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ORIGINAL PAPER

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Serum levels of anti-corona virus specific-IgG and -IgM antibodies in COVID-19 patients at admission and at discharge

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ABSTRACT

Introduction. Clear understanding of duration of antibody based protective immunity following natural infection with SARS-CoV-2 will give idea about the efficacy of proposed prophylactic vaccines against SARS-CoV-2, establishment of herd immunity and use of convalescent plasma.

Aim. This study clarified the kinetics and magnitude of the initial antibody response against SARS-CoV-2 in a cohort of symptomatic COVID-19 patients from Ibadan, Nigeria.

Material and methods. This study quantified immunoglobulin M (IgM) and G (IgG) antibodies recognizing the SARS-CoV-2 Spike (S) protein in 35 symptomatic COVID-19 patients at admission and at discharge using ELISA.

Results. CovIgG was positive in none (0)% and 20% of COVID-19 patients at admission and at discharge respectively while CovIgM was positive in 54% and 69% of COVID-19 patients at admission and at discharged respectively. The level of CovIgG was significantly higher in COVID-19 patients at discharge compared with the level at admission while the level of CovIgM was insignificantly reduced in COVID-19 patients at discharge compared with the level at admission.

Conclusion. The data indicates increased anti-SARS-COV-2 IgG Spike antibody in symptomatic COVID-19 at discharge, thus providing basis for antibody-based therapies to treat COVID-19 patients.

Keywords. anti-SARS-CoV-2 specific antibodies, convalescence plasma, COVID-19, spike protein, vaccine

Introduction

The novel SARS-CoV-2 is a recently emerging virus causing a human pandemic having symptoms ranging from mild to severe, eventually leading to death in some cases.[1] Currently, the lockdown imposed by many governments controls the spread, but there is neither a sufficiently effective antiviral drug to treat COVID-19 cases nor an approved vaccine.[2] In order to guide future vaccine design and antibody-based therapies for the management of SARS-CoV-2 disease, it is obligatory to understand duration of immunity against SARS-CoV-2 in infected individuals and whether antibodies produced in response to a natural infection provide protective immunity, which may prevent re-infection with SARS-CoV-2.[3] Therefore, there is an urgent need to characterize viral-mediated antibody responses, in order to develop therapeutic tools to efficiently cure COVID-19 patients. In this study the dynamics of the

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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anti-SARS-COV-2 IgG and IgM immune response in COVID-19 patients were measured.

Coronaviruses are enveloped, single-stranded positive-sense RNA viruses having spike (S), envelope (E), membrane (M), and nucleocapsid (N) structural proteins.[4] The SARS-CoV spike (S) protein is composed of two subunits (S1 and S2). The N-terminal S1 subunit contains a receptor-binding domain (RBD) binds the angiotensin-converting enzyme 2 receptor on human alveolar epithelial cells of the low respiratory tract while the C-terminal S2 subunit mediates fusion between the viral and host cell membranes. The S protein is highly immunogenic while M and E proteins are necessary for virus assembly.[5] This necessitates the choice by the author to determine the levels of anti-SARS-COV-2 IgM and IgG antibodies against S protein in COVID-19 patients.

Literatures reported that there are need to explore changes in anti-SARS-CoV-2 specific antibody response of follow-up COVID-19 patients, in order to guide vaccine design and antibody-based therapies for cheaper effective management of the disease.[1-3,6,7] However, the dynamics of the antibody response against SARS-CoV-2 are still under investigation and previous studies showed that CovIgM was detected earlier than CovIgG.[6-10] Xiao et al. reported that some SARS-CoV-2 laboratory confirmed cases were positive for IgM and IgG at week 3 post symptoms onset.[6] Concomitantly to IgM decrease, IgG levels raised gradually from week 3 to week 7. Guo et al. showed that 90.4% and the 93.3% COVID-19 patients had plasma IgM and IgA, respectively, and the 77.9% of plasma samples were positive for IgG against nucleocaspid protein of SARS-COV-2 at day 5 post symptom onset and day 14 post symptom onset for IgG.[7] Higher numbers of COVID patients were positive for IgG than IgM at the moment of hospitalization and 5 days later; moreover, they had an earlier IgG than IgM seroconversion.[8]

As shown in short-term studies, a seroconversion of IgG and IgM occurred about two to three weeks after disease onset while IgM levels dropped significantly earlier than IgG titers.[3] However, it is unclear which anti-SARS-COV 2 specific antibody type (CovIgG or CovIgM) perform best in the epidemiologic identification of convalescent patients. Some authors favoured IgG while other proposed a higher positivity rate for IgM.[9-11] In addition, the reported peak of IgM response was assigned to different time points ranging from two to five weeks.[9,11]

Aim

Thus, this study clarified the kinetics and magnitude of the initial antibody response against SARS-CoV-2 in a cohort of symptomatic COVID-19 patients from Ibadan, Nigeria. This might assist in crucial decision-making on vaccine development or antibody based therapy.

Material and methods

Study Population

Thirty-five symptomatic COVID-19 patients recruited from Infectious Diseases Isolation Center, Nigeria were enrolled into this study at admission and followed up till discharged. The clinical signs on admission were dry cough, high fever, sore throat and shortness of breath. The real-time reverse-transcriptase polymerase-chain reaction (RT-PCR) assay was used to confirm the status of all the study participants using nasal and pharyngeal swab specimens following WHO guideline.[12] COVID-19 patients were hospitalised until swab specimens were twice negative for SARS-COV-2 which lasted between 4-19 days. The control subjects were COVID-19 free apparently healthy individuals recruited from staff and students of University of Ibadan, Nigeria. They were age and sex-matched with COVID-19 patients. None of the controls was on compulsory medication and without communicable or non-communicable diseases. Five milliliters (5 ml) of venous blood was obtained from each subject and was dispensed into plain sample bottles to obtain sera as appropriate. Blood samples were collected on the day of diagnosis when admitted into the isolation center and on the day of discharge when the swab specimens were negative for SARS-COV-2. Enzyme Linked Immunosorbent Assay (ELISA) was used to determine levels of SARS-CoV-2 Spike protein IgM and IgG in the patients using optical density as specified by the kit manufacturer (Elabscience Biotechnology Inc, USA). Samples were analyzed in duplicates within 1 week of collection.

