	100	100	92.27
	Pleural fluid (120)	Xpert MTB/RIF	50	100	100	98.33
		EZN	50	100	100	98.33
	ETA* (11)	Xpert MTB/RIF	0	91.66	0	100
		EZN	0	100	0	100
	Gastric fluid (294)	Xpert MTB/RIF	64.28	100	100	98.27
		EZN	50	100	100	96.93
	Urine (215)	Xpert MTB/RIF	100	100	100	100
		EZN	50	100	100	99.06
Extra Pulmonary Samples (883)	Sterile body fluid (123)	Xpert MTB/RIF	100	99.18	66.66	100
		EZN	50	100	100	98.5
	Exudate (88)	Xpert MTB/RIF	100	95.4	55.55	100
		EZN	55.55	100	100	95.4
	CSF* (82)	Xpert MTB/RIF	100	100	100	100
		EZN	50	100	100	95.12
	Surgical material (81)	Xpert MTB/RIF	80	100	100	98.71
		EZN	50	100	100	95.06

*BAL: Broncho alveolar alavage, ETA: Endotracheal aspirate, CSF; cerebrospinal fluid.

4. Discussion

Rapid and accurate identification of mycobacterial infections is important to initiate appropriate treatment, contact precautions and prophylaxis (Kox et al., 1994; Field and Cowie, 2006; Mokaddas and Ahmad, 2007). Conventional diagnostic methods for detection of Mycobacterial infections are microscopic examination of samples with EZN staining and culturing on LJ medium (Mokaddas and Ahmad, 2007; Aryan et al., 2010).

Microscopic smear examination is a low-cost, rapid and easy to perform method while it has poor sensitivity and no distinctive specificity (Ritis et al., 2000). It is known that 5.000 - 10.000 bacteria per mL are required for staining and smear microscopy (Çöplü, 2002; Forbes et al., 2007; Babacan and Hasdemir, 2008). The gold standart for diagnosis of mycobacterial infections are culture method. It has high specificity and its sensitivity is considered to be about 100 folds more than that of microscopic examination. But there are some disadvantages of the culture method, such as its prolonged hands-on time and the need for good and specific laboratory infrastructure, which is limited to reference centers (Aryan et al., 2004).

PCR-based assays have been used to detect Mycobacterial DNA with high sensitivity and specificity for rapid detection of mycobacterial infections (Kox et al., 1994; Soini and Musser, 2001; Espasa et al., 2005; Mokaddas and Ahmad, 2007; Elbir et al., 2008; Davis et al., 2009; Gopinath and Singh, 2009; Coelho et al., 2010; Arjomandzadegan et al., 2011). In our study, we aimed to compare the EZN staining and culture with Xpert MTB/RIF system. Culture method was regarded as the gold standard of TB diagnosis.

The Xpert MTB/RIF system provides determination of *M. tuberculosis* complex and rifampin resistance in a single test in a short time (less than 2h) directly from clinical sample via a semiquantitative nested real-time PCR method. Since all reagents required for the test are kept in a closed cartridge, there is no cross-contamination possibility between clinical samples (Durmaz, 2010).

In the study by Bunsow et al. sensitivity, specificity, PPV, and NPV of the GeneXpert system for pulmonary samples and extrapulmonary samples were found to be 97.1%, 98.6%, 95.7%, and 99.1% and 33.3%, 99.7%, 80.0%, and 97.3% respectively (Bunsow et al., 2014). In their study of 521 extrapulmonary samples, Hillemann et al. compared the results of the Xpert MTB/RIF system with those of conventional liquid (MGIT 960) and solid (LJ) culture methods and found sensitivity and specificity as 77.3% and 98.2%, respectively. They expressed that the Xpert MTB/RIF system is a rapid and useful technique in the identification of extrapulmonary tuberculosis (Hillemann et al., 2014).

Ioannidis et al. compared the results of culture methods (LJ and MGIT 960) with those of the, Xpert MTB /RIF system and found sensitivity, specificity, PPV, and NPV as 90.6%, 94.3%, 93.5%, and 91.7% in respiratory samples and 100%, 91.6%, 50%, and 100% in nonrespiratory samples, respectively. At the end of the study, they concluded that the GeneXpert system, a NAA-based method, would be beneficial in treating tuberculosis (Ioannidis et al., 2011).

Özkütük and Sürücüoğlu compared Xpert MTB/RIF test results with culture results (BACTEC MGIT 960 and LJ medium). For pulmonary samples, specificity, sensitivity, PPV, and NPV were found to be

80.8%, 98.8%, 84.9%, and 98.4%, respectively. These values for nonpulmonary samples were 58.2%, 98.4%, 66.7%, and 97.7%, respectively. They suggested that Xpert MTB/RIF is a useful method for the diagnosis of tuberculosis (Özkütük and Sürücüoğlu, 2014).

In a meta-analysis about accuracy of Xpert MTB/RIF assay for extrapulmonary tuberculosis, thirty-six studies were identified, with a pooled sensitivity and specificity of respectively 77% (95% CI 66-85) and 97% (95% CI 94-98). Among site specific estimates for lymph, pleural fluid, cerebrospinal fluid, gastrointestinal and urinary samples, the pooled sensitivity was lower in pleural fluid (37%, 95% CI 26-50, meta-regression p, 0.001) and higher in lymph node samples (87%, 95% CI 75-95, meta-regression P¼ 0.03). And they reported that Xpert MTB/RIF has high specificity but limited sensitivity for the detection of extrapulmonary tuberculosis. In conclusion they reported that positive Xpert MTB/RIF test results may be useful in rapidly identifying the extrapulmonary tuberculosis, while negative test results provide less certainty for ruling out disease (Penz et al., 2015).

In a study by Luetkemeyer et al. Xpert MTB/RIF was compared with acid-fast bacilli (AFB) smear and mycobacterial culture using liquid and solid culture media, from participants with suspected pulmonary tuberculosis from the United States, Brazil, and South Africa. They found the sensitivity of the Xpert MTB/RIF result was 81.4% and sensitivity was 98.5% in AFB positive and 54.8% in AFB negative participants. Also they reported that the diagnostic performance of Xpert MTB/RIF in the United States was similar to higher-tuberculosis prevalence sites in Brazil and South Africa, and was comparable to other studies in higher-tuberculosis prevalence locations (Luetkemeyer et al., 2016).

In our study, sensitivity of Xpert MTB/RIF for pulmonary samples is higher than extrapulmonary samples but both of them have high sensitivity and specificity. The sensitivity of Xpert MTB/RIF for pulmonary samples is higher than a extrapulmonary samplesdue to the higher bacterial load in pulmonary samples. Also it was reported that sensitivity at extrapulmonary samples has differences among sample types, it is about 80% in lymph nodes however does not reach 50% at pleural fluids (Vergara Gomez et al., 2017). And in our result, one sample was positive for Xpert MTB/RIF but it was negative in culture. Sometimes due to the antituberculosis treatment growth on the culture cannot be detected but pcr assays can detect the bacterial DNA.

Our study have some limitations such as we only evaluated the microbiological findings, but not radiological, histological and clinical findings for the diagnosis of tuberculosis.

In summary, Xpert MTB/RIF is a rapid and reliable system that can be employed in the diagnosis of tuberculosis, and when utilized together with conventional tests, it can make significant contributions to tuberculosis diagnosis.

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Investigation of the antimicrobial effects of *Sapindus mukorossi* on endodontic pathogens

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Sapindus mukorossi

ABSTRACT

Endodontic infections have a polymicrobial nature. Thus, eliminating the microorganisms from infected root canals without damaging healthy surrounding tissue is a major concern. *Sapindus mukorossi* (*S. mukorossi*) is a natural product with potential antimicrobial effects. The aim of this study was to evaluate whether various extract solutions of *S. mukorossi* have an antimicrobial activity against specific endodontic pathogens. Extracts were obtained from *S. mukorossi* fruit pericarps using methanol, ethanol, butanol and distilled water solvents. The inhibition zone, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined with disc diffusion assay, broth microdilution assay and agar dilution assay. Antimicrobial inhibitory activity was observed with all four different solvent extracts of *S. mukorossi* against *Fusobacterium nucleatum* American Type Culture Collection (ATCC) 25586, *Porphyromonas gingivalis* ATCC 33277 and *Actinomyces odontolyticus* (clinical isolate). The MIC values were ranged 10.24-10.24, 0.01-0.64 and 1.28-2.56 mg/mL, respectively. The MBC value was not detected for *Fusobacterium nucleatum* ATCC 25586. The MBC values were 0.02-1.25 and 2.56-5.12 mg/mL for *Porphyromonas gingivalis* ATCC 33277 and Actinomyces odontolyticus (clinical isolate), respectively. Antifungal activities were also observed with the four different solvent extracts of *S. mukorossi* against *Candida albicans* ATCC 10231 and *C. albicans* clinical isolates 1, 2 and 3. The inhibition zone diameter values were in the range of 18-21 mm. The MIC values for *C. albicans* ATCC 10231 and *C. albicans* clinical isolates 1, 2 and 3 were 0.2-0.4 and MFC values were 0.4-0.8 mg/mL, respectively. The antimicrobial effects of the *S. mukorossi* fruit pericarp extract inhibited the growth of *P. gingivalis, A. odontolyticus, F. nucleatum, and especially the C. albicans strains. S. mukorossi* extract has interesting potential as an antimicrobial agent against endodontic pathogens.

1. Introduction

Microorganisms play an important role in the initiation and progression of tooth pulp diseases. In the endodontic applications, such as root canals treatment or regeneration procedures, chemomechanical preparation with an antimicrobial solution is an important stage for the treatment success. Bacterial flora in this region are normally mixed, with a predominance of anaerobic

and facultative species. The presence of bacterial residue in the root canal and periapical regions, even if the treatment is adequate, may result in treatment failure in the long term (Sundqvist, 1992). Studies have shown that bacteria inhibit differentiation of human periodontal stem cells and induce pro-inflammatory cytokines, which play an important role in delaying periodontal tissue regeneration (Graves et al., 2011; Kato et al., 2014). This is especially important in endodontic regeneration processes.

A wide variety of disinfection agents have been developed and used to eliminate necrotic tissues and microorganisms from the root canals. For example, sodium hypochlorite (NaOCl) and chlorhexidine (CHX) are frequently used for the elimination of necrotic tissues and microorganisms during root canal treatment. Despite NaOCl has been the conventionally primary choice in endodontic treatment over the years for its antimicrobial and tissue dissolving properties, alternative approaches are being sought mostly due to concerns regarding cytotoxicity, especially in cases of regeneration (Gatot et al., 1991; Onçağ et al., 2003; Spencer et al., 2007; Simbula et al., 2010). In recent years, the trend of using natural resources has led to investigation of alternative plant extracts, and many researchers have investigated the activity of new substances in root canal disinfection (Herrera et al., 2010; Castilho et al., 2013; Dutta and Kundabala, 2013; Caetano da Silva et al., 2014; Sathyaprasad et al., 2015).

Sapindus mukorossi (S. mukorossi) is a deciduous tree that is widely grown in tropical and sub-tropical regions of Asia, and it is a valuable medicinal plant. It is commonly known by several names, such as soap walnut, ritha, soapberry or washnut. The fruit of *S. mukorossi* (soap walnut) is used in folk medicine for the traditional treatment of diseases like excessive salivation, pimples, epilepsy, chlorosis, migraines, eczema and psoriasis (Shah et al., 2017). Phytochemical studies have shown the presence of saponins (10-11.5%), sugars (10%) and mucilage, with different levels of biological activity in the fruit. These substances give the plant important potential effects, such as antimicrobial, anti-inflammatory, antiprotozoal, anti-cancer, spermicidal and hepatoprotective actions (Ibrahim et al., 2006; Sharma et al., 2011; Upadhyay and Singh, 2012; Shah et al., 2017; Hu et al., 2018). Although some properties of *S. mukorossi* in the medical field have been investigated, comprehensive studies on its antimicrobial activity are still limited (Ibrahim et al., 2006; Sharma et al., 2013; Srinivasarao et al., 2015; Porsche et al., 2018).

The quantity of the active substances in the content of *S. mukorossi*, expecially saponin that can be responsible for antimicrobial effect may change depending on the method of extraction and the type of solvent (Huang et al., 2006; Ghagi et al., 2011). The aim of this study was to evaluate whether various extract solutions of *S. mukorossi* have the antimicrobial activity against some important bacterial and fungal strains that are responsible from endodontic infections.

2. Materials and methods

Preparation of *S. mukorossi* extracts

Extracts of *S. mukorossi* fruit pericarps in different solvents (ethanol, methanol, butanol or distilled water; Sigma-Aldrich, US) were obtained using the Soxhlet extraction method in the Chemistry Department of Akdeniz University. The type of solvents was selected based on previous study results by the researchers (Güçlüer et al., 2020). For this purpose, the dried pericarp of *S. mukorossi* fruits (Botanik City Centre, Ankara, Turkey) was pulverized, and the obtained powder (50 g) was placed in an extraction cartridge. Extraction was carried out for each solvent (700 mL) and completed after 20 cycles of solvent in the system. The remaining solvent was removed in the evaporator under reduced pressure, and the mixture was completely dried under a vacuum at 600°C for 24h. As a result, four different extracts were successively obtained using methanol, ethanol, butanol and distilled water solvents. The ultraviolet (UV) spectrum percentages of extracts in dimethyl sulfoxide (DMSO) were determined for quality control. For the antimicrobial tests, 204.8 mg/mL of stock solution was prepared from the extracts with 10% DMSO and distilled water.

Microorganisms

This study was approved by the Akdeniz University Faculty of Medicine Ethics Committee (No. 05.13.2015/238). Fourteen species comprising ten bacteria and four yeasts were included. Seven of them were obtained from the American Type Culture Collection (ATCC) and seven clinical isolates were obtained from the culture collection of the Department of Microbiology of Akdeniz University cryopreserved at -80°C. More specifically, the following strains were employed: *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Fusobacterium nucleatum* ATCC 25586, *Porphyromonas gingivalis* ATCC 33277, *Bacteroides fragilis* ATCC 25285, *Candida albicans* ATCC 10231, clinical isolates of *Actinomyces odontolyticus* (n=1), *E. faecalis* (n=3) and *C. albicans* (n=3) were used in this study.

Aerobic and facultative anaerobic isolates were grown on Colombia Agar with 5% Sheep Blood plates (Becton Dickinson [BD], Germany). Anaerobic isolates were grown on Brucella Blood Agar with Hemin and Vitamin K1 plates (BD, Germany; 5 μg/mL of hemin, 1 μg/mL of vitamin K1, 5% lysed sheep blood) and incubated in anaerobic conditions

(GasPak EZ, Anaerobe Gas Generating Pouch System with Indicator, BD, Germany) for 3-4 days at 37°C. The yeasts were grown in Sabouraud dextrose agar (SDA; BD). In addition, quality control study (with fluconazole) recommended by the Clinical and Laboratory Standards Institute (CLSI) were included to evaluate the suitability of the tests.

Disc diffusion assay (DDA)

The extracts were initially evaluated with Kirby-Bauer disc diffusion assay (DDA) for determining their antimicrobial activity according to the CLSI standards (CLSI M02-A12, 2015; CLSI M44-A2 2009). Aerobic bacteria suspension of 0.5 McF (1-2 x 108 colony forming unit; CFU/mL) turbudity was applied to Mueller-Hinton agar medium (BD, Germany). Twenty microlitres of 204.8 mg/mL of each extract solution were added and then placed in each sterile paper disc (6-mm diameter) (BD Sensi-Disc, Germany). The discs (4.1 mg extract/disc) previously inoculated with the test microorganisms (*E. faecalis, S. aureus, P. aeruginosa* strains) were placed in the Mueller-Hinton agar plates (BD, Germany). The inhibition zones were measured for each extract after incubation under aerobic conditions for 16-24h.

DDA was also performed as a preliminary assay for anaerobic bacteria. For this, 0.5 McF density bacterial suspensions from colonies (*F. nucleatum, P. gingivalis and B. fragilis*) were prepared in Brucella broth and placed in Brucella Blood Agar with Hemin and Vitamin K1 plates (BD, Germany). Discs (BD Sensi-Disc, 4.1 mg extract/disc) prepared as described above were placed in Brucella Blood Agar plates that were previously inoculated with the anaerobic test bacteria. The inhibition zones were measured for each extract after incubation under anaerobic conditions for 36-48 h. DMSO and distilled water were used as negative controls for all DDAs.

DDA prepared a 0.5 McF density suspension of Candida strains grown for 24 h on SDA plates and distributed on the Mueller-Hinton agar supplemented with 2% glucose and 0.5μg/ml methylene blue dye. Discs (BD Sensi-Disc, Germany) prepared as described above (4.1 mg extract/disc) were placed on the surfaces of Mueller-Hinton agar medium plates previously inoculated with the test microorganisms [*C. albicans* ATCC 10231 and *C. albicans* 1, 2 and 3 (clinical isolates)]. The inhibition zones were measured for each extract after incubation at 37°C under aerobic conditions for 16-24h.

Broth microdilution assay (BMA)

The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the *S. mukorossi* extracts were evaluated using the broth microdilution assay (BMA) under sterile conditions for aerobic/facultative anaerobic bacteria (CLSI M07-A9. 2015) and yeast according to CLSI (CLSI M27-A3. 2008). In the BMA for bacteria, stock extract solutions (204.8 mg/mL) were serially diluted twofold with cation-adjusted (Ca^{+2} and Mg^{+2}) Mueller-Hinton broth (CAMHB; BD, Germany), and each dilution was dispensed into the wells. Aerobic bacterial stock solutions (0.5 McF; 1-2 x 108 CFU/mL) were prepared from fresh colonies with 0.9% NaCl, then diluted with CAMHB and was inoculated in sterile, U-bottom 96-well microplates. The last bacterial inoculum density was 5 x 105 CFU/mL and final volume was 100 µL in each test well. The microplates were incubated at 35°C for 24 h. The amount of growth in each well was compared with the positive control and the MIC values were obtained the lowest concentration that completely inhibits growth, and 100μLbacterial suspensions from inhibited test wells were sub-cultured in 5% blood agar to evaluate the MBC.

A similar procedure to the BMA for bacteria was used for yeast. Briefly, stock extract solutions (204.8 mg/mL) were serially diluted twofold (between 25.6 - 0.05 mg/ml) with Roswell Park Memorial Institute (RPMI) 1640 broth (buffered with 3-(N-Morpholino) propane sulfonic acid, MOPS; Sigma-Aldrich, USA), and each dilution was dispensed into sterile 96-well microplates. Yeast suspensions from overnight cultures were prepared with an initial density of 0.5 McF (1-5 x 106 CFU/mL). The inoculum was adjusted to a density of 0.5-2.5 x 103 CFU/mL with RPMI 1640 broth and tested against each extract concentration.

The microplates were incubated at 35°C for 24 h. The MIC values were determined as the smallest extract concentration at which no increase is apparent in visual turbidity (approximately 80% growth reduction compared with the positive control). The MFC values were determined by plating 10 μL from each negative well and from the positive control on SDA. MFC was defined as the lowest concentration yielding negative subcultures or only one colony. Fluconazole powder (Fluka Analytical, Sigma) was included in the test for quality control.

Agar dilution assay (ADA)

The MIC and MBC of extracts of *S. mukorossi* were determined using the agar dilution test for anaerobic strains in the sterile conditions according to CLSI (M11-A8. 2012). The bacteria were grown under anaerobic conditions in Brucella Blood Agar supplemented with Hemin and Vitamin K1 plates (5 μg/mL of hemin, 1 μg/mL of vit K1, 5% lysed sheep blood; BD, Germany). For ADA, Brucella agar media (BD) plates were prepared that included different concentrations (20.48-0.01 mg/ml) of extracts.

Anaerobic bacteria solutions (1-2 x 108 CFU/mL) were diluted with Brucella broth medium (BD) over 36-48 h of culturing of bacterial colonies, and were inoculated in the prepared Brucella agar plates containing extracts, then were incubated under anaerobic conditions (GasPak EZ, Anaerobe Gas Generating Pouch System, BD, Germany). The MIC (80% reduction of bacterial growth) and MBC (99.9% reduction of bacterial growth) values were determined by comparing the colonies with the control plate after 48 h of incubation at 35°C. If necessary, the incubation time was prolonged to 72h (for *P. gingivalis*, ATCC 33277). The standard *B. fragilis* ATCC 25285 strain was tested for ampicillin-sulbactam susceptibility as a quality control strain.

3. Results

The antibacterial activities observed with all four solvent extracts of *S. mukorossi* fruit pericarp against *F. nucleatum* ATCC 25586 (Fig. 1), *P. gingivalis* ATCC 33277 and *A. odontolyticus* (clinical isolate). The zone diameters are presented in Table 1. The MIC and MBC values for *P. gingivalis* ATCC 33277 and A. odontolyticus (clinical isolate) with ADA are presented in Table 2. Although growth inhibition exhibited a relatively high concentration in *F. nucleatum* ATCC 25586 with ADA, there was no bactericidal activity (Table 2).

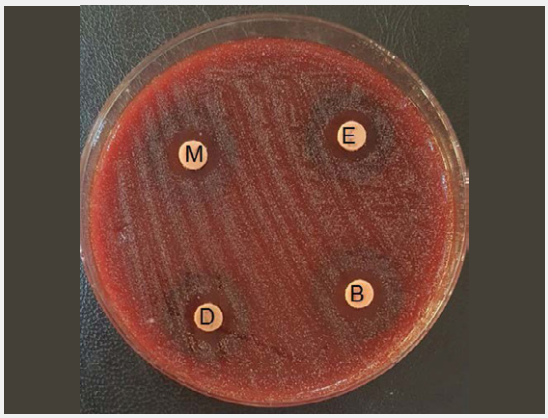

Fig. 1. DDA for *Fusobacterium nucleatum* (ATCC 25586); discs including methanol (M), ethanol (E), butanol (B) and distilled water (D) extracts.

When the different solvent extracts were compared, larger zone diameters with ethanol and methanol solvent extracts were observed against *F. nucleatum* ATCC 25586. Similarly, the extracts obtained with ethanol and methanol solvents were found to be more effective for *A. odontolyticus* (clinical isolate); the MIC and MBC values were determined to be twofold lower. For *P. gingivalis* ATCC 33277, the antibacterial activity of the extracts obtained with methanol, butanol, and ethanol solvents was found to be more effective (the zone diameters were higher and MIC and MBC values were lower compared to the distilled water extract).

Antifungal activity was observed against *C. albicans* ATCC 10231 (Fig. 2) and *C. albicans* clinical isolates 1, 2 and 3, as presented in Table 1, and higher inhibition zone diameter values were detected. At the same time, fungicidal activity was observed against *C. albicans* ATCC 10231 and all *C. albicans* clinical isolates and the MIC and MFC values are presented in Table 2.

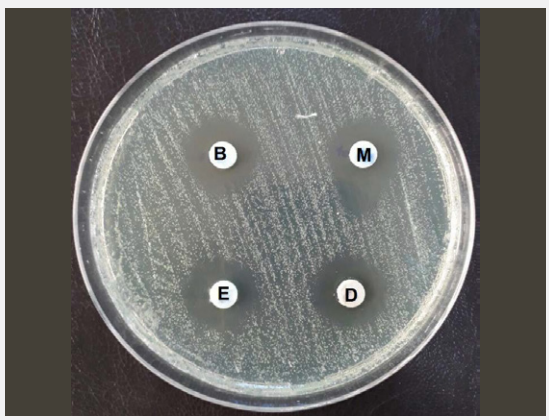

Fig. 2. DDA for *Candida albicans* (ATCC 10231); discs including methanol (M), ethanol (E), butanol (B) and distilled water (D) extracts.

When compared the extracts obtained with different solvents, the extracts obtained with ethanol and methanol solvents showed greater zone diameters for *C. albicans* strains with DDA. However, no difference was found between the MIC and MFC values.

According to the test results, no antimicrobial effect was detected against *E. faecalis* ATCC 29212 or E. faecalis clinical isolates 1, 2 and 3; *S. aureus* ATCC 29213; or *P. aeruginosa* ATCC 27853, even at the

Table 1. Inhibition zone diameter values (mm) of *S. mukorossi* extracts in Disc Diffusion Assay*.

S. mukorossi extracts	*F. nucleatum* (ATCC 25586)	*P. gingivalis* (ATCC 33277)	*C. albicans* (ATCC 10231)	*C. albicans* (clinical isolate 1)	*C. albicans* (clinical isolate 2)	*C. albicans* (clinical isolate 3)
Ethanol	14	15	19	20	21	19
Methanol	14	15	19	20	21	19
Butanol	12	15	18	19	19	18
Aqueous	12	14	18	19	19	18

*Disc Diffusion Assay not applied for A. odontolyticus. The results of bacteria for which inhibition zone was not created are not shown in this table.

Table 2. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration.

	Ethanol extract		Methanol extract		Butanol extract		Distilled water extract	
	MIC (mg/ml)	MBC or MFC*	MIC (mg/ml)	MBC or MFC*	MIC (mg/ml)	MBC or MFC*	MIC (mg/ml)	MBC or MFC*
F. nucleatum (ATCC 25586)	10.24	Ineffective¥	10.24	Ineffective¥	10.24	Ineffective¥	10.24	Ineffective¥
P. gingivalis (ATCC 33277)	0.32	0.64	0.01	0.02	0.01	0.02	0.64	1.28
A. odontolyticus (clinical isolate)	1.28	2.56	1.28	2.56	2.56	5.12	2.56	5.12
C. albicans (ATCC 10231)	0.4	0.8	0.4	0.8	0.2	0.4*	0.4	0.8
C. albicans (clinical isolate 1)	0.4	0.4*	0.4	0.4*	0.2	0.4*	0.4	0.4*
C. albicans (clinical isolate 2)	0.2	0.4*	0.4	0.4*	0.2	0.4*	0.2	0.4*
C. albicans (clinical isolate 3)	0.4	0.8*	0.4	0.4*	0.4	0.4*	0.4	0.8*

* Minimal fungicidal concentrations for C. albicans strains.
¥ Bactericidal effect was not detected with evaluated concentrations (51.2 mg/mL).

highest concentration (51.2 mg/mL) of *S. mukorossi* extract used in this study. Similarly, an antimicrobial effect was not observed against *B. fragilis* ATCC 25285 with DDA (containing 4.1 mg/disc) or ADA (51.2 mg/mL) with *S. mukorossi* extract.

4. Discussion

The presence of infection agents inside the root canal system or periapical tissue can alter the success rate of endodontic treatment. It has already been well established that the common endodontic irrigation solutions (e.g., NaOCl and CHX) are cytotoxic to pulp stem cells and oral tissues. It is evident that antimicrobial and biocompatible irrigants are needed for many endodontic applications, and many studies have been carried out in this field (Herrera et al., 2010; Trevino et al., 2011; Dutta and Kundabala, 2013; Sathyaprasad et al., 2015). In studies about *S. mukorossi,* its fruit pericarps have been reported to have anti-oxidant and anti- inflammatory activity, as well as a protective capacity on some tissue cells (Upadhyay and Singh, 2012; Shah et al., 2017; Ali et al., 2018). These effects can make the plant useful for endodontic applications. In this study, the antibacterial and antifungal effects of fruit pericarp extracts of *S. mukorossi* were investigated against significant endodontic infection agents in vitro and obtained a useful perspective on the antimicrobial efficacy.

Some solvents are used during the preparation of the plants to evaluate their in vitro properties. In the previous studies, ethanol (Ibrahim et al., 2006; Sharma et al., 2011; Srinivasarao et al., 2015; Hu et al., 2018), chloroform (Ibrahim et al., 2006; Porsche et al., 2018) and methanol (Shah et al., 2017; Porsche et al., 2018) were frequently used as a solvent for *S. mukorossi*. Although many researchers obtained the extracts of *S. mukorossi* using different solvents, these studies have reported limited information on differences in antimicrobial efficacy of this solvent extraction methods. Ibrahim et al. reported that chloroform and ethanolic extracts of *S. mukorossi* exerted antibacterial effects on *H. pylori*, while petroleum ether and benzene extracts did not show any antibacterial effect (Ibrahim et al., 2006). In this study, antimicrobial effect of *S. mukorossi* extracts obtained with ethanol, methanol, butanol or distilled water was evaluated against significant pathogen microorganisms of endodontic infections. First, DDA was applied for the preliminary study. The antibacterial (for *F. nucleatum)* and antifungal activities (for *C. albicans*) of the extracts with ethanol and methanol solvents were found to be slightly higher in DDA. Similarly, lower MIC and MBC values were determined for *A. odontolyticus* (clinical isolate) and P. gingivalis ATCC 33277 with extracts obtained from ethanol, methanol and butanol solvents. In this study, the extracts obtained with ethanol and methanol solvents were found to be more effective against related microorganisms.

The endodontic microbiota were predominantly anaerobes and facultative species. The bacterial species tested in this study, *F. nucleatum* and *P. gingivalis*, are Gram-negative, anaerobic bacterial species located in flora, and they may cause periapical infections from the pulpitis (Martinho et al., 2016). *A. odontolyticus* is another bacteria in this study, described as a Gram-positive anaerobic specie, highly detected in infected root canals and has pathogenic potential in periapical diseases (Abou-Rass and Bogen, 1998).

Previous studies about the activity of antimicrobial substances, such as plants, are usually expressed as inhibition zones by DDA in vitro. In previous studies the antimicrobial activity of plant extracts and phytochemicals were evaluated with antibiotic susceptible and resistant microorganisms. In this study, many plant extracts showed antibacterial activity against specific microorganism with zones of inhibition of 7 mm and above is acceptable (Nascimento et al., 2000; Dutta and Kundabala, 2013; Srinivasarao et al.,

2015). Srinivasarao et al. interpreted an 8 mm zone of inhibition for a plant extract as antimicrobial. The extracts of *S. mukorossi* showed inhibition zones of 12-14 and 14-15 mm for *F. nucleatum* ATCC 25586 and P. gingivalis ATCC 33277, respectively (Srinivasarao et al., 2015). Thus, it includes effective antibacterial compounds.

The determination of antimicrobial activity with MIC is accepted as a significant quantitative technique for in vitro studies with crude extracts (Alves et al., 2008). In this study, DDA was applied as a preliminary study and the MIC values were determined with ADA, which is the gold standard for anaerobic bacterial species. The sensitivities were observed for *F. nucleatum, P. gingivalis* and *A. odontolyticus*, which are three important periodontal bacterial pathogens, and the MIC values were determined as 10.24-10.24, 0.01-0.64 and 1.28-2.56 mg/mL, respectively (with different extracts). Aligiannis et al. accepted MIC values 0.28-1.27 mg/mL an extreme activity against a bacteria for a plant (Aligiannis et al., 2001). The bactericidal effect and MBC values for *P. gingivalis* ATCC 33277 and *A. odontolyticus* were also determined in our study. It appeared that *S. mukorossi* has a bacteriostatic effect against *F. nucleatum* ATCC 25586 compared with the other two anaerobic bacteria. Hence, sub-fractionationof this extract may be better for explaining the antibacterial effect.

It has been reported that *S. mukorossi* ethanol and chloroform extracts inhibit the growth of H. pylori in vitro (10-200 μg/mL by DDA) and in vivo (Ibrahim et al., 2006). In the literature, there has been no study evaluating the efficacy of *S. mukorossi* against Fusobacterium spp. and Actinomyces spp. and our study is the first to report the antibacterial effect of this plant's extracts on *F. nucleatum* and *A. odontolyticus*. Bacterial species evaluated in this study are representative of the different groups causing infections in root canals. *E. faecalis, S. aureus and P. aeruginosa* are usually associated with periapical lesions. *B. fragilis* is a Gram-negative anaerobic bacteria commonly found in endodontic infections (Pallotta et al.,, 2007). In this study, antibacterial effects of *S. mukorossi* extracts could not be detected against these bacteria. Srinivasarao et al. reported that *S. mukorossi* extract obtained with the thin layer chromatography method, showed antibacterial effect (8-mm inhibition zone) for *Bacillus subtilis, B. cereus, Pseudomonas aeruginosa and Escherichia coli*. It is thought that saponins, which are components of *S. mukorossi*, are responsible for its antimicrobial activity and other useful effects (Upadhyay and Singh, 2012; Srinivasarao et al., 2015; Ali et al., 2018; Hu et al., 2018). Another study reported that ethanolic extract of *S. mukorossi* has an antibacterial effect against the same bacterial species using the well diffusion method, drawing attention to the importance of saponin content in the plant (Sharma et al., 2013). There is also a study which the oleanane-type saponin fraction exhibits moderate antibacterial activity against Gram-positive bacteria (*S. aureus*) and no activity against Gram- negative bacteria (*P. aeruginosa and E. coli*). (Tamura et al., 2012). Overall, there has been a limited number of studies in this area. In our study, it could not be determinated the sensitivity to these bacteria. The reason for this may be that the extract did not divided into fractions (e.g., by the chromatographic method). Another possible reason may be that there was a relatively low amount of components responsible for antibacterial effects, such as saponins, in the extract.