The test principle

This ELISA kit uses Indirect-ELISA as the method to qualitatively detect the level of anti-SARS-CoV-2 Spike protein -IgG or -IgM in the sample. The micro-ELISA plate is pre-coated with purified SARS-CoV-2 Spike protein antigen. On adding samples and controls to wells, the SARS-CoV-2 Spike protein -IgG or -IgM antibody in the samples bind the pre-coated SARS-CoV-2 Spike protein antigen in the wells of the plate. After washing, Horseradish Peroxidase (HRP) conjugated mouse anti-human antibody added will combine with SARS-CoV-2 Spike protein -IgG or -IgM antibody. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) which is directly proportional to the level of anti-SARS-CoV-2 Spike protein -IgG or -IgM antibody is measured spectrophotometrically at a wavelength of 450nm wavelength.

Assay procedures

One hundred μL of controls (positive and negative) and samples were added into appropriate wells in duplicates. The plate was covered and incubated for 45 minutes at

37°C. After decanting, 350 µL of wash buffer was added to each well, washed 3 times and 100 µL of HRP Conjugated Mouse anti-human -IgG or -IgM was added which was incubate for 30 minutes at 37°C. The solution was decanted from each well, washed 5 times and 90 µL of Substrate Reagent was added to each well, incubated for 15 minutes at 37°C away from light. After which 50 µL of Stop Solution was added to each well and the OD value which was proportional to the level of CovIgG or CovIgM of each well was measured with a micro-plate reader set to 450 nm wavelength.

Calculation

Cut Off for SARS-CoV-2 Spike protein IgM was calculated thus.

Cut Off for CovIgM = 0.10 + negative control (NC) average A450. When NC average A450<0.10 = 0.10; while 0.10≤NC average A450≤0.20 = actual value.

Positive result was taken as sample absorbance ≥ Cut Off. Negative result was taken as sample absorbance < Cut Off.

Calculation of the Cut Off for SARS-CoV-2 Spike protein IgG was calculated thus:

Cut Off(C.O.) = 0.13 + NC average A450. When NC average A450<0.05=0.05; while 0.05≤NC average A450≤0.10 = actual value.

Positive control (PC) A450>0.60. Negative control (NC) A450≤0.10.

Negative result was taken as sample absorbance < Cut Off.

Statistical Analysis

The positivity and negativity of sera of COVID-19 patients were presented as frequencies (percentages) and were analyzed using X^2. Optical density of all samples was presented as mean and Standard Deviation. Student *t*-test was used to analyze the differences between two mean values. *P*-value less than 0.05 was considered as statistically significant.

Results

None of the COVID-19 patients was positive for CovIgG at admission while 20% of the patients were positive for CovIgG at discharge. The difference was significant (p<0.01). Fifty-four (54)% of the COVID-19 patients were positive for CovIgM at admission while 69% of the patients were positive for CovIgM at discharge. The difference was not significant (Table1). As shown in the Table 2, the mean CovIgG was significantly increased in COVID patients at discharge than at admission. However, mean CovIgM level was reduced though not significant at discharge compared with mean level at admission.

Table 1. Prevalence of anti-SARS-COV-2 specific -IgG or -IgM antibody in COVID patients at admission and at discharge

CovIgG		
Positive	Negative	
At admission = 0 (0%)	At admission = 35 (100%)	$X^2 = 7.79$, p < 0.01
At discharge = 7 (20%)	At discharge = 28 (80%)	
CovIgM		
Positive	Negative	
At admission = 19 (54%)	At admission = 16 (46%)	$X^2 = 1.56$, p > 0.10
At discharge = 24 (69%)	At discharge = 11 (31%)	

* significant at p<0.05

Table 2. Mean Levels of anti-SARS-COV-2 specific -IgG or -IgM antibody in COVID patients at admission and at discharge

CovIgG			
At admission (n=35)	At discharge (n=35)	t-,	p-value
0.042±0.022	0.090±0.087*	-2.793	0.009
CovIgM			
At admission (n=35)	At discharge(n=35)	t-	p-value
0.395±0.487	0.323±0.215	0.940	0.354

* significant at p<0.05

Discussion

The recent COVID-19 pandemic caused by SARS-CoV-2 infection calls for urgent need for therapeutic interventions to manage the outcome of the disease.[1] The characterization of the humoral immune response of COVID-19 patients will elucidate the mechanism of natural protection and will guide through the use of SARS-CoV-2 specific antibodies as prophylactic and therapeutic options to manage the disease, which may contribute to the possibility of vaccine efficacy and herd immunity.[2,3] To the best of author's knowledge, this is the first study demonstrating dynamism in the SARS-CoV-2-specific IgG and IgM recognizing S protein in Nigerian COVID- 19 at the point of admission and at discharge.

In the present study, COVID-19 patients at admission had higher positivity and level of IgM recognizing S protein compared with patients at discharge. This might be related to the fact that COVID-19 patients at admission experienced higher virus replication leading to the expression of more virus antigens, eliciting strong primary humoral immune responses. Thus, suggesting that CovIgM antibodies are involved in immunopathology rather than antiviral effects. Contrary to this, the present study also reported higher positivity and level of CovIgG recognizing S protein in COVID 19 patients at discharge compared with at admission. This highlights the relevance of CovIgG against S protein as correlate of protection in humans as

previously elucidated, thus, suggesting sustained antiviral effects of CovIgG antibodies in COVID-19 patients.[13,14] Previous study showed that IgG against receptor binding domain of S protein has neutralizing activity and that CoV specific IgG has been correlated with a neutralising function which persisted for 24 months, despite the declining titers.[3,14-17] Another study showed the 74.2% and the 83.9% of the patients were positive for IgG and neutralizing antibodies 36 months post symptom onset.[18] An observational cohort study including 16 COVID-19 patients whose serum samples were collected 14 days post symptom onset showed that the majority of patients harboured neutralizing IgG against both NP and receptor binding domain.[19] Nucleocapsid protein (NP) is highly immunogenic, although smaller than S, lacks of glycosylation sites, and induces antibodies earlier than S during the infection, thus contributing to neutralization; therefore, anti-NP-specific antibodies might play a key role during the early stages of acute infection.[20]

Neutralizing antibodies (NAbs) play critical roles in blocking viral infections, thus contributing to viral clearance during acute infection or controlling disease progression during chronic phase. These antibodies are, therefore, useful tools for the protection from viral infection and for the development of effective treatments. CovIgG which was found to be raised in COVID-19 patients considered for this study had been shown to have neutralizing activity.[3,14-23] It was previously reported that NAbs in the plasma of convalescent COVID-19 patients were successfully employed in the passive antibody therapy to treat 10 severe cases of SARS-CoV-2 infection.[22]

The present data strongly suggests that the deeper characterization of plasma from recovered patients might give important information for the development of effective antibody-based therapies to treat COVID-19 patients. However, the rapidly declining Cov specific antibodies from 6 months provoked doubts and anxiety about the long duration of COVID vaccine effectiveness and usefulness of antibody therapy.[3] The present study therefore suggests collection of blood sample for the purpose of convalescent plasma therapy in selected COVID patients at discharge.

This study has some limitations, viz: small sample size and need for longer follow-up of COVID-19 patients to give opportunity for sub-grouping COVID-19 patients into days post-discharge.

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ORIGINAL PAPER

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Anxiety states and knowledge of COVID-19 among pregnant women during the pandemic in Turkey – a cross-sectional study

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ABSTRACT

Introduction. Infectious outbreaks have negative effects not only on the physical health of the society but also on the mental health.

Aim. To evaluate the anxiety states and knowledge of COVID-19 during the pandemic in pregnant women.

Material and methods. Cross-sectional study conducted in a university hospital in Turkey. A total of 199 pregnant women were included in the study. The State-Trait Anxiety Inventory (STAI), two questionnaires to evaluate the knowledge about COVID-19, and COVID-19-related anxiety were applied to all the women.

Results. The highest level of COVID-19-related anxieties were about their spouses or newborns contracting COVID-19, effects of drugs on fetus and contracting COVID-19 during delivery. There was a negative correlation between gestational week and the questionnaire of COVID-19-related anxieties (r=-0.152, p=0.037). STAI total score was 76.48±14.11, and STAI-T scores (42.39±7.66) were higher than STAI-S scores (34.09±8.77). Although their general knowledge about the disease was relatively good, their level of knowledge on issues that pertained specifically to pregnancy was low.

Conclusion. These findings indicated more than four months had passed since the pandemic came to the country but, pregnant women were very worried and did not have enough information about the disease.

Keywords. anxiety, knowledge, pandemic, pregnancy

Introduction

Coronavirus disease (COVID-19) first appeared in Wuhan, China's Hubei province in December 2019.[1] In a cluster of patients followed up for pneumonia of unknown cause, a novel coronavirus detected which was different from severe acute respiratory failure syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV).[2,3] The coronavirus disease started to spread around the world from China and was defined as an international emergency threat in

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January 2020 and was declared as a pandemic in March 2020 by the World Health Organization (WHO).[1]

While infectious outbreaks threaten the health of the whole society; the diseases can be more severe in people with some medical and physiological conditions such as chronic disease, advanced age, and obesity.[4] Pregnancy is also one of the risky conditions for viral diseases.[5] Studies conducted during past outbreaks had shown that pregnant women had more hospitalization, the outbreaks were more common cause of death in pregnant women and caused negative fetal outcomes such as stillbirth, prematurity and congenital malformations.[6-8] Additionally, there are also data indicating that drugs used in the treatment of infections have teratogenic effects.[9] The current knowledge of the effects of COVID-19 infection in pregnancy are limited. However, preliminary data showed that pregnant women have a similar clinical presentation and severity to non-pregnant adults.[10]

Infectious outbreaks have negative effects not only on the physical health of the society but also on the mental health.[11] The studies evaluating the effect of the epidemic/pandemic diseases on the mental state of pregnant women are limited.[12,13] During the 2003 SARS epidemic in Hong Kong, pregnant women worried about themselves, their spouses, and newborns getting infected with SARS and the negative effects of antiviral agents on the fetus.[12] The same study showed an increase level of general anxiety in pregnant women.[12] Another study during the SARS outbreak in the pregnant population found that about a quarter of pregnant women had high anxiety levels.[13] Similar findings were found in the COVID-19 pandemic.[14-18] Two separate studies were done in Canada and Turkey found increased anxiety levels in pregnant women compared to the pre-pandemic period.[14-15] Pregnant women reported they were concerned about the negative consequences of COVID-19 on their own, baby's and family's health and pregnancy.[16-18] However, our knowledge about the impact of the pandemic on mental health of pregnant women is still limited.