C. albicans is one of the most common microorganisms in the flora of periapical lesions (Dutta and Kundabala, 2013; Sathyaprasad et al., 2015). In our study, the antifungal activity of *S. mukorossi* against *C. albicans* strains was determined with all extracts obtained with different solvents.

Aligiannis et al. and Tsuzuki et al. presented suggestions a classification for in vitro antimicrobial plant studies (Aligiannis et al., 2001; Tsuzuki et al., 2007). Briefly, with a MIC value up to 0.5 mg/mL, the antifungal activity is considered reliable. If the extracts show a MIC between 0.6 mg/mL and 1.5 mg/mL, the antifungal activity is moderate and MIC above 1.6 mg/mL, the antifungal activity is considered weak (Aligiannis et al., 2001; Duarte et al., 2005; Tsuzuki et al., 2007).

In this study according to the criteria outlined above, the MIC values determinated against *C. albicans* strains (0.4 and 0.2 mg/mL) suggested that *S. mukorossi* has promising antifungal activity. Indeed, this action was better than the observed antibacterial effects of all the extracts. These results also support the disc diffusion test results (18-21 mm). Studies have shown that triterpenoid saponins in the structure of *S. mukorossi* exhibited antifungal activity against *C. albicans* (Aneja et al., 2010; Upadhyay and Singh, 2012; Hu et al., 2018). In addition, the saponin fraction inhibited the dermatophyte fungi *T. rubrum, T. mentagrophytes, Sabouraudites canis, Epidermophyton floccosum* and *C. albicans* (Tamura et al., 2012). In another study, the efficacy of saponins isolated from *S. mukorossi* with the liquid chromatography technique was also shown against plant fungi (Porsche et al., 2018). The results of our study are consistent with other studies on the efficacy of *S. mukorossi* against *C. albicans* species.

Although many phytochemicals present in *S. mukorossi* have been isolated and identified by researchers, pharmacological studies on these components have been limited so far, and there is a need for extensive supplementary research on *S. mukorossi* to explain its mechanism of action. In addition, detailed analysis is required for isolating phytoactive components from *S. mukorossi* and

tracing their biological activities. If the phytoactive components are purified, the substances responsible for the antimicrobial effects can be detected more accurately. These results were obtained in vitro, and thus, they may not exactly reflect in vivo interactions. Safety assess and in vivo studies should be conducted to elucidate the effectiveness of *S. mukorossi*.

Conclusions

In this study, the antibacterial and remarkable antifungal activities of *S. mukorossi* fruit pericarps were determined. It was demonstrated that the fruit extracts of *S. mukorossi* inhibited the growth of *F. nucleatum, P. gingivalis, A. odontolyticus and C. albicans*. The extracts of *S. mukorossi* fruit pericarps were most effective against *C. albicans* strains. If advanced susceptibility tests are carried out separating the active substances, it may help to identify these effects better. These substances can be combined with different chemicals to increase their efficiency, especially when interacting with mixed bacterial flora, which are involved in most root canal infections.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication that could have influenced its outcome.

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Effects of leptin on the viability of MCF-7 and T47D cells at different glucose concentrations

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ABSTRACT

Obesity is associated with increased risk of breast cancer. Leptin is a well-known factor involved in obesity and its serum levels are increased in breast cancer. Hyperglycemia is another significant risk factor for breast cancer. Consistently, high glucose induces proliferation and invasion of breast cancer cells and in-vivo calorie restriction reduce tumorigenesis in rodent models. The aim of this study was to investigate the effect of leptin on the viability and mode of cell death in breast cancer cells incubated in different glucose concentrations to represent caloric restriction. For this purpose, MCF-7 and T47D breast cancer cells incubated in different glucose concentrations for a total of 72 hours were treated with or without leptin either for one hour or 24 hours and the ratio of apoptotic, necrotic and alive cells were analyzed by flow cytometry. Our data revealed that glucose incubation significantly decreased apoptosis and necrosis, while increasing viability in both cell lines in a dose dependent manner. One-hour leptin treatment significantly decreased viability, and increased apoptosis but did not significantly affect necrosis in T47D cells incubated in 2.5 mM glucose. In MCF-7 cells, one-hour leptin incubation significantly increased necrosis but its effects on apoptosis and viability were not significant. In conclusion, although glucose induces cell death by apoptosis and necrosis in T47D and MCF-7 cells respectively in a dose dependent manner , the overallviability is still increased in both cell lines. One-hour leptin treatment reverses the effect of low glucose incubation on apoptosis of T47D and necrosis of MCF-7 cells. Moreover, the effect of one-hour leptin treatment on apoptosis or necrosis is significantly higher than that of 24-hour leptin treatment.

1. Introduction

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer related death among women worldwide (Niu et al., 2013). A number of meta-analyses revealed that obesity and body weight (BW) gain are associated with an increased breast cancer risk, especially for post-menopausal women (Keum et al., 2015). Obesity has been reported to be related to an increased risk of disease recurrence (Ligibel, 2011) and a poorer survival rate (Chan et al., 2014) in both pre- and post-menopausal women diagnosed with breast cancer. Although there are several previous studies investigating the association between obesity and breast cancer development, the molecular mechanism of the association remains largely unknown (Dogan et al., 2007; Dogan et al., 2011; Tuna et al., 2017). In this context, adipokines are suggested as important molecules linking obesity to breast cancer (Dogan et

al., 2010; Cicekdal et al., 2019; Cicekdal et al., 2019). Leptin, a 16 kDa peptide hormone, is an adipokine secreted mainly by the adipose tissue as a product of the obese (Ob) gene. There is a well-established link between leptin and obesity (Artac and Altundag, 2012) as leptin is a key appetite-regulating hormone which plays critical roles in appetite control, energy intake and energy expenditure (Amitani et al., 2013). Both leptin and its receptors were reported to be overexpressed in breast cancer tissue (Ishikawa et al., 2004). Consistently, serum leptin levels have also been reported to be significantly higher in breast cancer patients compared to that of healthy individuals (Wu et al., 2009). Besides, circulating leptin levels are higher in breast cancer patients with lymph node metastasis relative to the benign disease breast cancer patients (Niu et al., 2013). Effects of leptin on the proliferation and survival of anchorage independent growth of breast cancer cells have also been demonstrated in vitro (Hu et al., 2002).

Calorie restriction, defined as reducing the energy intake without causing malnutrition, is one of the most accepted preventive approaches for breast cancer development in many species (Dogan et al., 2010; O'Flanagan et al., 2017). In vitro cell culture models may allow for a more controllable calorie intake and also provide quantification of inputs and outputs on a molecular level in a shorter period of time. Therefore, in vitro cell culture experimental design of calorie intake (lower or higher), which is controlled by the glucose concentration in cell culture, may lead to a better understanding of the association between leptin and breast cancer cell proliferation.

Leptin plays an important role in the regulation of glucose metabolism independent of its effect on BW. Inadequate leptin signaling has a pivotal role in hyperglycemia induction, persistence of which may lead to diabetes (Amitani et al., 2013). Consistently, chronic hyperglycemia is a common characteristic of mice lacking the leptin encoding gene (ob/ob) (D'souza et al., 2017) and is alleviated by administration of low doses of leptin that do not significantly affect BW in ob/ob mice (Pelleymounter et al., 1995; Hedbacker et al., 2010). However, not much is known about the effect of leptin on the viability and mode of cell death in breast cancer cells especially in the presence of different glucose concentrations in-vivo or in vitro.

Here, we investigated the effect of leptin on viability and cell death using MCF-7 and T47D breast cancer cell lines cultured in different glucose concentrations (0 mM, 2.5 mM, 5 mM (normal glucose levels in cell medium), 25 mM, 50 mM), which mimics in-vivo calorie intake conditions (lower or higher) in-vitro condition. The present study is the continuation of our previous research which was focusing on the effects of glucose or leptin on cancer cell growth either in-vitro or in-vivo conditions (Cicekdal et al., 2019; Demirel et al., 2019; Tuna et al., 2020). To the best of our knowledge, this is the first study to report the effects of leptin on breast cancer cell viability and mode of cell death in the presence of different glucose concentrations in-vitro.

2. Materials and methods

Cell culture

Estrogen receptor (ER) positive breast cancer cell lines (T47D, MCF-7) were obtained from the American Type Culture Collection (ATCC, VA, USA). These two cell lines differ by their TP53 characteristics. MCF-7 cells have wild type TP53 while T47D cells have mutant TP53 (Neve et al., 2006). Breast cancer cell lines (MCF-7 and T47D) were chosen to be studied because the focus of our group has been breast cancer and obesity in mouse models (Dogan et al., 2007; Demirel et al., 2019). These two cells lines have different characteristics for intracellular apoptotic signaling proteins. Although T47D cells have caspase 3 activity, MCF-7 cells don't have caspase 3 activity, suggesting that leptin may be triggering a different apoptotic pathway(s) (Mooney et al., 2002). Cells were grown in complete DMEM medium (Gibco, NY, USA) containing 5 mM glucose, 10% heat inactivated FBS (Gibco, NY, USA), 0.25% Insulin (Gibco, NY, USA), 1% Pen/Strep (Gibco, NY, USA) then incubated in 5% CO_2 at 37°C until they reached 70% confluency prior to the experiments.

Experimental design

After reaching confluency, T47D (passage 29) and MCF-7 (passage 19) cells were washed with PBS and then trypsinized at 37°C to collect cells in normal media. Cells were centrifuged at 1000 rpm for 7-10 min, resuspended in medium, an equal number of cells (8.5 x 105 cells/plate) were placed in petri dishes and incubated overnight to allow the cells to attach. On the following day, cell medium was removed and replaced with fresh DMEM containing differing amounts of glucose (0 mM, 2.5 mM, 5 mM, 25 mM, and 50 mM) and incubated at 37°C for 72 hours. Normal glucose concentration in a cell medium is 5 mM. Therefore, two of the glucose concentrations tested were below the normal glucose concentration (0 mM, 2.5 mM,) while the other two concentrations were above the normal glucose levels (25 mM, 50 mM). To see the effects of leptin on breast cancer cells in different glucose concentrations, 100 ng/ml leptin (Cell Sciences, MA, USA) was added to the cell medium either at the end of either 48 or 71 hours after the start of incubation of the cells with the medium containing different glucose concentration. Cells were further incubated in medium with or without leptin until 72 hours from the start of glucose incubation. In other words, the cells were incubated with leptin either for 24 hours or one-hour.

Thus, total glucose (Sigma, MO, USA) incubations were 72 hours at each glucose concentration tested (either 48 hours glucose incubation +/- 24-hour leptin treatment or 71 hours glucose incubation +/- one-hour leptin treatment). Study design is shown in Fig. 1. For each set of experiments on a given day, two to three different petri dishes were used for each glucose concentration and the average of these replicates was taken as one sample (duplicate or triplicate).

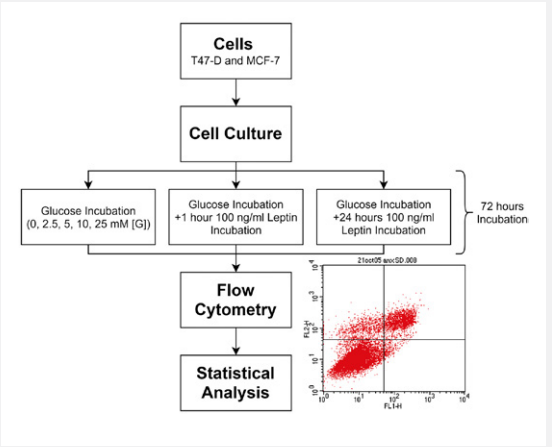

Fig. 1. Experimental study design.

Flow cytometry analysis of apoptosis

Following the 72 hour-incubations, T47D or MCF-7 cells were trypsinized (Gibco, NY, USA) and harvested. Cells were then centrifuged, washed with PBS and labeled with Annexin V-Fluorescein Isothiocyanate and PI (Propidium Iodide) (Annexin V-FITC Apoptosis Detection Kit, MBL International Corp., MA, USA). The distribution pattern of alive and dead (apoptotic/necrotic) cells were determined by FACS analysis (Becton-Dickinson FACS Calibur flow cytometer, BD Biosciences, CA, USA). The FITC and PI signals were detected in FL1 and FL2 channels, respectively. Small debris were excluded by gating intact cells in the FSC/SSC plot. Cells in the lower left panel of the FL1/FL2 dot plot (labeled with low Annexin V-FITC and PI) were considered as viable, cells in the right panel (labeled with Annexin V-FITC) were considered to be apoptotic, and cells in the upper left plot (labeled with PI only) were considered to be in necrosis.

Statistical analysis

Two tailed Mann Whitney test and Kruskal-Wallis test with Dunn's multiple comparison test were performed to determine statistical differences among the groups. All analyses were conducted in Graphpad Prism 7.0. Data represent mean ± standard deviation (SD). Differences were accepted as statistically significant when $p<0.05$. "n value" for each comparison is given in the figure legends.

3. Results

Glucose decreases cell death in T47D and MCF-7 cells in a dose dependent manner

MCF-7 and T47D cells were incubated in medium containing different glucose concentrations for 72 hours and necrotic/apoptotic cells were analyzed using flow cytometry to investigate the effect of glucose on breast cancer cell death (Fig. 2). All tested glucose concentrations (2.5 mM, 5 mM, 25 mM, and 50 mM) significantly decreased cell death in both T47D and MCF-7 cells compared to the glucose free group ($p<0.05$, Fig. 2). Although incubation in each glucose concentration led to a significant decrease in apoptosis in both T47D and MCF-7 cells ($p<0.05$, Fig. 2A, 2B), the levels of apoptosis in T47D cells were lower than that of MCF-7 cells for 2.5 mM glucose concentration ($p<0.0001$ for T47D cells, $p<0.05$ for MCF-7 cells).

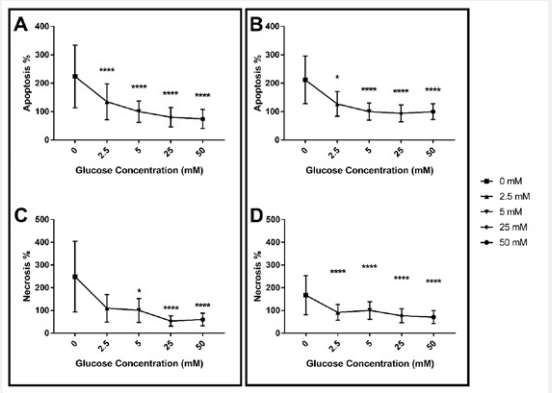

Fig. 2. Effect of different glucose concentrations on cell death in breast cancer cells. A) Apoptosis of T47D (n=30) B) Apoptosis of MCF-7 (n=29) cultured in 0 mM, 2.5 mM, 5 mM, 25 mM, or 50 mM glucose. C) Necrosis of T47D (n=29-31) D) Necrosis of MCF-7 (n=29) cultured in 0 mM, 2.5 mM, 5 mM, 25 mM, or 50 mM glucose for 72 hours. n represents the number of independent experiments. Data represent mean ± S.D. *, $p<0.05$, **** $p<0.0001$.

All glucose concentrations significantly decreased necrosis compared to 0 mM glucose except for 2.5 mM in T47D cells ($p<0.05$, Fig. 1C). On the other hand, all glucose concentrations decreased necrosis significantly compared to the 0 mM glucose group in MCF-7 cells ($p<0.0001$, Fig. 2D). However, the effect of 5 mM glucose on necrosis was more drastic in MCF-7 cells compared to T47D cells ($p<0.0001$ for MCF-7 cells, $p<0.05$ for T47D cells, Fig. 1D). Consistently, glucose incubation significantly increased viability of both MCF-7 and T47D cell lines in a dose dependent manner ($p<0.05$, Fig. 3). Notably, the positive effect of 2.5 mM glucose incubation on viability was more apparent in MCF-7 cells compared to T47D ($p<0.01$ for MCF-7 cells, $p<0.05$ for T47D cells, Fig. 3).