The outbreaks have caused fear and panic in the general population. This panic and fear can cause the public to have inappropriate attitudes and behaviors regarding the disease. What we learned from the past epidemic diseases showed us that education about the disease was necessary to implement preventive measures and reduce the negative psychological reactions of the public.[19] Previous studies showed that knowledge of the disease was important in the adoption of preventive measures, the risk perception of the disease, and the level of anxiety.[20,21] There are a limited number of studies on the level of knowledge of pregnant women about COVID-19. Lee et al. stated the rate of correct answers to questions about COVID-19 was 76.4% in their study with Chinese pregnant women.[17] A cross-sectional survey from Nigeria reported pregnant women had adequate knowledge about COVID-19.[22] However, a study conducted with a Turkish sample found majority of pregnant women was not knowledgeable about the effect of COVID-19 related to the birth.[23]

Anxiety and stress during pregnancy might cause negative outcomes like preeclampsia, preterm birth, low birth weight, low APGAR scores, increased nausea, and vomiting.[24] Prenatal anxiety, depression and stress could be associated with cognitive, behavioral, and emotional problems in children.[25] In this context, determining anxiety levels and factors related to anxiety levels in pregnant women during pandemics may be important in terms of decreasing the negative results related to the mothers and their babies.

Aim

This study aimed to evaluate the general anxiety levels, COVID-19 related anxiety, and level of knowledge of the COVID-19 of the pregnant women during the COVID-19 pandemic.

Material and methods

This cross-sectional survey study was carried out in 1-31 July 2020.The study sample consisted of the pregnant women who visited the Department of Obstetrics and Gynecology (O&G) of Giresun University for their antenatal consultation and agreed to participate in the study after informing about the study. All pregnant women (n=250) who visited for antenatal consultation were offered to participate in the study. One hundred ninety-nine pregnant women participated in the study by completing the study questionnaires. The questionnaires were filled out by the participants before the examination. The routine pregnancy USG was performed in all cases at least once before the visit they participated in the study. We excluded the pregnant with confirmed infection of COVID-19. The participants completed two questionnaires about COVID-19 which one evaluating their knowledge of the disease and the other evaluating their anxiety related to the disease. And they also fulfilled The State-Trait Anxiety Inventory (STAI) to assess their anxiety levels. All participants gave written and verbal approval to be enrolled into the study.

A power analysis was conducted to determine the sample size, through calculations using the publicly available statistical software Open Epi, version 3 (http://www.openepi.com). This analysis was done using a significance level of 5%, an effect size of 21% and an ability to represent the population of 80% (power). It was shown that the sample size needed to be at least 98.

The Sociodemographic form designed by authors to provide demographic information like the participants' age, educational levels, household income, their pregnancy such as parity, gestational age of fetus.

We designed two questionnaires to evaluate the knowledge about COVID-19, and COVID-19 related anxiety in pregnant women. These questionnaires were designed by authors based on the questionnaires used in previous studies during outbreaks.[12,13] The questionnaire of COVID-19 related anxiety was a 4-point Likert-type scale (not at all, slightly, moderately, very much) consisted of 10 questions which evaluate anxiety about herself, her family, baby and pregnancy related to the outbreak. Cronbach's alpha and Spearman Brown coefficients were calculated to evaluate reliability of the questionnaire. Cronbach's alpha coefficient was calculated as 0.911, so we could say our questionnaire has very good internal consistency and Spearman-Brown coefficient was calculated as 0.949. The questionnaire of knowledge about the COVID-19 had questions regarding sign and symptoms, transmission routes, prevention and COVID-19 in pregnancy.

STAI is a self-report rating scale developed by Spielberger et al.[26] consisting of 40 Likert- type questions. The first 20 questions evaluate the state anxiety and the other 20 questions the continuous anxiety. Each item is scored between 1-4 points, and there are 17 reverse items. The scale indicates the level of anxiety rather than makes a diagnose and higher scores representing higher anxiety levels. Turkish translation and adaptation of the scale was done by Oner and Le Compte.[27]

Approval for the study was granted by a local internal review board (ethical committee) (date: 03.07.2020, nº: 2020/30).

Statistical Analysis

All analyses were performed on SPSS v21 (SPSS Inc., Chicago, IL, USA). Histograms and Q-Q plots were used to determine whether variables are normally distributed. Data are given as mean ± standard deviation (minimum - maximum) for continuous variables and as frequency (percentage) for categorical variables. Pearson or Spearman correlation coefficients were calculated to evaluate relationships between variables depending normality of distribution. Two-tailed p-values of less than 0.05 were considered statistically significant.

Results

We included 199 pregnant women into our study. The mean age of the participants was 28.30±4.67. The mean number of pregnancy was 2.09±1.11, more than half of the pregnant women (68.58%) had their first or second pregnancy. The mean gestational week was found as 30.76±8.46. The educational level and family income of participants were presented at Table 1. STAI mean total score was 76.48±14.11 and STAI-State scores (34.09±8.77) were higher than STAI-Trait scores (42.39±7.66) (Table 1).

Table 1. Summary of sociodemographic attributes and State-Trait Anxiety Inventory scores

Age	28.30±4.67 (18-42)
Education status	n (%)
Primary school	14 (7.11)
Secondary school	49 (24.87)
High school	50 (25.38)
University	84 (42.64)
Monthly income*	n (%)
< 1500	39 (20.42)
1500 - 3000	80 (41.88)
3000 - 5000	41 (21.47)
> 5000	31 (16.23)
Number of pregnancy	2.09±1.11 (1-6)
	n (%)
1	71 (37.17)
2	60 (31.41)
3	39 (20.42)
≥ 4	21 (10.99)
Gestational week	30.76±8.46 (7-41)
State-Trait Anxiety Inventory	
State score	34.09±8.77 (20-56)
Trait score	42.39±7.66 (24-62)
Total score	76.48±14.11 (44-117)

Data are given as mean ± standard deviation (minimum - maximum) for continuous variables and as frequency (percentage) for categorical variables

More than half of women worried moderately or very much about their spouses or newborns contracting COVID-19, while they were less worried about themselves (52.76% vs 51.26% vs 38.69, respectively). Forty-six (23.12%) pregnant women were very worried with regards to their babies would have disability if they would receive drugs for COVID-19. Approximately one-third of participants (33.17%) were worried moderately or very much about having an abortion due to COVID-19 while 73 (36.69%) about having a preterm delivery. More than a quarter of women (27.14%) were very worried about contracting COVID-19 during delivery. About 35 percent of pregnant women were moderately or very worried about going out, but less worried at home. More than one-fifth of women (21.61%) were very worried about going to the hospital because of COVID-19 (Table 2).

We graded questions zero (not at all) to three (very much) points so the minimum possible total score was zero and the maximum possible total score was 30. The mean total score of the questionnaire was calculated as 14.39±7.11. Five (2.51%) women had the maximum point and four (2.01%) women had the minimum point (Table 2).

Table 2. The questionnaire of COVID-19 related anxiety of pregnant women

Question (Q)	Not worried n (%)	Slightly worried n (%)	Moderately worried n (%)	Very worried n (%)	Mean	Standard deviation
1. Contracting COVID-19-herself	21 (10.55)	101 (50.75)	44 (22.11)	33 (16.58)	1.45	0.89
2. Contracting COVID-19-spouse	12 (6.03)	85 (42.71)	48 (24.12)	54 (27.14)	1.72	0.93
3. Contracting COVID-19-baby	18 (9.05)	76 (38.19)	53 (26.63)	52 (26.13)	1.70	0.96
4. Fetal malformation if drugs are needed for treatment	30 (15.08)	65 (32.66)	58 (29.15)	46 (23.12)	1.60	1.00
5. COVID-19 leading to miscarriage	63 (31.66)	70 (35.18)	40 (20.10)	26 (13.07)	1.15	1.01
6. COVID-19 leading to preterm birth	49 (24.62)	77 (38.69)	38 (19.10)	35 (17.59)	1.30	1.03
7. Contracting COVID-19 during birth	20 (10.05)	77 (38.69)	48 (24.12)	54 (27.14)	1.68	0.98
8. Worry even at home	79 (39.70)	86 (43.22)	19 (9.55)	15 (7.54)	0.85	0.88
9. Going out of the home	35 (17.59)	93 (46.73)	43 (21.61)	28 (14.07)	1.32	0.93
10. Going to hospital	18 (9.05)	82 (41.21)	56 (28.14)	43 (21.61)	1.62	0.92
Total Score					14.39	7.11

Data are given as mean ± standard deviation (minimum - maximum) for continuous variables and as frequency (percentage) for categorical variables

Fever (91.21%), dyspnea (78.02%) and cough (76.37%) were the most common answers about COVID-19 symptoms. 73 women had knowledge of major mode of transmission (droplet spread). While the ideal hand wash time was known by three-fourths of the participants (75.88%), the quarantine period was known by almost all of them (94.47%). More than half of women (64.82%) believed that they had more risk to contracting COVID-19 than the others. One hundred and sixteen women (58.59%) thought that COVID-19 could be transmitted to baby from mother. More than half of the participants did not know that what would happen to a 28-week pregnant woman if she contracts COVID-19 (57.07%) or whether mother with COVID-19 could breastfeed her baby (52.76%) (Table 3).

When we evaluated relationships between variables, we found that the questionnaire of COVID-19 related anxiety total scores had a low positive correlation with STAI state scores (r=0.219, p=0.002), trait scores (r=0.281, p<0.001) and total scores (r=0.298, p<0.001). In addition, we found a low negative correlation between education status and STAI trait scores (r=-0.144, p=0.044), also low negative correlation between gestational week and questionnaire scores (r=-0.152, p=0.037) (Table 4).

Discussion

In this study, we evaluated the anxiety states and knowledge regarding COVID 19 in pregnant women who applied to a University Hospital during the outbreak. We found a significant proportion of pregnant women were worried about contracting COVID-19, impact of COVID-19 on pregnancy outcomes, the negative effects of drugs to treat COVID-19 on the fetus, and going to the hospital. We also found that although their general knowledge about the disease was relatively good, their level of knowledge on issues that pertained specifically to pregnancy was low.

Thirty-nine percent of pregnant women were moderately or very much worried about contracting COVID-19. Similarly, Lee et al. found that 37.7 % of prenatal and postnatal women were worried about contracting COVID-19.[17] We also found they worried about their spouse and children more than themselves. Likewise, Rivaldi et al. reported pregnant women were less worried about their own health than the health of others.[16] They explained this situation as the instinct of women to protect their offspring, which was potentially present in them, could be exacerbated by the pandemic, and caused them to act protective among people around them.[16] A similar explanation may apply to our findings. More than half of the pregnant women stated that they were worried about miscarriage or premature birth. Approximately half of pregnant women were worried much or very much about effects of drugs on fetus. Similar to our finding Nanjundaswamy et al. reported about half of the pregnant women were concerned about the effects of COVID-19 on pregnancy outcomes.[18] Mappa et al. stated 46.6% of pregnant women were afraid of COVID-19 related fetal structural anomalies, and more than half of pregnant women were afraid of fetal growth restriction (65.2%) and preterm birth (51.1%).[28] During the SARS pandemic, it was found 68.8% of pregnant women were concerned that antiviral drugs could cause fetal malformation.[12] It was also reported COVID-19 causes fetal complications such as miscarriage, intrauterine growth restriction, and preterm birth.[29] The effects of antiviral drugs on the fetus vary. While ribavirin therapy has a teratogenic effect, remdesivir and lopinavir-ritonavir appear to be safe in pregnancy.[30-32] It is recommended to decide on the choice to use the drug by taking into account benefits and possible side effects in