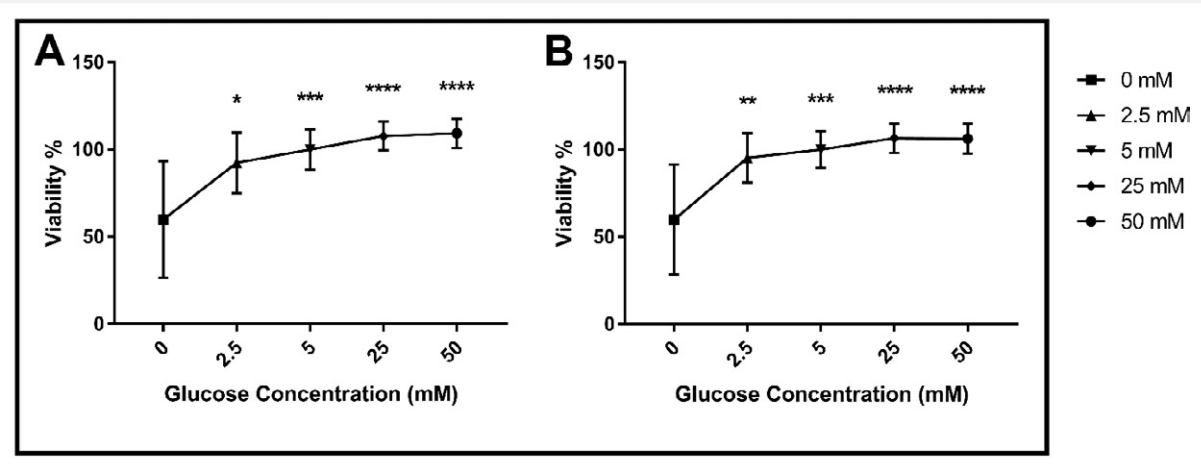

Fig. 3. Effect of different glucose concentrations on the viability of breast cancer cells. A) Viability of T47D (n=30) and B) Viability of MCF-7 (n=28) cultured in various glucose concentrations for 72 hours. n represents the number of independent experiments. Data represent mean ± S.D. *, p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Leptin enhances the effect of glucose on cell death in T47D and MCF-7 cells

In order to examine the effects of leptin on the viability of breast cancer cells cultured in medium with different glucose concentrations, T47D and MCF-7 cells incubated in 0 mM, 2.5 mM, 5 mM, 25 mM, or 50 mM glucose for 72 hours in total were treated with or without leptin (100 ng/ml) for either one-hour or 24 hours and necrotic and apoptotic cells were analyzed by flow cytometry (Fig. 4). One-hour leptin incubation significantly increased apoptosis (p<0.05, Fig. 4A), but did not dramatically affect necrosis in T47D cells cultured in medium with 2.5 mM glucose (p>0.05, Fig. 4B). Consistently, viability was significantly lower in one-hour leptin treated cells cultured in 2.5 mM glucose compared to leptin untreated cells cultured in 2.5 mM glucose (p<0.05, Fig. 4C). Notably, the positive effect of one-hour leptin treatment on the apoptosis of T47D cells in 2.5 mM glucose was significantly higher compared to 24-hours leptin treatment (p<0.05, Fig. 4A). However, the effect(s) of one-hour leptin treatment on the necrosis of T47D cells was not significantly different compared to the 24-hour leptin treatment (p>0.05, Fig. 4B). In support of these data, viability was significantly decreased in T47D cells incubated in medium with 2.5 mM glucose and then treated with leptin for one-hour compared to 24-hour leptin treatment (p<0.01, Fig. 4C). Similar to 2.5 mM glucose cultured cells, one-hour leptin treatment significantly increased apoptosis in T47D cells in 5 mM glucose (p<0.05, Fig. 4A) but, it did not have a significant effect on necrosis in T47D cells incubated in 5 mM glucose (p>0.05, Fig. 4B). Although not statistically significant, one-hour leptin treatment decreased viability of T47D cells incubated in 5mM glucose (p>0.05, Fig. 4C). Besides, compared to the 24-hour leptin treatment, the negative effect of one-hour leptin treatment on viability of T47D cells incubated in either 2.5 mM or 5mM glucose was significantly higher (p<0.05, Fig. 4C).

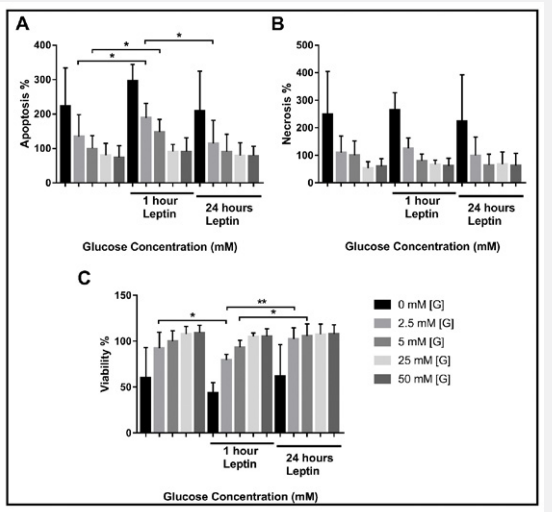

Fig. 4. Effect of leptin on the viability of T47D cells cultured in different glucose concentrations. A) Apoptosis of T47D B) Necrosis of T47D C) Viability of T47D cultured in different glucose concentrations in the presence or absence of leptin (100 ng/ml) (n=7-29). n represents the number of independent experiments. Data represent mean ± S.D. * p<0.05, ** p<0.01.

Unlike T47D cells, one-hour leptin treatment significantly increased necrosis in MCF-7 cells (p<0.001, Fig. 5B), but did not have a significant effect on apoptosis in 2.5 mM glucose (Fig. 5A). Although not statistically significant, viability of one-hour leptin treated MCF-7 cells cultured in 2.5 mM glucose was

lower compared to leptin untreated cells ($p>0.05$, Fig. 5C). The positive effects of one-hour leptin treatment on necrosis of MCF-7 cells incubated in either 2.5 mM or 5 mM glucose were significantly higher compared to 24-hour leptin treated cells ($p<0.05$, Fig. 5B), although apoptosis was not significantly different in these groups ($p>0.05$, Fig. 5A). Consistently, one-hour leptin treatment significantly decreased viability of MCF-7 cells in either 2.5 mM or 5 mM glucose relative to the 24-hour leptin treatment ($p<0.01$, Fig. 5C). Compared to leptin untreated cells, one-hour leptin treatment significantly ($p<0.05$) reduced viability of MCF-7 cells incubated in 5 mM glucose, however, it did not have a significant effect on either necrosis or apoptosis (Fig. 5). One or 24-hour leptin treatment did not have any significant effect on modes of cell death or viability in either MCF-7 or T47D cells (Figs. 4, 5).

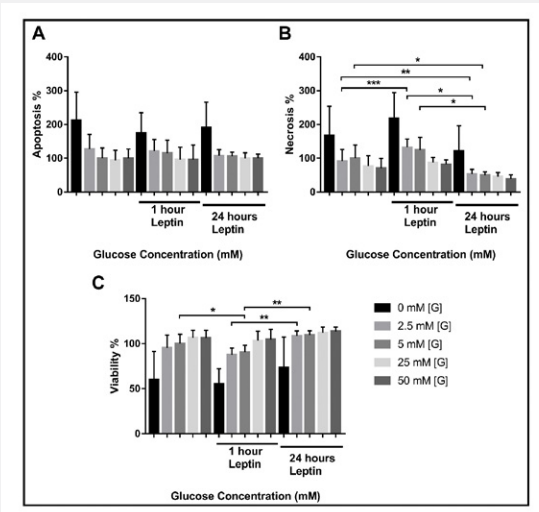

Fig. 5. Effect of leptin on the viability of MCF-7 cells cultured in different glucose concentrations. A) Apoptosis of MCF-7 B) Necrosis of MCF-7 C) Viability of MCF-7 cultured in different glucose concentrations in the presence or absence of leptin (100 ng/ml) (n=4-30). n represents the number of independent experiments. Data represent mean ± S.D. *, $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Our data showed that glucose significantly decreased cell death while increasing viability in both MCF-7 and T47D cell lines in a dose dependent manner. Additionally, one-hour leptin treatment significantly increased apoptosis in T47D cells cultured in the presence of 2.5 mM glucose, however it significantly increased necrosis in MCF-7 cells. Moreover, one-hour leptin treatment increased necrosis more significantly in 5 mM glucose cultured MCF-7 cells relative to T47D cells. Besides, the effect of one-hour leptin treatment of MCF-7 and T47D cells cultured in low (2.5 mM) and physiological (5 mM) doses of glucose was more significant compared to that of 24-hour leptin treatment.

4. Discussion

Hyperglycemia, defined as increased glucose levels in blood, is a significant risk factor for breast cancer development especially for post-menopausal women (Boyle et al., 2012). Several studies have suggested that hyperglycemia may contribute to cell proliferation, apoptosis, metastasis, and chemotherapy resistance of cancer cells (Vigneri et al., 2009; Johnson et al., 2012; Duan et al., 2014; Ryu et al., 2014; Demirel et al., 2019). Consistently, glucose and other factors involved in glucose metabolism including insulin and insulin-like growth-factors (IGFs) contribute to breast cancer development (Muti et al., 2002; Dogan et al., 2011). In addition, incubation in high glucose containing medium induced proliferation and invasion of breast cancer cells in-vitro (Wei et al., 2017; Demirel et al., 2019). On the other hand, leptin, a key appetite-regulating hormone, is recognized as another important risk factor for breast cancer development and prognosis since both leptin and its receptors are reported to be overexpressed in breast cancer (Ishikawa et al., 2004). In this study, we examined the effect of leptin on cell death and viability in T47D and MCF-7 breast cancer cell lines cultured in cell media with different glucose concentrations to mimic leptin's effect on calorie intake (lower or higher) in-vitro.

Although there are studies performed in high glucose medium and assessing cell proliferation, to our knowledge there are no previous studies investigating the effects of leptin at different glucose concentrations, specifically lower than normal glucose concentration (5 mM glucose) in cell media. In this context, high glucose levels were previously demonstrated to promote proliferation of breast cancer cells (Yamamoto et al., 1999; Okumura et al., 2002; Hou et al., 2017; Wei et al., 2017; Demirel et al., 2019). Consistently, we showed that glucose increases viability and decreases cell death in both T47D and MCF-7 cells in a dose dependent manner (Figs. 2 ,3). Specifically, high glucose concentrations (25 mM and 50 mM) decrease necrosis and apoptosis significantly in both MCF-7 and T47D cell lines. However low glucose (2.5 mM), which was used to mimic calorie restriction in-vitro, significantly induced apoptosis in T47D and necrosis in MCF-7 cells. Additionally, physiological normal glucose (5 mM) increases necrosis more significantly in MCF-7 cells compared to T47D cells (Fig. 2A, 2B). In this context, although Krętowski et al. reported a decrease in the percentage of apoptotic cells in low glucose (2.8 mM) compared to the high glucose concentration of 25 mM (Krętowski et al., 2016), our data indicated that apoptosis was lower in high glucose cultured cells (25 mM and 50 mM) compared to low glucose (2.5 mM) in both MCF-7 and T47D cells (Figs. 2, 3). A major difference between Krętowski et al.'s study and ours that might have contributed to the different findings is

in the duration of the glucose incubations: Krętowski et al. examined the apoptotic cells following a 48-hour incubation, whereas we applied a 72-hour glucose incubation prior to examining apoptosis.

Okumura et al., previously reported that 5 day-treatment of 10-9 or 10-8 M leptin in the presence of physiological glucose concentration (5.5 mM) did not significantly increase proliferation of MCF-7 cells (Okumura et al., 2002). Consistently, we also did not observe a significant effect on the viability of MCF-7 cells following the 24-hour treatment of 6.25x10-9 M leptin. However, one-hour leptin (6.25x10-9 M) treatment significantly decreased viability in 5 mM glucose-cultured MCF-7 cells, indicating that leptin might have different effects on the viability of MCF-7 cells dependent on the dose and duration of the treatment. Other previous studies have also reported different results regarding hyperleptinemia both in-vitro and in-vivo studies. One of the reasons for this could be leptin resistance effects especially in obese subjects.

Our data revealed that glucose incubation significantly reduced apoptosis and necrosis, while increasing overall viability in the T47D cell line in a dose dependent manner. Additionally, one-hour leptin treatment of T47D cells significantly increased apoptosis in 2.5 mM glucose. In MCF-7 cells, glucose incubation decreased both apoptosis and necrosis in a dose dependent manner, thus increasing overall viability. One-hour leptin treatment significantly increased necrosis in 2.5 mM glucose cultured MCF-7 cells. In both cell lines, the effect of one-hour leptin treatment on apoptosis or necrosis was significantly higher compared to 24-hour leptin treatment. It's noteworthy that leptin at low and physiological glucose concentrations increase apoptosis in T47D cells which lack P53, suggesting that leptin may be triggering a P53-independent apoptotic pathway(s) (Mooney et al., 2002). Although Okumura et al., reported that 5-day treatment of 10-6 M leptin enhanced proliferation of MCF-7 cells incubated in 25 mM glucose, in the present study we did not observe any significant effect of one or 24-hour 6.25x10-9 M leptin treatment on the viability of T47D and MCF-7 cells incubated in 25 mM or 50 mM glucose compared to 5 mM (Okumura et al., 2002). The different effects of leptin and glucose concentrations on the mechanisms of cell death in T47D and MCF-7 cell lines might be due to the genetic background differences. It is also not unusual to see different responses for leptin and/or glucose in different cell lines. Therefore, these results should be tested in healthy breast cell lines to see whether leptin effects are specific to cancerous cells.

These results reveal that leptin effect for cell viability, apoptosis and necrosis are secondary in the presence of enough levels of glucose in breast cancer cell lines in in-vitro conditions. However, leptin may play a glucose like role in the absence of glucose in cancer cell growth. More mechanistic studies are needed to clarify the cross-talk between leptin and glucose signaling pathways in regard to cancer cell proliferation.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Association of angiotensin-converting enzyme and nitric oxide synthase genes polymorphisms with the risk of myocardial infarction in Bangladesh

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ABSTRACT

Myocardial infarction is one of the leading manifestations of illness and death worldwide. The aim of the study was to find out the associations of ACE (I/D) and NOS3 (G894T & 4b/a) genes polymorphisms with the occurrence of myocardial infarction (MI) in Bangladeshi population. The study was conducted on 100 cardiac patients experiencing MI and 150 healthy volunteers with no complications. The genotyping was done using PCR and PCR-RFLP methods and the biochemical parameters were measured using auto biochemistry analyzer. Over all, the serum Troponin I, AST and ALP levels were significantly ($p<0.01$, and 0.001, respectively) higher and the albumin level was significantly ($p<0.001$) lower among the patients. The percentage of DD genotypes of ACE gene was significantly ($p<0.05$) higher in patients. The individual with DD allele was at 3.28-fold increased risk (OR=3.28; 95% CI=1.6 to 6.7; $p < 0.01$) of experiencing MI while individual with ID genotype was at lower risk. In addition, the cigarette smokers with DD genotypes were found to have a 4.1-fold increased risk to develop cardiac disease (OR=4.1; 95% CI=1.5 to 11.2; $p<0.01$). The frequencies of NOS3 (G894T & 4a/b) genotypes in the patients and controls were almost similar. There were no significant differences among the biochemical parameters for different genotypes. Thus our recent study suggested that the ACE (I/D) gene may have strong associations with the occurrence of MI and DD genotype would be considered as a risk factor.

1. Introduction

Myocardial infarction (MI) is one of the major manifestations of coronary artery disease (CAD). CAD is considered to be one of the main causes of morbidity around the world (Gouvinhas et al., 2013). According to the World Health Organization (WHO, 2019), every year approximately 17.9 million people die of cardiovascular diseases worldwide that is 31% of all deaths and most of these occur in developing countries. CAD is a multifactorial disorder that is a consequence of an interaction between genetic background and environmental factors such as diet, smoking and physical activity (Smith et al., 1997; Prins et al., 2012). The genes conferring susceptibility to coronary artery disease are largely unknown. Atherosclerotic plaque formation, hypercholesterolemia, hypertension, and

diabetes are considered as major risk factors for CAD (Smith et al., 1997). Nitric oxide (NO), an important relaxation factor in the human body, plays a key role in the relaxation of vascular smooth muscle, inhibits adhesion of platelets and leukocytes to the endothelium, reduces vascular smooth muscle cells migration and proliferation, and limits the oxidation of atherogenic low-density lipoproteins (Schmidt and Walter, 1994). Thus intracellular NO has been considered as an inhibitor of atherosclerotic plaque formation and reduces the risk of CAD. NO is synthesized by the action of nitric oxide synthase (NOS) from L-arginine. There are at least three isoenzymes of NOS: Inducible NOS, neuronal NOS, and endothelial NOS (eNOS/NOS3) (Nathan and Xie, 1994).

The NOS3 gene is located on chromosome 7q35-36, consists of 26 exons with a total size of 21 kb, encodes for intracellular NO production (Marsden et al., 1993). This gene is expressionally and functionally regulated through multiple regulatory steps, and entails several polymorphisms, some of which have functional consequences (Hingorani et al., 1999). Study conducted by Hingorani et al. first described that exchange of guanine to thymine at nucleotide 1917 in exon 7, replaces glutamic acid to aspartic acid of NOS3 gene which has been associated with coronary spasm (Hingorani et al., 1995; Yoshimura et a., 1998), essential hypertension (Miyamoto et al., 1998) and the risk of acute myocardial infarction (AMI) (Hingorani et al., 1999). Several variable number of tandem repeats (VNTRs) such as polymorphic repeats close to the 5' end, the 27 base pair (bp) repeat in intron 4 are the most studied. The resulting rare 4-repeat allele (4a/b) has been shown to be associated with CAD among subjects belonging to European ancestry (Wang et al., 1996). This 4a/b mutation of NOS3 gene was found to be associated with MI, among subjects of Turkish descent (Cine et al., 2002). Several studies reported that significant association of the 4a/b polymorphism with CAD and MI has been found in several ethnic populations (Wang et al., 1996; Park et al., 2000), though a few groups have also reported lack of association of this SNP (Single Nucleotide Polymorphism) with CAD (Granath et al., 2001; Jaramillo et al., 2010).

The renin-angiotensin system is one of the key regulatory systems, play important role in cardiovascular physiological processes such as cardiovascular remodeling, sodium homeostasis and maintenance of vascular tone (Dzau, 1994). The angiotensin-converting enzyme (ACE) is a major component of the renin-angiotensin system, which is found in the lungs, kidneys, cardiomyocytes and other tissues. ACE converts angiotensin I to angiotensin II a potent vasoconstrictor and also inactivates bradykinin a potent vasodilator (Murphey et al., 2000; Cicoira et al., 2001). Elevated levels of angiotensin II and decreased bradykinin levels is accompanied by the inactivation of ACE can cause increased vascular resistance and high blood pressure found in CAD. The ACE gene is located on chromosome 17q23, and bears 26 exons and 25 introns (Hubert et al., 1991). Even though the human ACE gene contains a large number of polymorphic regions that can be used in the genetic analysis of populations (Reider et al., 1991). The insertion/deletion (I/D) polymorphism of ACE, present in intron 16, where 287 Alu repeat is deleted, has been extensively investigated (Howard et al., 1990). It has been reported that ACE genotypes affect the levels of ACE, which has been consider as a high risk factor for the development of MI (Leatham et al., 1994). It is considered that I allele has a sequence that silences ACE enzyme causing low activity while D allele lacking silencer sequence causes higher ACE activity (Choi et al., 2004). To the best of our knowledge there is no study presenting the possible relation of NOS3 and ACE (I/D) polymorphism with MI in Bangladesh yet. The aim of the present study was to find out the effects of NOS3 and ACE gene (I/D) polymorphisms in the risk of Myocardial Infarction in Bangladeshi population.

2. Materials and methods

The study was a case-control study conducted in 250 subjects (Table 1). The case group comprises 100 cardiac patients who have experienced MI one or more times. The studied MI patients were referred by their treating physicians, and severe chest pain and serum high troponin I level were also measured to include the patients as MI groups. The cardiac patients were recruited for this study immediately after being hospitalized with MI symptoms at Coronary Care Unit (CCU) of Sir Salimullah Medical College Hospital without any medical history of other chronic diseases. A total of 150 healthy controls with no history of cardiac or chronic diseases were recruited from different hospitals of Dhaka city where they came for regular health check-up (Table 1). There was no significant difference in baseline characteristics between control and cases (Table 1).

All participants were given an explanation of the nature of the study, and informed consent was obtained. They completed a structured questionnaire covering information on age, gender, medical, family history of chronic diseases and smoking status. Smoking status was summarized as smoker or nonsmoker (Table 1). The study was approved by the departmental ethical committee. The study was conducted in accordance with the declaration of Helsinki and its subsequent revisions (World Medical Association, 2013).

Sample collection

Approximately 5.0 mL of venous blood was drawn from each individual following all aseptic precautions

with the help of a trained person, using a disposable syringe. About 2.0 mL of drawn blood was immediately transferred into a tube containing EDTA (1.20 mg/ml) and remaining blood into a plain tube for the separation of serum and transported to the laboratory using ice-box. The blood samples of EDTA tubes were stored at –20°C until genomic DNA extraction. The serum was isolated by centrifugation and the biochemical parameters were measured by Dimension Xpand auto biochemistry analyzer.

Allele genotyping

Genotyping of ACE (I/D) gene

The ACE (I/D) genotypes were determined using the previously described PCR method (Uddin et al., 2007). The genomic DNA was extracted from peripheral leukocytes according to our previous method (Hosen et al., 2015). Then polymerase chain reaction (PCR) was performed to amplify the genomic DNA. PCR conditions and primer sequences were used according to the method of Uddin et al. (2007). PCR was carried out using Go Taq polymerase (Go Taq® Flexi, Promega Corporation, USA). Approximately 0.5 µg of genomic DNA was added to a PCR mix composed of 2.5 units Taq polymerase, 200 µmol dNTPs, 50 pmol of each primer, and PCR buffer composed of 50 mol/mL KCl, 10 mol/mL Tris-HCl (pH 8.3), and 2.5 mol/mL MgCl2 in a volume of 50 µL. The PCR products were separated by electrophoresis and visualized under UV light after ethidium bromide staining. Presence of 490bp and 190bp fragment demonstrates insertion (I) and deletion (D) variants respectively while presence of both bands indicates I/D variants of ACE gene (Fig. 1).

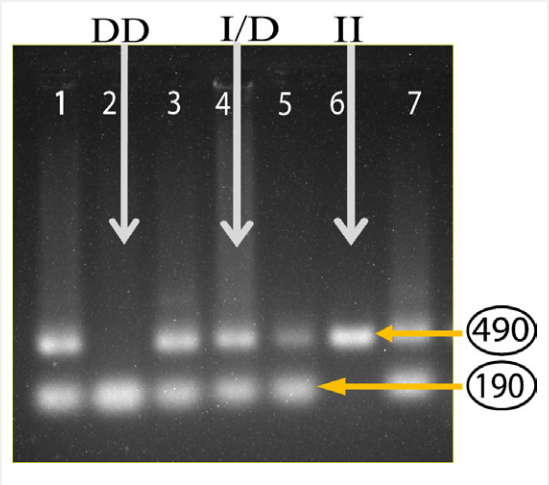

Fig. 1. Polymorphic variants of ACE (I/D) alleles. Both 490 bp and 190 bp fragments at well 1, 3, 4, 5 and 7 demonstrate insertion/deletion (ID) variants; 190 bp fragment at well 2 indicates DD variants and 490 bp fragment at well 6 depicts II variants of ACE gene.

Genotyping of NOS3 (G894T & 4a/b) gene

The genomic DNA amplification for NOS3 G894T and 4a/b genotyping were done by PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) and PCR methods respectively, using our newly designed primers and PCR was carried out similar ways as described for ACE genotyping (Hosen et al., 2015). The forward and reverse primers for G894T genotyping were 5'-TCCCTGAGGAGGGCATGAGGCT-3' and 5'-TGAGGGTCACACAGGTTCCT-3' respectively and the forward and reverse primers for 4a/b genotyping were 5'-AGGCCCTATGGTAGTGCCTT-3' and 5'-TCTCTTAGTGCTGTGGTCAC-3' respectively. About 5.0 μL PCR product (457bp) of G894T genotype was digested with Msp I restriction enzyme (Fig. 2a). The presence of 320bp and 137bp fragments demonstrate GG genotype, presence of 457bp, 320bp and 137bp bands indicate GT genotypes while only one band of 457bp depicts TT genotype (Fig. 2a). For 4a/b genotyping, the PCR product of 393bp indicates "a" genotype and the insertion of 27bp VNTR (420bp) indicates "b" genotype and presence of both 393bp and 420bp fragments demonstrate a/b genotypes (Fig. 2b).

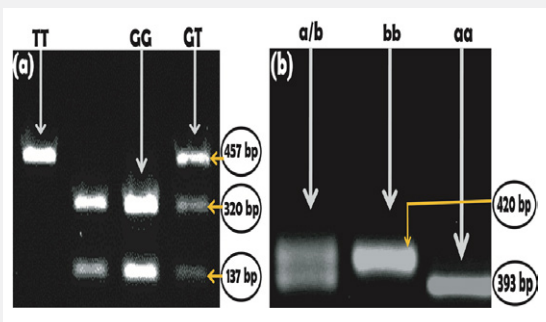

Fig. 2. Polymorphic variants of NOS3 (G894T & 4a/b) alleles. (a) After Msp I digestion, 320 bp and 137 bp fragments demonstrate GG alleles, presence of 457bp, 320 bp and 137 bp bands indicate GT alleles while only one band of 457 bp depicts TT alleles. (b) Presence of both 420 bp and 393 bp fragments indicates a/b variants whereas only 393 bp band demonstrates aa variants and only 420 bp band bb variants.

Statistical analysis

Statistical Package for Social Science (SPSS), windows version 17.0 and GraphPad Prism software were used to perform statistical analyses. The relative association between cases and controls were assessed by calculating the odds ratio (OR). ORs, as a measure of relative risk, at 95% confidence intervals (95% CI) were estimated using logistic regression models. Data were considered as statistically significant according to P values < 0.05. One way ANOVA test was used to estimate the levels of biochemical parameters for different genotypes.

Table 1. Baseline characteristics and clinical parameters.

	Healthy Control (n=150)	CAD Patients (n=100)	p value
Age	53 ± 17	55 ± 12	ns
Gender (n, %)			
Male	113 (75)	76 (76)	ns
Female	37 (25)	24 (24)	
Smoking Status			
Non-Smoker	60 (40)	41 (41)	ns
Smoker	90 (60)	59 (59)	
Family history			
Parents Affected		28 (28)	
Siblings Affected		23 (23)	
No comments		22 (22)	
No History		27 (27)	
Biochemical parameters			
Troponin I (ng/mL)	0.028 ± 0.002	22.3 ± 4.3	<0.01
Total Protein (g/dL)	6.95 ± 0.2	6.89 ± 0.15	ns
ALT (IU/L)	34.4 ± 3.2	27.6 ± 2.2	ns
AST (IU/L)	29.1 ± 2.2	55.2 ± 4.8	<0.001
ALP (IU/L)	74.5 ± 3.8	92.3 ± 3.1	<0.001
ALB (g/dL)	4.11 ± 0.1	3.3 ± 0.1	<0.001

Results are expressed as Mean ± SEM and as number (percentage). Student t-tests were performed to estimate the level of significances. p<0.05 was taken as level of significance. ns; Non-significant. ALT; Alanine aminotransferase, AST; Aspartate aminotransferase, ALP; Alkaline phosphatase, ALB; Albumin.

3. Results

In this study, we examined the association of ACE (I/D) and NOS3 (G894T and 4a/b) genes polymorphisms with the susceptibility of MI in Bangladeshi population. The genotypic distribution (p<0.01) and the allelic frequency (p<0.001) of ACE (I/D) genotypes among the study subjects were significantly different (Table 2). In contrast, the genotype proportions of different NOS3 (G894T and 4a/b) genotypes in both groups were not significantly different (Table 2). The percentages of homozygous insertion, homozygous deletion and heterozygous insertion/deletion alleles were 36.7%, 42.5% and 20.8% respectively in control subjects and 22%, 37% and 41% respectively in MI patients. On the other hand, the percentages of different variants of NOS3 gene were almost similar among the study subjects.

Table 2. Genotypic frequencies of ACE and NOS3 genes in study subjects.

Genes	Controls subjects	CAD Patients (n=100)	p value
ACE			
Genotype (I/D) frequency			
II	**55 (36.7)**	22 (22)	
ID	63 (42.5)	37 (37)	<0.01
DD	32 (20.8)	41 (41)	
NOS3			
Genotype (G894T) frequency			
GG	67 (44.7)	47 (47)	
GT	81 (54)	51 (51)	>0.05
TT	02 (1.3)	02 (02)	
Genotype (VNTR Variants; 4a/b) frequency			
bb	101 (67.3)	69 (69)	
a/b	45 (30)	27 (27)	>0.05
aa	04 (2.7)	04 (04)	

Results are expressed as number (percentage). Chi-square test was performed. p<0.05 was taken as level of significance.

Genotypic analysis of ACE (I/D) and NOS3 (G894T & 4a/b) genotypes

The risk of myocardial infarction associated with the ACE (I/D) and NOS3 (G894T & 4a/b) genotypes were estimated and presented in Table 3. In consideration of ACE (I/D) genotypes, there were four genotyping groups while the subjects with II genotypes was considered the reference group. Individual with DD genotypes was in high risk of experiencing myocardial infarction when compared (OR, 3.28; 95% CI, 1.60-6.70; p<0.01) to the control. In addition, patients having either DD or ID genotypes showed higher risk for MI compared to control group (OR=2.05; 95% CI=1.20-3.70; p< 0.05). On the other hand, association of ID genotypes with MI was not statistically significant (OR, 1.50; 95% CI, 0.75-2.80, p>0.05).

Table 3. Odds ratios of developing MI for ACE and NOS3 genotypes.

Gene	Control subjects (n=150)	CAD Patients (n=100)	P value	Odd ratio (95% CI)
ACE				
Genotypes (Insertion/Deletion)				
II	55	22	-	1 (Ref.)
ID	63	37	ns	1.5 (0.75 to 2.8)
DD	32	41	<0.01	3.28 (1.6 to 6.7)
ID+DD	95	78	<0.05	2.05 (1.2 to 3.7)
NOS3				
Genotypes (G894T)				
GG	67	47	-	1 (Ref.)
GT	81	51	ns	0.92 (.5 to 1.5)
TT	02	02	ns	1.4 (0.2 to 10.5)
Genotypes (VNTR Variants; 4a/b)				
bb	101	69	-	1 (Ref.)
a/b	45	27	ns	0.88 (.5 to 1.5)
aa	04	04	ns	1.5 (0.35 to 6.1)

Odds ratios (OR) and 95% confidence interval (95% CI), OR adjusted for ages and gender. Fisher's exact test was performed. p<0.05 was taken as level of significance.

The NOS3 G894T and 4a/b alleles are considered to be rare alleles and their relationship with study variables was analyzed and presented in Table 3. The GG and TT variants at 894 position of NOS3 did not exert any relation (OR, 1.40; 95% CI, 0.20-10.50, p >0.05) with myocardial infarction in Bangladeshi population. On the other hand, the results showed weak association (OR, 1.50; 95% CI, 0.35-6.10, p >0.05) between the 4a allele and the risk of myocardial infarction compared with the b allele.

The risk of MI with the combination of ACE (I/D) gene and smoking status

The risk associated with the combination of ACE (I/D) genotypes and smoking status was estimated (Table 4). There were six combined groups while the non-smoker group with II genotypes was considered the reference group. The risk of having myocardial infarction was low for both smokers and non-smokers

with ID genotypes (OR, 1.1; 95% CI, 0.40-3.0, p>0.05; OR, 2.3; 95% CI, 1.0-8.50, p>0.05; respectively) and smokers with II genotypes and non-smoker with DD genotypes (OR, 1.1; 95% CI, 0.5-3.1, p>0.05; OR, 2.3; 95% CI, 0.7-7.2, p>0.05, respectively). On the other hand, the risk of occurrence of myocardial infarction was significantly higher among smokers (OR, 4.1; 95 % CI, 1.5-11.2, p<0.01) with DD genotypes.

Table 4. Risk of MI with the combination of ACE (I/D) gene and smoking status.

Genotypes	Non-Smoker	Smoker
II	**1 (19/09)**	1.1, 0.4 to 3.1 (25/13)[a]
ID	**2.3, 1.0 to 8.5 (18/20)[a]**	**1.1; 0.4 to 3.0 (32/17)[a]**
DD	2.3, 0.7 to 7.2 (11/12)[a]	4.1; 1.5 to 11.2 (15/29)*

Odds ratios (OR) and 95 % confidence interval (95 % CI), OR adjusted for ages and gender. Fisher's exact test was performed. p<0.05 was taken as level of significance. aNon-significant; *p<0.01.