Table 3. Knowledge of COVID-19 of the pregnant women

			n (%)
Symptoms	Fever		166 (91.21)
	Cough		139 (76.37)
	Dyspnea		142 (78.02)
	Fatigue		112 (61.54)
	Myalgia		50 (27.47)
	Diarrhea		47 (25.82)
	Headache		55 (30.22)
	Skin eruption		10 (5.49)
Mode of transmission	Droplet spread		142 (73.20)
	Body secretions		91 (46.91)
	Stools		4 (2.06)
	Blood		25 (12.89)
	Don't know		20 (10.31)
Ideal hand wash time	5 seconds		0 (0.00)
	10 seconds		24 (12.06)
	20 seconds		151 (75.88)
	1 minute		21 (10.55)
	Unknown		3 (1.51)
Quarantine period for suspicious persons	7 days		0 (0.00)
	10 days		11 (5.53)
	14 days		188 (94.47)
	1 month		0 (0.00)
	Unknown		0 (0.00)
Probability of catch the disease in pregnants	Same with others		45 (22.61)
	More than others		129 (64.82)
	Less than others		8 (4.02)
	Unknown		17 (8.54)
Intervention to a 28 week pregnant with COVID-19	Caesarean		34 (17.17)
	No intervention		51 (25.76)
	Unknown		113 (57.07)
	Yes	No	Unknown
Transmission of disease from mother to baby	116 (58.59)	32 (16.16)	50 (25.25)
Mother with COVID-19 can breastfeed her baby	27 (13.57)	67 (33.67)	105 (52.76)

Data are given as frequency (percentage)

each single case.[32] Taking into account all of these data, the concerns of pregnant women on this issue could be understood.

Quarantine and social isolation are important methods to prevent the spread of infections. Physical isolation methods such as staying at home, not going to crowded places are common practices towards the outbreaks.[33] Studies showed pregnant women were anxious when went out of the home during the outbreaks.[12] Similarly, in our study, it was found that pregnant women were less anxious at home and were anxious about going out. Hospitals are considered risky areas during the outbreaks and people would stay away from hospitals to avoid contracting infections.[33] An American study showed 56.2% of the pregnant women in the third trimester changed their birth plans due to the anxiety related to COVID-19.[34] A study from Israel stated 66.7% of pregnant women had much or very much anxiety about going to the hospital.[35] Similarly, a study in Italy reported that 75% of pregnant women had fears associated with going to the hospital.[28] In another study, pregnant women and postpartum women in India showed 72.65% of the participants were concerned about going to hospital visits.[18] In our study, the rate of having moderately or very much anxiety about going to the hospital was 49.75%. The fact that conducting the study in the normalization process of the country regarding the pandemic and in the hospital which was not a pandemic hospital might have been effective in the relatively low rate.

In previous studies, increased anxiety levels in pregnant women during the pandemic were reported.[14,15] In the current study the level of trait anxiety according to STAI was similar to a study in Turkey, but the level of state anxiety was lower.[15] In contrast to the two studies that found STAI-S score higher than STAI-T scores, the STAI-T score was higher than the STAI-S score in our study.[28,36] Considering the concerns of pregnant women about going to hospital, it could be expected that the state anxiety levels were higher in the hospital environment where the questionnaires were filled. However, pregnant women might feel safer due to being in the hospital with the doctor. We did not know the anxiety levels of women in the pre-pandemic period. This situation may make it difficult to interpret our findings on the effect of the epidemic on the general anxiety levels of pregnant women. It was observed that the relationship between state and trait anxiety levels and levels of anxiety about the disease was significant. It could be expected that people who were generally concerned would be more concerned about the COVID-19. Consistent with this finding Lebel et al. showed that COVID-19-related anxieties are associated with depression and anxiety in pregnant women.[14]

Although socioeconomic status and the history of pregnancy could have an effect on anxiety, our study did not find a significant relationship between these variables and total anxiety, and COVID-19-related anxiety.[14,37] Nanjundaswamy et al. reported more COVID-19 related anxiety to pregnant women in the first trimester. Conversely we found a negative correlation between gestational age and COVID-19 related anxiety.[28] The effects of COVID-19 such as miscarriage and the increase in the probability of the baby's survival as gestational age increases might be effective in this finding.[29,38]

Another issue we examined in our study was the level of knowledge of the COVID-19 of the pregnant women. While fever, cough, fatigue are more common in COVID-19, symptoms such as headache, rash are less

Table 4. Correlations between State-Trait Anxiety Inventory, questionnaire scores and pregnants attributes

		State-Trait Anxiety Inventory scores			Questionnaire
		State	Trait	Total	
Questionnaire	r	0.219*	0.281*	0.298*	-
	p	0.002	<0.001	<0.001	-
Age	r	0.087	0.014	0.050	-0.073
	p	0.225	0.844	0.486	0.314
Education status	r	0.000	-0.144*	-0.078	0.048
	p	0.997	0.044	0.275	0.505
Monthly income	r	0.061	-0.132	-0.030	0.101
	P	0.401	0.069	0.682	0.166
Numberof pregnancy	r	0.015	0.011	0.022	-0.012
	p	0.838	0.883	0.763	0.873
Gestational week	r	0.060	0.004	0.042	-0.152*
	p	0.417	0.954	0.566	0.037

R: Correlation coefficient, * Correlation is significant at the 0.05 level (2-tailed).
Questionnaire: The questionnaire of COVID-19 related anxiety of pregnant women

common symptoms.[39] Although common symptoms in COVID were mostly known there were significant knowledge gaps in terms of symptoms of COVID-19. Similar to our findings, Aniwke et al. reported that the most common symptoms known by pregnant women were fever and cough.[22] More emphasis on common symptoms may have been effective in this situation. Approximately three-fourths of the women were aware of the main route of transmission. Considering the time elapsed since the beginning of the epidemic, the rate was low. Droplet transmission is a medical term in the transmission method questions in our survey. The fact that the participants did not understand this term may have been effective in this result. The level of knowledge about the quarantine period and importance of hand washing were very high in studies.[17,40] In our study, a satisfactory correct rate of knowledge about quarantine period and hand washing time was also obtained. Since the virus originates from abroad, it was recommended that people coming from abroad should stay in quarantine for 14 days at the beginning of the outbreak.[41] Turkish Ministry of Health established The 14-Day Rule for returns from abroad.[42] These rules included the quarantine period and the isolation and hygiene recommendations and were conveyed to the public through printed and visual media. Efforts to inform the public about the 14-Day Rule may have been effective in this regard.

Most of the pregnant women believed the probability of getting the disease themselves was higher than the general population. Similarly, Lee et al. reported in their study that most pregnant women thought they were more susceptible to COVID-19 than the general population.[17] However, there is not enough evidence to support that the risk of contracting COVID-19 for pregnant women is higher than the general population.[10,43] In addition, a multi-center study found maternal mortality due to COVID-19 was lower than the non-pregnant population.[44] Pregnant women would think it will be risky about going to hospital. And as a result, they could not benefit from health services sufficiently. It was reported that women who experienced a decrease in fetal movements were reluctant to contact the hospital for help due to the risk of COVID-19 exposure in hospital.[45] Women did not have enough information about how the course of pregnancy would be if they caught COVID-19. Studies showed pregnancy could be continue even if the pregnant had COVID-19. The decision to delivery or the mode of delivery is made according to obstetric factors and clinical situation. And vaginal delivery is not contraindicated since vertical transition is not proven.[43,44] Although it is still not known clearly whether there is a vertical transition or not, no viruses were found in amniotic fluid and placenta.[43] The multi-center study found only one infant born from a pregnant woman with COVID-19 was found positive to COVID-19 and the authors stated the vertical transition was negligible in COVID-19.[45] Current evidence suggests that the virus is not transmitted in breast milk and breastfeeding could continue.[46] Uncertainty is a situation that triggers anxiety and stress in people. Having accurate information can reduce their anxiety.

With the rapid spread of COVID-19 from China to the whole world, countries started to take measures to prevent the spread of the disease. In this context, Turkey banned travel abroad and people returning abroad were being quarantined for 14 days. However, despite all the measures first case was detected in Turkey in Istanbul on 11 March 2020, and the number of cases started to increase.[47] The Turkish Minister of Health publishes daily update on TV, including number of tests performed and

confirmed patients. The government has imposed travel bans and lockdown of several cities.[48] Considering the country's advances in fighting the COVID-19, the normalization process had started in Turkey in the month of June as the government decided to ease restrictions related to COVID-19. Our study was conducted after the normalization process has started. Despite entering the normalization process, most of the pregnant women were found to be anxious about the pandemic in our study. These findings indicated more than 4 months had passed since the pandemic came to the country but, pregnant women did not have enough information about the disease.

Limitations

This study has a number of limitations. First is small sample size and single-center study design. Our participants were not representative of the Turkish population of pregnant women because data were collected only one city. Another limitation is related to the questionnaires used in this study. The instruments could be developed to evaluate both knowledge and anxiety levels more comprehensively.

Conclusion

These findings showed us that pregnant women have high anxiety levels related to the pandemic and that they have lack of information about COVID-19. Considering the negative effects of anxiety and stress on pregnancy and fetus, it may be important to provide psychological support to pregnant women during the pandemic period and to expand their knowledge about the disease.

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ORIGINAL PAPER

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The expression of CD44, CD90 and CD133 in response to cisplatin in hepatocellular cancer cells

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ABSTRACT

Introduction. Cancer is a leading cause of mortality. Hepatocellular cancer is one of the malignancies associated with poor outcome and resistance to pharmacotherapy. Cancer stem cells (CSCs) contribute to resistance to therapy and hence lead to the treatment failure of tumors.

Aim. This study aims to explore the expression of CSCs in response to cisplatin treatment in HepG2 hepatocellular cancer cell line.

Material and methods. Cell proliferation test, CCK-8, was used to evaluate the cell proliferation following cisplatin treatment for 72 hours. The expressions of CSC markers CD44, CD90, and CD133 were assessed by flow cytometric analysis.

Results. The results showed a dose-dependent decrease in cell proliferation and increased expression of CSC markers CD44 and CD90 in response to cisplatin.

Conclusion. Understanding the roles of CSC markers may point to new targets and therapeutic strategies to predict and overcome cisplatin resistance.

Keywords. cancer, cisplatin, hepatocellular, stem cells

Introduction

Cancer is a leading cause of mortality despite evolving strategies to treat. The mainstream treatment approach is surgery, radiotherapy, and/or pharmacotherapy. Non- response to pharmacotherapy might be associated with drug resistance, which contributes to failure in the treatment. Drug resistance is a multifactorial phenomenon that involves patient-related factors, tumor-related factors, and surrounding factors.[1] Intrinsic factors or acquired factors during the pharmacotherapy may alter drug response.[2]

Cisplatin (CIS) is the first metal-based antineoplastic drug, which is still one of the most widely used platinum-based anticancer agents in various types of solid cancers.[3] Co-administration of cisplatin with other drugs has clinical importance due to the decreased toxicity and drug resistance.[4]

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Domnez Cakıl Y, Ozunal ZG, Kayalı DG, Aktas RG. *The expression of CD44, CD90 and CD133 in response to cisplatin in hepatocellular cancer cells*. Eur J Clin Exp Med. 2021;19(1):18–22. doi: 10.15584/ejcem.2021.1.3

Hepatocellular cancer (HCC), the most frequent type of primary liver cancer, is one of the neoplasms associated with poor outcomes, especially for its non-surgically removable advanced stage. Poor outcome is mainly due to the potential resistance to tumor pharmacotherapy.[5] Therefore, different administration routes are applied to increase the efficacy of the treatment and decrease systemic toxicity. CIS is within the therapeutic approaches with direct hepatic arterial infusion.[6] CIS is still explored for combination therapies with other drugs to ameliorate the efficacy in HCC.