Biochemical parameters and different genotypes (I/D, and G894T & 4a/b)

The Table 1 represents the significance levels of biochemical parameters. The serum Troponin I, AST and ALP levels were significantly (p<0.01, 0.001 and 0.001, respectively) higher among the patients compared to the control subjects whereas the albumin level was significantly (p<0.001) lower (Table 1). On the other hand, total protein and ALT levels were not significantly different. One way ANOVA tests were performed to estimate the levels of biochemical parameters for different genotypes and there were no significant differences exist.

4. Discussion

Myocardial infarction (MI) is a devastating disorder that evolved from the interaction of several genetic and environmental factors. These factors differ in various ethnic groups. Though numerous investigation have been performed to find the relation of environmental and genetic factors with MI, the underpinning mechanism of these disorders is still elusive. There are several candidate genes have been documented to be associated with MI worldwide (Cicoira et al., 2001; Cine et al., 2002; Oniki et al., 2008). Sequence variants of the components of the RAS system and the nitric oxide synthase are suggested to have significant influences on cardiovascular homeostasis. The polymorphisms of ACE and NOS3 genes have been investigated and reported to be involved with coronary artery disease and myocardial infarction in several studies (Cambien et al., 1992 ; Wang et al., 1996). In our present study, we have investigated the relationship between the most common polymorphisms of ACE (I/D) and NOS3 (G894T and 4a/b) genes and MI in Bangladesh.

In this study, the ACE (I/D) genotypes and allele frequencies were significantly different between two groups and D allele was more frequent in patients with MI than normal subjects. The risk of occurring MI was 3.28-fold (OR, 3.28; 95% CI, 1.60-6.70; p<0.01) in patients with DD allele while the patients with D allele (ID+DD), was 2.05 times more prone (OR = 2.05; 95% CI = 1.20-3.70; p < 0.05) to experience MI. Moreover, we found strong association of cigarette smoking with MI, where smokers with DD genotypes were 3.91-fold (OR, 3.91; 95% CI, 1.33-11.5, p<0.05) likely to develop cardiovascular diseases.

An association between the polymorphism in the ACE gene and the risk of MI was first reported by Cambien et al. (1992). Some follow-up studies have also shown a significant association between the ACE DD genotype and an increased risk of MI (Rigat et al., 1990; Seckin et al., 2006). A meta-analysis carried out by Samani et al. on 3,394 MI cases and 5,047 controls also showed a high frequency of DD genotypes in MI patients (Samani et al., 1996). Studies from different part of India have revealed that the ACE DD genotype is a risk factor for coronary artery disease and hypertension, which is also a potent risk factor for MI (Bhavani et al., 2004; Dalal et al., 2006). In addition, Firouzabadi et al. in Iran, and Uemura et al. in Japan have also shown association between DD variants of ACE gene and MI (Uemura et al., 2000; Firouzabadi et al., 2012).

However, some studies have not been able to establish an association between ACE gene polymorphism and MI (Keavney et al., 2000; Abdelhedi et al., 2013). A study conducted by Lindpaintner et al., showed no association between the ACE I/D polymorphism and the risk of CAD (Lindpaintner et al., 1995). In addition, Pandey et al. in India and Basol et al. in Turkey also reported lack of association between ACE I/D polymorphism and MI (Pandey et al., 2011; Basol et al., 2014). Therefore ethnic differences may be considered as contributing factor for these discrepancies.

Though several studies across the world have shown association between NOS3 (G894T and 4a/b) gene polymorphism and MI, we found no significant association in Bangladeshi population. Our findings are in agreement with two different studies conducted in South Indian population (Syed et al., 2010; Narne et al., 2013). Study conducted in Italian population also reported lack of association between NOS3 (G894T and 4a/b) genes and CAD (Colomba et al., 2008). In addition, Karvonen et al. also stated that the G894T variant of the NOS3 gene was not a major risk factor for cardiovascular alterations (Karvonen et al., 2002).

In contrast, several studies in different countries supported the hypothesis about the association between NOS3 (G894T and 4a/b) polymorphism and MI. Abolhalaj et al. (2013) found significant association between NOS3 4a/b gene polymorphism and CAD patients in Iran. Li et al. study also suggested that

NOS3 polymorphism is one of the contributing factors for the predisposition of hypertension in the Han population in southwestern China (Li et al., 2011). Moreover, Colombo et al. also reported the evidence of association between NOS3 (G894T) polymorphism MI (Colombo et al., 2003).

In this study, we have found increased levels of Troponin I, AST and ALP as well as decreased level of Albumin among the patients while we could not found any association between ALT and MI. A population based cohort study in the United State also reported no association between MI and ALT, while several investigations showed that ALT and AST are associated with myocardial infarction (Ruhl et al., 2009; Moon et al., 2014; Gao et al., 2017). A recent meta-analysis study conducted on over 9.24 million participants and reported contradictory results (Kunutsor et al., 2014).

However, our study was conducted on small number of samples which was one of the main limitations that necessitate careful interpretation of results. Studies on larger population would certainly be more conclusive. In conclusion, we found the association between ACE (I/D) gene polymorphism and MI in Bangladeshi population. The D allele was significantly higher in MI patients compared to control subjects. Thus, DD genotype would be considered as a risk factor and II genotype would be a molecular marker of reducing CAD. On the other hand, the G894T and 4a/b polymorphic genotypes of the NOS3 gene were not found to be a risk factor for MI in Bangladeshi population. Therefore, genotyping of ACE (I/D) gene would be a biomarker of early diagnosis of CAD and also be helpful to intervene personalized medicine as a novel treatment of CAD.

Conflict of interest

The authors reported that there is no conflict of interest.

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Comparison of the protective effects of betamethasone, dexamethasone and methylprednisolone in ischemia/reperfusion injury of rat ovary

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ABSTRACT

Our aim is to evaluate the protective effect of dexamethasone, methylprednisolone and betamethasone treatment against ischemia-reperfusion damage created experimentally in rat ovaries. For this study, 30 female Wistar albino rats were used and the rats were separated randomly into five groups consisting of six rats each: Normal ovary, torsion-detorsion, torsion-detorsion + betamethasone 3 mg/kg, torsion-detorsion + dexamethasone 4 mg/kg and torsion-detorsion + methylprednisolone 10 mg/kg. Except for the normal group, an ovarian torsion procedure was implemented in all other groups for three hours. Then, a detorsion procedure was implemented to the groups for three hours. Medications were given intraperitoneally, 30 minutes before the detorsion procedure. Ovaries of all rats were removed and anti-mullerian hormone (AMH) levels were examined. The methylprednisolone treatment seems to be protective for the damage in terms of vascular congestion (p=0.238), inflammation (p=0.575), edema (p=0.118) and cellular degeneration (p=0.523) by preventing the meaningful increase. The dexamethasone and betamethasone treatment seems to be protective for tissue damage in inflammation (p=0.575, 0.299), cellular degeneration (p=0.575, 0.368) and edema (p=0.212, 0.162). For all steroid groups, preantral+antral follicle decrease and atretic follicle increase were prevented. AMH decline was prevented and levels were similar to normal group (methylprednisolone, betamethasone and dexamethasone p values, respectively; 0.872, 0.064, 0.335). In ischemia / reperfusion injury due to ovarian torsion, steroid use reduces damage and protects ovarian reserves. There was no significant difference between dexamethasone, betamethasone and methylprednisolone in terms of success.

1. Introduction

Ovarian torsion refers to a complete or partial rotation of the infindibulopelvic or uteroovarian ligament, resulting in ischemic changes in the ovary. Ovarian

torsion accounts for 2.7% of all the gynecologic emergencies (Aslan et al., 2017). Torsion could occur in all ages, but it is more common in reproductive ages, especially early 20s and mid-30s (Pinar et al., 2017). Early diagnosis and treatment is essential for protecting ovarian injury and fertility (Huang et al., 2017). In case of delayed diagnosis and intervention, it may cause deterioration or loss of fertility (Sintim-Damoa et al., 2017). Ovarian damage can decrease the reserves, cause infertility and also early menopause risk increases in later periods (Oktem and Oktay, 2007). Ovarian reserves can be assessed by serum markers or follicle counts (Yeral et al., 2019).

The process of ovarian torsion / detorsion is called ischemia / reperfusion (I/R) damage (Behroozi-Lak et al., 2017). I/R injury cause inflammation, including migration of leukocytes and macrophages, and production of proinflammatory cytokines. I/R induced hypoxia damages the vascular endothelial cells, which may consequently lead to leukocyte-endothelial cell adhesion and neutrophil migration through the endothelial barrier results (Auphan et al., 1995; Luscinskas et al., 2002; Uchiyama et al., 2016). Glucocorticosteroids primarily acts by binding to the steroid receptor and regulating the activity, promoter sequences and gene expression as a nuclear transcription factor. Inducible nitric oxide synthase inhibits the transcription of selectins and adhesion molecules and neutralize transcription factors activator protein-1 and nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) (De Bosscher and Haegeman, 2009; Wystrychowski et al., 2018). Corticosteroids are potent anti-inflammatory and immunosuppressive agents. They inhibit the synthesis of almost all known cytokines and cell surface molecules (Uchiyama et al., 2016).

Recently, the beneficial effects of steroids on I/R injury had been shown in many different tissues. Corticosteroids had been shown to be effective in the prevention of renal tissue, brain tissue, also of myocardial tissue after cardiopulmonary bypass and of liver tissue during the hepatic ischemic events (Wang et al., 2001; Glanemann et al., 2004; Saidi et al., 2007; Wang et al., 2008; Subhas et al., 2010; Uchiyama et al., 2016). In a study, oxidative stress markers in I/R injury were investigated. High-dose methylprednisolone was found to improve the histopathological damage. They stated that the effect of I/R injury on rats was effective but there is need for future studies for the clarification of the protective mechanisms (Osmanagaoglu et al., 2012).

To the best of our knowledge, in the literature, there is not any research investigating the protective effects of steroids on ovary follicles against ischemia-reperfusion damage and the effects on the level of AMH. Our aim in this study is to evaluate the benefits of steroids on I/R injury and to compare the effects of the agents.

2. Materials and methods

This study was conducted at the Animal Testing Laboratory of Marmara University after the approval of the Ethics Committee (dated on November 5, 2018; protocol No. 101.2018.mar).

Laboratory animals and the care of animals in research

Female Wistar albino (Rattus Norvegicus spp.) rats, 12-weeks-old, were used and the rats weighed 200 to 250 grams. The rats were exposed to light for 12 hours per day (08:00 to 20:00), access to food and drinking water without restriction (standard rodent pellet, tap water), and held in rooms with a humidity of between 40 - 50% with room temperature of 21 to 23° C, and 4 or 5 per cage. The number of rats was selected based on previous studies (Celik et al., 2014;Tokgoz et al., 2018; Yildirim et al., 2018). The rats were randomly divided into five groups with 6 mice per group. The rats were not fed for 6 hours prior to laparotomy to empty the intestines and ease the surgery, but they had access to drinking water.

Groups

For this study, 30 female Wistar albino rats were used, and the rats were separated randomly into five groups consisting of six rats each: Normal, torsion-detorsion, torsion-detorsion + betamethasone, torsion-detorsion + dexamethasone and torsion-detorsion + methylprednisolone.

Group 1 (normal ovary group): This group of rats underwent laparotomy once. During the laparotomy, one of the ovaries was removed and fixed in 10% formaldehyde. And at least 1 ml of blood sample was taken for AMH test.

Group 2 (torsion ovary group): Laparotomy was performed and one of the ovaries was twisted 720 degrees and untwisted 3 hours later and the surgical wound was closed without administering any medicine. A second surgery was performed 3 hours later and both ovaries were removed. And at least 1 ml of blood sample was taken for AMH test.

Group 3 (betamethasone group): At the first laparotomy one of the ovaries was twisted 720 degrees. Betamethasone 3 mg kg (Celestone® ampule, Schering Plough Tibbi Urunler Ticaret A.S., Istanbul, Turkey) was administered intraperitoneally 30 minutes before detorsion. At the second laparotomy, the ovaries were detorsioned and reperfusion was maintained for 3 hours. At the third laparotomy rats were sacrificed and at least 1 ml of blood was taken for AMH testing and both ovaries were removed by laparotomy.

Group 4 (dexamethasone group): At the first laparotomy one of the ovaries was twisted 720 degrees. Dexamethasone 4 mg/kg (Dekort® ampule, DEVA Holding, Istanbul, Turkey) was administered intraperitoneally 30 minutes before detorsion. At the

second laparotomy, the ovaries were detorsioned and reperfusion was maintained for 3 hours. At the third laparotomy rats were sacrificed and at least 1 ml of blood was taken for AMH testing and both ovaries were removed by laparotomy.

Group 5 (methylprednisolone group): At the first laparotomy one of the ovaries was twisted 720 degrees. Methylprednisolone 10 mg/kg (Prednol® ampule, Mustafa Nevzat, Istanbul, Turkey) was administered intraperitoneally 30 minutes before detorsion. At the second laparotomy, the ovaries were detorsioned and reperfusion was maintained for 3 hours. At the third laparotomy rats were sacrificed and at least 1 ml of blood was taken for AMH testing and both ovaries were removed by laparotomy.

Surgical procedures

Sterile, powder-free latex gloves were preferred for surgery. For laparotomy anesthesia, a dose of 10% ketamine hydrochloride at 80 mg/kg per rat and 2% hydrochloride xylazine (Rompun, Bayer Health Care LCC, Kansas City, KS) at a dose of 15 mg / kg (Ketalar; ECZACIBAŞI) Warner Lambert, Istanbul, Turkey) were used. The procedure was performed while rats were lying in a supine position. 10% povidone-iodine solution (Batticon; Adeka Laboratories, Istanbul, Turkey) was used for shaving before the procedure. To enter the abdominal cavity, a median (approximately 5 cm, on the line between the xiphoid process and pubis) incision was applied, and the right ovary was twisted 720 degrees along with tubo-ovarian blood vessels (Fig. 1). To fix the ovary to the abdominal muscles, 5/0 silk sutures were used and the abdominal wall (peritoneum, fascia and skin) was closed in two layers using running locking sutures with 2/0 polyglactin 910, following bleeding control. Each surgical procedure lasted 15 to 20 minutes to protect the drying effect of the room air and the rats were allowed to wake up.

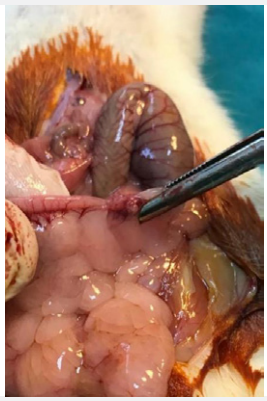

Fig. 1. Torsion of ovaries.

Histopathological examinations

Surgically excised ovaries were fixed in 10% formalin. Paraffin blocks were prepared 24 hours after the oophorectomy procedure. Tissue sections of 5 micrometers were taken and follicular activity was assessed in 5 randomly selected samples from each ovary. Slides were stained with hematoxylin eosin and examined under the light microscope. The paraffin blocks were sectioned using a microtome blade (Leica, Nussloch, Germany). Every slide was blindly assessed by the same pathologist. A light microscope (Olympus Clinical Microscope, Tokyo, Japan) was used to analyze the sections.

Edema, vascular congestion, inflammation, cellular degeneration and hemorrhage were examined as histopathological injury scores (Fig. 2). The scores were evaluated as described by Celik et al. (Celik et al., 2014). Pathological findings were rated. Grade 0 indicated normal alterations, no abnormal findings; Grade 1 indicated mild edema, mild vascular congestion, absence of hemorrhage or leukocyte infiltration; Grade 2 indicated moderate edema, moderate vascular congestion, absence of hemorrhage or leukocyte infiltration; Grade 3 indicated severe edema, severe vascular occlusion, minimal hemorrhage and minimal leukocyte infiltration, Grade 4 indicated severe edema, severe vascular occlusion, hemorrhage and leukocyte infiltration.

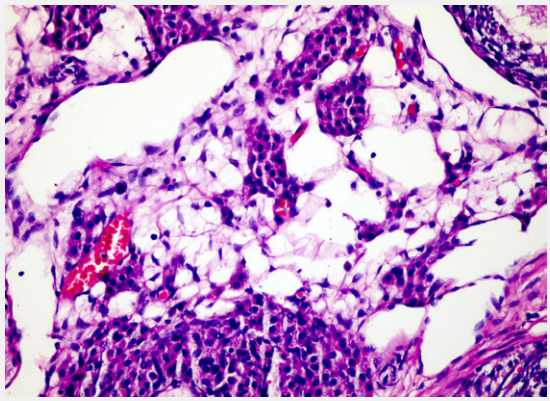

Fig. 2. Moderate edema x400 hematoxylin eosin.

All follicles were counted to assess ovarian reserve. Primordial, primary, secondary (pre-antral) and tertiary (antral) follicles were counted. Follicles were evaluated as described by Parlakgumus et al. (Parlakgumus et al., 2014). Primordial, primary, secondary (pre-antral) and tertiary (antral) follicles were counted. Primordial follicle is described as an oocyte with surrounded only one layer of epithelial cell layer, primer follicle is surrounded with one or more layer of cuboidal granulosa cells. Secondary/pre-antral follicle is surrounded with more than two cell layers and consists of antrum folliculi and zona pellucida. Tertiary follicle is defined as possessing antrum, stratum granulosum and surrounding cumulus oophorus layers (Fig. 3, 4).

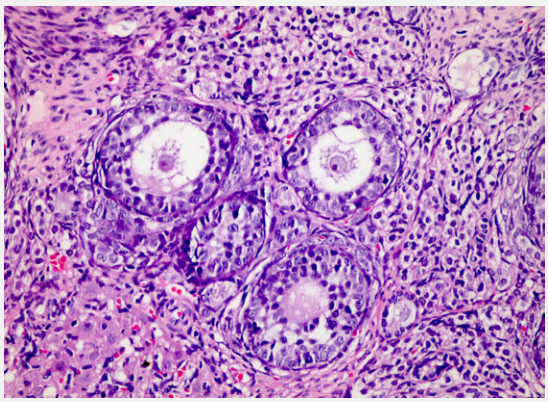

Fig. 3. Preantral follicle x400 hematoxylin eosin.

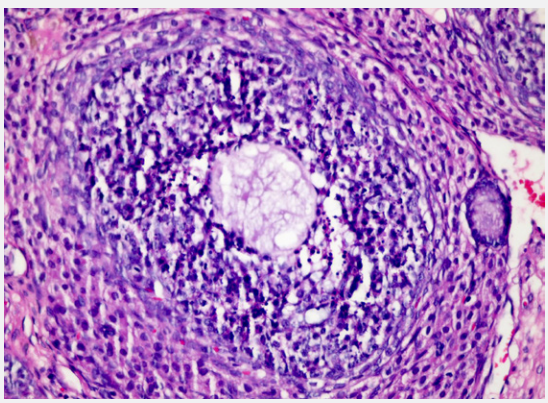

Fig. 4. Degenerated follicle x400 hematoxylin eosin.

AMH assays

Blood samples were collected into tubes containing lithium heparin (BD Vacutainer Plasma tubes®, Manchester, England). The concentration of the Lithium Heparin additive in these tubes is 17 international units of heparin/ml of blood. Blood samples were centrifuged within 30 minutes of sampling. After 15 minutes of centrifugation at 1000xg, serum was removed and remaining plasma was transferred into an eppendorf tube and stored frozen at -20°C until the time of analysis AMH concentrations were measured in "ng/ml" plasma using ELISA method. The rat AMH kit used in study had a sensitivity of 0.10 g/mL, a detection range of 0.16 to 10 ng/mL and a coefficient of variation less than 10% (Elabscience®, Rat AMH kit; Houston, Texas, ABD). The laboratory technician of the laboratory of the university hospital was blinded to the study groups and unaware of which samples belonged to which rat. All samples were analyzed in the same assay.

Statistical analysis

Statistical analyses were performed using the SSPS Version 15.0. The Kolmogorov-Smirnov test and histograms were used to assess the normality of the distribution of variables. The mean ± standard deviation or median [interquartile range] were used to present descriptive analyses. One-way ANOVA test was used to analyze normally distributed numerical data and the Kruskal-Wallis test was used to analyze non-normally distributed numerical data. The limit of statistical significance was set at p-values <0.05.

3. Results

Histopathological damage scores

According to the histopathological damage scores, minimum damage was seen in the normal group and maximum damage was seen in the torsion-detorsion group. There was an increase in histopathological damage scores (edema, cellular degeneration, hemorrhage) in the torsion group which was not given any drug (p scores, respectively; 0.011, 0.011, 0.003). The methylprednisolone treatment seems to be protective for the damage in terms of vascular congestion (p=0.238), inflammation (p=0.575), edema (p=0.118) and cellular degeneration (p= 0.523) by preventing the meaningful increase (Table 1). Its protection was not sufficient only in case of hemorrhage. Four of the five parameters were found to be successful (4/5). Dexamethasone and betamethasone treatment seems to be protective for tissue damage in inflammation (p=0.575, 0.299), cellular degeneration (p=0.575, 0.368) and edema (p=0.212, 0.162). Three of the five parameters were found to be successful (3/5). Dexamethasone and betamethasone treatment could not provide successful protection in vascular congestion

Table 1. Comparison of normal ovarian tissue and histopathological damage scores of torsion and torsion + steroid treated groups.

	Steroids	Normal	Torsion	P****	Detortion+ Dexamethasone	P*	Detortion+ Betamethasone	P**	Detortion+ Methylprednisolone	P***
Edema	Mean SD	1.33±0.52	2.50±0.55	**0.011**	1.83±0.75	**0.212**	2.00±.89	**0.162**	0.67±0.82	**0.118**
	Median- IQR	1.00 (1.00-2.00)	2.50 (2.00-3.00)		2.0 (1.0-2.0)		2.0 (1.0-3.0)		0.5 (0.0-1.0)	
Vascular congestion	Mean SD	0.83±0.98	1.33±1.03	**0.403**	2.17±0.75	**0.036**	3.00±0.00	**0.002**	1.50±0.84	**0.238**
	Median- IQR	0.50 (0.00-2.00)	1.00 (1.00-2.00)		2.0 (2.0-3.0)		3.0 (3.0-3.0)		1.0 (1.0-2.0)	
Inflammation	Mean SD	0.50±0.55	1.00±0.00	**0.056**	0.33±0.52	**0.575**	1.00±0.89	**0.299**	0.33±0.52	**0.575**
	Median- IQR	0.50 (0.00-1.00)	1.00 (1.00-1.00)		0.0 (0.0-1.0)		1.0 (0.0-2.0)		0.0 (0.0-1.0)	
Cellular degeneration	Mean SD	0.33±0.52	1.83±0.98	**0.011**	0.50±0.55	**0.575**	1.00±1.26	**0.368**	0.17±0.41	**0.523**
	Median- IQR	0.00 (0.00-1.00)	1.50 (1.00-3.00)		0.5 (0.0-1.0)		0.5 (0.0-2.0)		0.0 (0.0-0.0)	
Hemorrhage	Mean SD	0.33±0.52	2.50±0.55	**0.003**	2.00±1.26	**0.028**	2.83±0.41	**0.002**	1.67±1.21	**0.042**
	Median- IQR	0.00 (0.00-1.00)	2.50 (2.00-3.00)		2.5 (1.0-3.0)		3.0 (3.0-3.0)		1.5 (1.0-3.0)	

*p **p ***p ****p Mann Whitney U Test

and hemorrhage. Methylprednisolone was the only effective agent in vascular congestion. It is noteworthy that no steroid can provide successful protection in hemorrhage.

Ovarian follicle counts

Primordial and primer follicle counts were statistically different between normal and the torsion group (p=0.010). Primordial follicles were significantly decreased in all steroid groups, (dexamethasone p= 0.004, betamethasone p=0.004, methylprednisolone p=0.004) highest decrease in median and median values was in the group 5. Primary follicles were also found to be significantly decreased in all steroid groups (dexamethasone p=0.006, betamethasone p= 0.010, methylprednisolone p=0.003), highest decrease in mean and median values was also in the group 5. Secondary (preantral) follicles decreased significantly only in group 5 (p=0.033), it was observed that follicles were similar to group 1 in other steroid groups (Table 2). Tertiary (antral) follicles were found to be in similar rates with group 1 in all steroid groups. Atretic follicle counts were statistically different between normal and torsion groups (p=0.002). On the other hand, methylprednisolone (p=0.317), dexamethasone (p=0.056) and betamethasone (p=0.140) group did not statistically differ from the normal group. In the group 1, no atresic follicle was detected, whereas the most atresic follicle was in the group 2. It was determined that atresic follicle increase was prevented in all steroid groups and was similar to normal group.

AMH levels

Besides AMH levels were statistically different between torsion and detorsion, whereas methylprednisolone, betamethasone and dexamethasone groups were similar to normal group (p values respectively 0.872, 0.064, 0.335) (Table 2). It was determined that decrease in AMH was also prevented in all steroid groups and was similar to normal group.

4. Discussion

In this experimental study, it is seen that ischemia / reperfusion injury was successfully achieved and caused a significant increase in histopathological damage scores in the torsion group, a significant decrease in primordial and primary follicles, an increase in atresic follicles and a decrease in AMH levels.

All of the steroids used to prevent this damage provided significant protection against edema, inflammation and cellular degeneration damage. While methylprednisolone was the only one that is successful in vascular congestion injury, no steroid agent was found to provide significant protection in hemorrhage injury.

I/R injury due to torsion of ovarian tissue can be detected by histopathological examination. Edema, vascular congestion, inflammation, cellular degeneration and hemorrhage may be seen in the damaged tissue. It is possible to come across numerous studies in the literature investigating these outcomes. In different studies, it has been determined that there are different degrees of damage at different damage scores. In some studies, I/R resulted in an increase in all damage scores in ovaries, while in some studies an increase in only one or several damage scores was observed (Celik et al., 2014; Parlakgumus et al., 2014; Aslan et al., 2017; Behroozi-Lak et al., 2017; Pinar et al., 2017; Tokgoz et al., 2018; Yildirim et al., 2018; Yeral et al., 2019).

The exact mechanisms of corticosteroids have not yet been elucidated. Inhibition of inflammatory cytokines, improvement of blood flow, modulation of immune/inflammatory cells, prevention of extracellular and intracellular Ca^2 + flow and cell degeneration have all been proposed (Kahraman et al., 2007). The anti-inflammatory effects of glucocorticoids are primarily via the glucocorticoid receptors. This effect may be related with the inhibition of the expression of phospholipase A2 and cyclo-oxygenase-2 that leads to reduction of inflammation-induced prostaglandin production or can be related with the direct effect on

Table 2. Comparison of normal rat AMH levels and ovarian follicle counts with torsion and torsion + steroid treated groups.

		Normal ovary	Torsion ovary	P*	Detortion+ Dexamethasone	P**	Detortion+ Betamethasone	P***	Detortion+ Methylprednisolone	P****
Primordial follicle count	Mean SD	14.83±3.54	5.50±3.99	**0.010**	4.2±3.5	**0.004**	4.2±2.6	**0.004**	3.0±1.4	**0.004**
	Median- IQR	15.50 (11.00-18.00)	4.50 (2.00-8.00)		3.0 (1.0-8.0)		4.00 (2.00-7.00)		3.0 (2.0-4.0)	
Primer follicle count	Mean SD	16.83±3.19	9.33±3.27	**0.010**	8.8±4.0	**0.006**	9.8±4.6	**0.010**	5.0±2.4	**0.003**
	Median- IQR	18.00 (13.00-18.00)	9.00 (6.00-12.00)		9.5 (5.0-12.0)		9.5 (8.0-12.0)		4.0 (3.0-8.0)	
Secondary (preantral) follicle count	Mean SD	7.83±3.92	9.17±2.93	**0.466**	5.8±3.7	**0,569**	5.2±2.6	**0.196**	3.5±1.6	**0.033**
	Median- IQR	7.50 (4.00-12.00)	8.50 (8.00-12,.00)		5.5 (4.0-7.0)		5.0 (3.0-7.0)		3.5 (3.0-4.0)	
Tersier (antral) follicle count	Mean SD	4.17±1.33	4.67±2.16	**0.739**	5.5±3.1	**0.616**	5.5±2.1	**0.211**	5.0±2.4	**0.513**
	Median- IQR	5.00 (3.00-5.00)	4.50 (3.00-6.00)		4.5 (3.0-7.0)		5.5 (4.0-6.0)		5.0 (3.0-7.0)	
Athresic follicle count	Mean SD	0.00±0.00	2.50±0.55	**0.002**	0.5±0.5	**0.056**	1.0±1.7	**0.140**	0.2±0.4	**0.317**
	Median- IQR	0.00 (0.00-0.00)	2.50 (2.00-3.00)		0.5 (0.0-1.0)		0.0 (0.0-2.0)		0.0 (0.0-0.0)	
AMH level (ng/ml)	Mean SD	2.64±0.95	0.84±0.25	**0.004**	1.95±0.57	**0.335**	1.82±0.95	**0.064**	2.77±0.98	**0.872**
	Median- IQR	2.59 (1.64-3.70)	0.92 (0.67-1.00)		1.85 (1.74-1.98)		1.39 (1.25-2.25)		2.98 (2.22-3.50)	

*p **p ***p ****p Mann Whitney U Test

vascular permeability and edema. Inhibition of lipid peroxidation was assumed to be the most protective effect of glucocorticoids, and methylprednisolone appears to be particularly effective when compared to other glucocorticoids (Osmanagaoglu et al., 2012).

In our study, methylprednisolone was successful in four of the five parameters (4/5) of the histopathological damage scores. Methylprednisolone was the only one that is successful in vascular congestion injury, no steroid agent was found to provide significant protection in hemorrhage injury. All of the steroids used to prevent the damages provided significant protection against edema, inflammation and cellular degeneration damage. Their direct effect on edema appears that it helped to prevent this damage in all steroid groups successfully.

Anti-mullerian hormone is produced by granulosa cells of preantral follicles and small antral follicles. AMH is used as a marker in the assessment of responsiveness of ovarian follicles. It is known as an indicator of the size of growing follicles pool. The level of AMH reaches the maximum in preantral follicles and small antral follicles. However, the production of AMH cannot be detected in follicles in response to FSH and disappears in atresia follicles (Yuan et al., 2014). In our study, we observed that there were significant decreases due to I/R damage in primordial and primary follicles and these decreases could not be prevented by steroid use. Decreases occurred in all steroid groups.

However, it was determined that preantral and antral follicles were significantly protected in all steroid groups (except methylprednisolone preantral follicle group) and follicular reduction was prevented. In addition, atretic follicle increase was also prevented in all steroid groups. As a result of all follicle examinations, it can be concluded that all steroid agents are effective in protecting follicle damage with the preservation of preantral + antral follicles and the prevention of atresic follicle increase. With the prevention of follicle damage, the decrease in AMH level was prevented in all groups and the AMH levels were found to be similar to the normal ovarian group.

In the literature, in different studies, it was found that AMH values decreased after 3 hours, 24 hours or 7 days of reperfusion following 3 or 6 hours of ovarian torsion. AMH decrease seems to be not primarily dependent with the torsion time and reperfusion time. If I/R damage occur, AMH value decreases. An effective therapeutic agent should be used to reduce this damage (Ozler et al., 2013; Kaya et al., 2014; Parlakgumus et al., 2014; Sahin Ersoy et al., 2016). Kaya et al. found significant difference in AMH values in all groups after 3 hours of reperfusion following 3 hours of ischemia (Kaya et al., 2014). They stated that in the control group there was no significant difference in preoperative and postoperative AMH values of detorsion and detorsion+enoxaparin groups, but AMH level decrease was less in the enoxaparin group. In another similar study, significant decrease in preantral + antral follicle count and AMH value was observed after 3 hours of ischemia followed by 3 hours of reperfusion. It has been stated that the decrease in AMH can be reduced by the use of N-acetyl cysteine and enoxaparin (Ersoy et al., 2016).

This is the first comprehensive study on the protective effects of steroids on ovarian I/R injury. All the agents were found to be effective in reducing I/R damage with similar efficacy and they did not show a significant difference in success.

In conclusion, in ischemia / reperfusion injury due to ovarian torsion, steroid use reduces damage and protects ovarian reserves. There was no significant difference between dexamethasone, betamethasone and methylprednisolone in terms of success.

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Reactivation of Epstein-Barr virus in aplastic anemia: A clinical challenge

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ABSTRACT

Acquired aplastic anemia is an unusual disease associated with pancytopenia characterized by hypocellular bone marrow. Aplastic anemia is an auto-immune disorder wherein patients would show a antithymocyte globulin (ATG)- induced hematological response after T-cell reduction. The combination of cyclosporine A and ATG as immunosuppressive therapy is considered as the standard treatment approach for patients with aplastic anemia. Epstein-Barr virus (EBV) infection in a patient with aplastic anemia is an unusual clinical presentation. A 49-year-old Asian female was presented to our hospital with dizziness and fatigue. The patient's platelet count was extremely low. A hypocellular marrow with lymphocytosis was observed with the help of a bone marrow aspirate and biopsy. The patient was given cyclosporine and eltrombopag as a bridge to primary therapy, i.e. antithymocyte globulin (ATG)/allogenic transplant considering she had pancytopenia. The patient developed platelet refractoriness. EBV polymerase chain reaction (PCR) was performed, considering the patient's atypical presentation. As per the results, it was significantly positive with 2250 copies/ul. A diagnosis of aplastic anemia with EBV infection was made. This is an unusual case of EBV in a patient with aplastic anemia. EBV infection can thwart the management of patients with AA.

1. Introduction

Acquired aplastic anemia (AA) is an uncommon disease associated with pancytopenia characterized by the hypocellular bone marrow. If left untreated, AA could be fatal for some patients. In most cases, patients with AA would show a typical response by antithymocyte globulins (ATG) induced T-cell reduction (Young et al., 2006). AA is considered as an immune-facilitated disorder.

The standard therapy for the treatment of young patients, specifically those with a suitable donor includes hematopoietic stem cell transplantation (HSCT). However, immunosuppressive therapy (IST) such as cyclosporine A (CsA) or ATG are considered as key treatment approaches in older patients or among those where HSCT is non-beneficial (Takahashi et al., 2015).

Epstein-Barr virus (EBV) is a type of γ-herpes virus

comprising of a linear DNA molecule of approximately 172 kb in length. EBV affects nearly 90% of the adult population worldwide. Infectious mononucleosis is often experienced by those exposed to the virus, specifically if the infection does not become clinically silent. EBV infection is lifelong. However, reactivation of EBV or a long latency can cause several lymphoproliferative lesions as well as hematologic malignancies (Stanfield, 2017). The scale of EBV-associated B-cell lymphoproliferative disorders (LPDs) is extensive, ranging from lymphomas to reactive lymphoproliferative lymphadenitis (Ok et al., 2015).

EBV associated diseases have been associated in people with immune deficiency. Patients who undergo allogeneic hematopoietic stem cell transplantation (HSCT) have a high risk of EBV reactivation including the development of EBV-related LPD (Van et al., 2011). Some of the key factors that influence the development of EBV-LPD in such patients include the use of T-cell depleted transplantation and ATG. EBV-LPD is an uncommon complication with rare cases becoming fatal. There is strong evidence citing that reactivation of EBV occurs in the majority of patients with severe AA who were treated with ATG (Scheinberg et al., 2007). Herein, we report an unusual case of EBV in a patient with aplastic anemia after treatment immunosuppressive therapy.

2. Case

A 49-year-old Indian female presented to our hospital with fatigue and dizziness. The patient also complained of dyspnea. However, the patient did not complain of of night sweats, fevers, weight loss, or chills. The physical examination was normal except pallor. The patient had no history of smoking tobacco use or any other substance abuse. A complete blood count was suggestive of pancytopenia with a hemoglobin of 4 grams per deciliter. The patient had a low total leucocyte count, 3800 per microliter (Neutrophil 12%, lymphocyte 80%, Monocyte 8%). The patient's platelet count was extremely low, i.e. 11.000 per microliter.

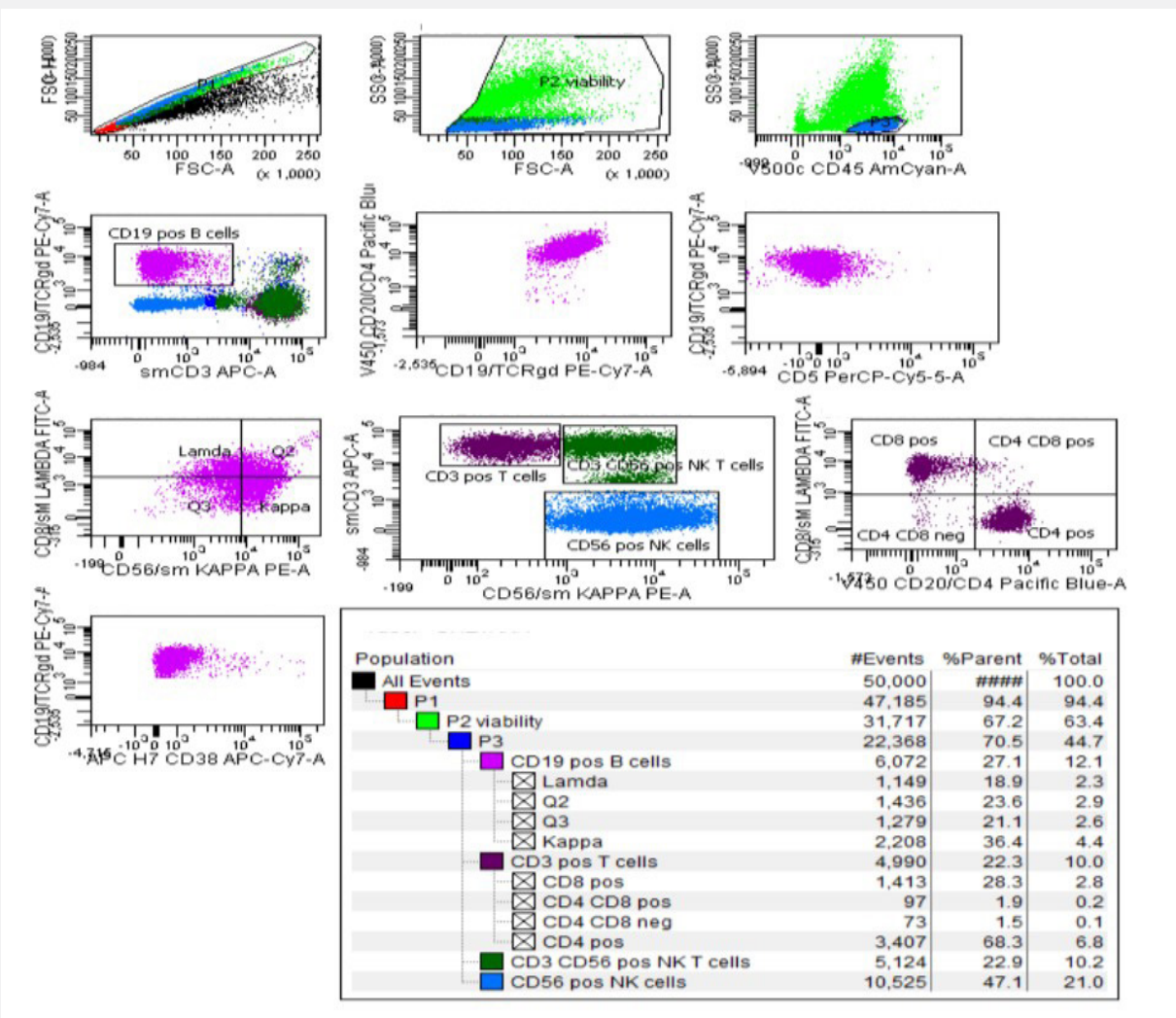

Fig. 1. Flow cytometry: 26% of the population comprised of NK cells (CD56+ve/sCD3).

A bone marrow aspirate and biopsy was performed that revealed a hypocellular marrow with relative lymphocytosis. Flow cytometry was performed to rule out malignancy. As per the results, 26% of the population comprised of monoclonal NK cells (CD56+ve/sCD3) (Fig. 1).

A bone marrow biopsy was performed that was hypocellular with normal immunohistochemistry. As the patient had severe pancytopenia, she was given cyclosporine and eltrombopag as a bridge to primary therapy, i.e. antithymocyte globulin (ATG)/allogenic transplant. Our differential diagnosis included aplastic anemia, viral infection-related pancytopenia, and lymphoproliferative disorders.

As the patient had lymphocytosis, a Positron Emission Tomography/Computed Tomography (PET-CT) was performed to look for any evidence of the lymphoproliferative disorder. PET-CT revealed activity in spleen without enlargement (Fig. 2). There was tiny cervical, inguinal, retroperitoneal and mesenteric lymphadenopathy. An increase in bone marrow activity was also observed. The patient's liver function tests (LFT) were deranged with indirect hyperbilirubinemia. The deranged LFT was attributed to eltrombopag. The patient eventually developed platelet refractoriness. The patient had poor platelet increments post single donor platelet (SDP) infusion. Unfortunately, we could not use the Human leucocyte antigen (HLA) matched platelets. As salvage, we used Intravenous immunoglobulin (IVIG) 400 mg/kg/day. After four days, her platelets improved significantly.

As per our multidisciplinary decision, we decided to commence therapy for aplastic therapy. As per the patient's atypical presentation we suspected and for EBV infection. Other vital viral tests such as cytomegalovirus infection (CMV) and parvovirus 19 were also considered. However, due to the patient's financial limitations, we relied on EBV tests only. EBV polymerase chain reaction (PCR) was performed and results were significantly positive with 2250 copies/ul.

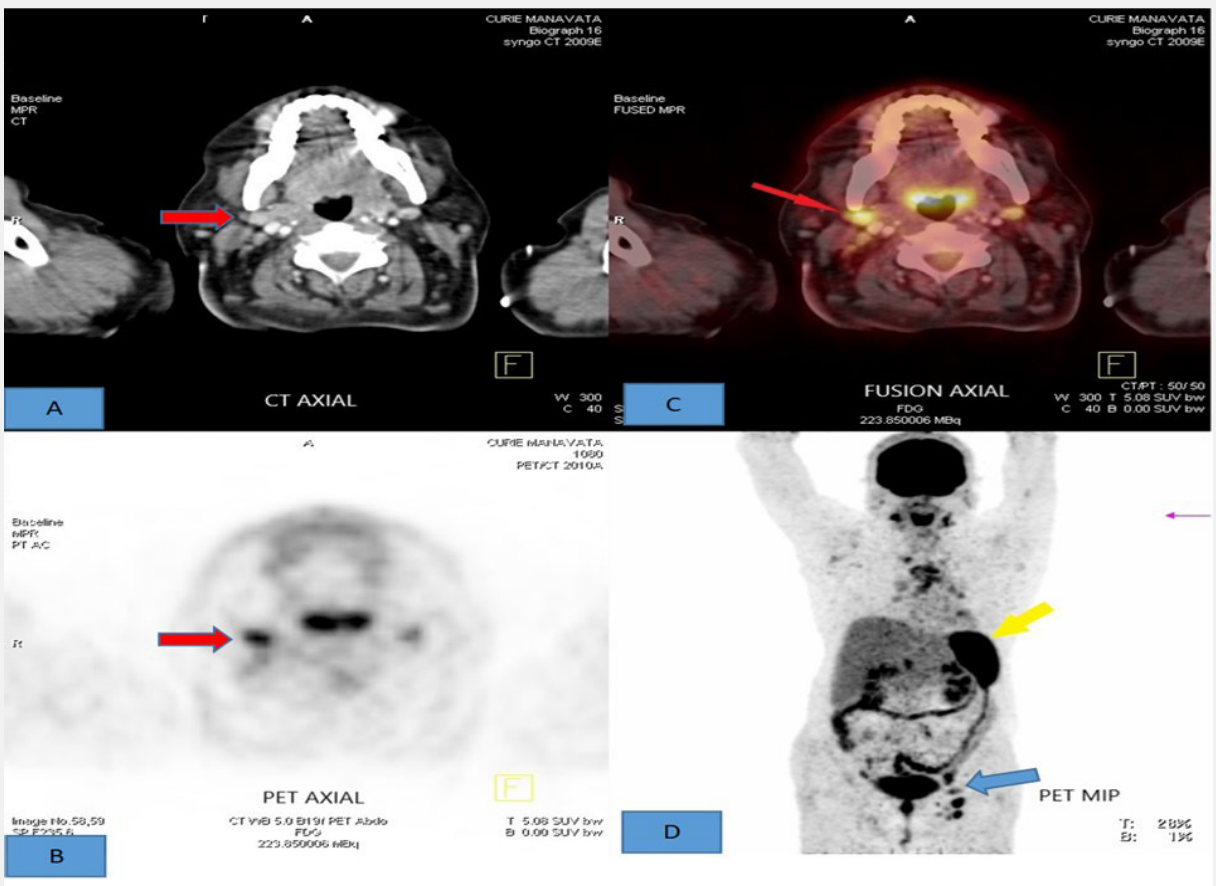

Fig. 2. Positron emission tomography.
A: CT Axial showing tiny cervical lymphadenopathy (red arrow).
B: PET Axial showing tiny cervical lymphadenopathy (red arrow).
C: Fusion Axial showing tiny cervical lymphadenopathy (red arrow).
D: PET WB-MIP revealed activity in spleen without enlargement (Yellow arrow).
It also shows Inguinal lymphadenopathy (Red arrow in D).

A diagnosis of aplastic anemia with EBV infection was made. The patient was on supportive care treatment along with a course of intravenous immunoglobulin (IVIG) and not received any antiviral treatment. After 4 weeks, EBV-PCR was repeated and titers were reduced to 50 copies/ul. Based on the reports, the patient was given successful definitive therapy in the form of ATG plus CsA. The patient has shown partial remission after 3 months' post-ATG and CsA treatment.

3. Discussion

Aplastic anemia is a bone marrow hematopoietic debacle due to several factors such as bone marrow fibrosis and bone marrow infiltration. Some of the most common clinical indicators of AA include infections, severe anemia, bleeding, and high risk of mortality. The pathogenesis of AA can be attributed to several underlying genetic factors. In the past few years, the activation of AA has been associated with environmental factors (viruses, drugs, antigens, or chemicals). Many scholars have cited the activation of lymphocytes and allied immune responses, specifically viral infections (Ihumura et al., 2010; Schenke, 2010; Khurana, 2014; Patel et al., 2017).

The EBV is a known human herpesvirus, discovered in 1964 by Epstein et al. in their study of malignant lymphoma in African children. EBV infections are common and span across many countries (Mashima et al., 2017). In the past few years, AA caused due to EBV infection has become a common observation. In a recent retrospective study, scholars have indicated that EBV plays a key role in the overall pathogenesis of AA (Zhang et al., 2018).

In patients with no pre-existing immunodeficiency, EBV infection is seldom complication by pancytopenia. The overall course of the disease is transient (Lazarus and Baehner, 1981; Purtilo et al., 1982; Anderlini et al., 1999). There have been a few cases of primary EBV infection in patients with AA (Shadduck et al., 1979; Ahronheim et al., 1983; Cabot et al., 1984; Grishaber et al., 1988). In all these patients, immunosuppressive treatment was the standard of care as in our case.

In our case, the patient had a hypoplastic bone marrow. She also presented with pancytopenia. Chromosomal analysis of the patient appeared to be normal. As per the bone marrow aspiration study, monoclonal lymphocytes were observed on flow cytometry. The patient had no hepatosplenomegaly, lymphadenopathy, or fever. However, reactivation of EBV occurred primarily before starting treatment. Severe immunosuppression was caused primarily due to CsA. It led to aggravated EBV-lymphoproliferation manifested in the form of severe thrombocytopenia not responding to the platelet transfusion. Flow cytometry had revealed NK cells. The EBV-driven NK-lymphocytosis may not necessarily be monoclonal. Post-IGV treatment, the EBV viral load had decreased significantly (50 copies/ul). In patients with aplastic anemia who undergo HSCT, EBV infection is likely to occur due to lymphoid or plasmacytic proliferations attributed to immunosuppression. They also include EBV-induced infectious mononucleosis-type polyclonal proliferation (Swerdlow et al., 2008).

The dilemma in diagnosis was created due to EBV reactivation which was giving the impression of lymphoproliferative disorder in this case. But, after treatment with IVIG, the platelet refractory had improved and EBV titer reduced significantly. Since the patient had limited finances, a follow-up PET-CT could not be obtained. The pathogenesis of EBV remains unclear. However, since EBV as an autoimmune disorder associated with the hyper-functioning of T lymphocytes, the correlation between the pathogenesis of AA and EBV infection requires further investigation. EBV infection can hinder the management of patients with AA. It can give a false impression of lymphoma. The clinician should be always kept in mind the possibility of viral infections before going with the management of such cases. Herein, we report an unusual case of fatal EBV infection in a patient with AA after being treated with Eltrombopag and CsA.

Disclaimer

None to declare.

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None.

Conflict of Interest

None.

Ethical Approval

All procedures performed on the patient were in accordance to the ethical standards of the institution research committee.

Informed Consent

Written informed consent was obtained from the patient for publication of this case in the text.

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