Increasing evidence supports the presence of a small subset of cancer cells with self-renewal and differentiation properties, the so-called cancer stem cells (CSCs). In the liver, CSCs show tumorigenicity and metastasis. Surface molecules; MDR, CD13, CD44, CD45, CD90, CD105, CD133, CD24, EPCAM are linked to CSC traits in HCC.[7,8] CD44, a transmembrane glycoprotein, is the most commonly observed CSC marker.[9] Tumorigenic capacity, an important feature of cancer cells, is associated with CD90 presence in HCC cell lines.[10] Furthermore, cisplatin resistance is highly associated with the biomarker CD133 in various cancers.[11] Overexpression of the CSC markers has been reported to be associated with poorer response to treatment in HCC patients and might have a role in the prediction of drug response.[12]

Aim

This study aimed to investigate the roles of CD44, CD90 and CD133 markers in cisplatin response in HCC by exploring their dose-dependent expression in HepG2 cells.

Material and methods

Cell culture

Human hepatocellular carcinoma cell line HepG2 (American Type Culture Collection) was cultured in 10% fetal bovine serum (PAN-Biotech GmbH, Germany), and 1% antibiotics (streptomycin 10 mg/ml, penicillin 10.000 U/ml, PAN-Biotech GmbH, Germany) containing Dulbecco's Modified Eagle's Medium (Biosera LM-T1720/100, France). Cells were incubated at 37°C in a CO_2 incubator (5%). When they reach 70-80% confluency, they are subcultured with trypsinization.

CCK8 cell proliferation test

The effect of cisplatin (Glentham Life Sciences, UK) on cell proliferation was determined with Cell Counting Kit-8 (CCK8, Abbkine, USA). CIS concentration ranged between 30-4 μM with a 3⁄4 dilution ratio. Cells were incubated for 72 hours after treatments. The optical density of soluble CCK-8 material in each sample is measured with a Synergy Microplate Reader (BioTek, Japan). Each concentration was repeated four times within the plate and three independent experiments were performed.

Determination of cancer stem cell marker expressions by flow cytometry

Following incubation with CIS for 72 hours, drug administered cells and control group were harvested and incubated with BB515 labeled-CD44 (1: 100 dilution), PE-labeled CD133 (1:50 dilution), and APC labeled-CD90 (1:50 dilution) (BD Pharmingen, BD Biosciences, USA). After 30 minutes of incubation at RT, cells were washed with PBS. The pellet was resuspended in PBS and the measurements were carried out in a BD AccuriC6 + flow cytometer (BD Biosciences, USA). At least 20.000 events were collected. The results were analyzed using BD Accuri C6 + software and depicted as dot plots and overlay histograms.

Statistical analysis

All data are the mean of the three independent experiments. CCK8 cell proliferation test results are shown as mean ± standard deviation (SD). Results of CSC marker expression are shown as mean ± standard error of the means (SEM). One-way ANOVA and posthoc Tukey tests were used to identify statistical significance among the groups. GraphPad Prism V.8.2.0 was used for conducting the statistical tests and creating the figures.

Results

Effect of cisplatin on the proliferation of HepG2 cells

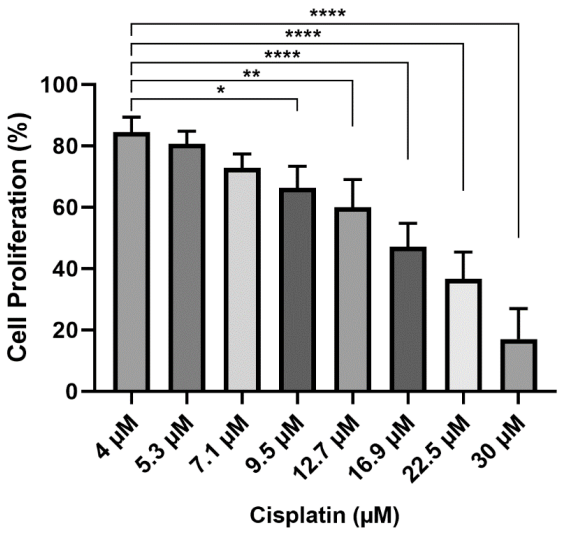

Fig. 1. Effect of treatment with increasing cisplatin doses (μM) for 72 h on HepG2 cell proliferation (%). Statistical evaluation was carried out by one-way ANOVA and post-hoc Tukey analyses were performed. *P<0.05; **P <0.01; ***P< 0.001, ****P< 0.0001 compared with proliferation at 4 μM CIS.

The incubation with decreasing doses of CIS (30-4 μM) for 72 hours exerted antiproliferative effects when compared to the control group (Figure 1). The inhibition was dose-dependent. The effect of the lowest administered

dose, 4 μM, was compared with that of the increasing doses. CIS at 5.3 μM and 7.1 μM did not exert a significant decrease in cell proliferation when compared with CIS at 4 μM. Starting with a dose of 9.5 μM, CIS inhibited cell proliferation significantly in comparison to 4 μM.

Administration of 4 μM, 5.3 μM, and 7.1 μM CIS resulted in 84.5%, 80.6%, 72.9% viability respectively. The highest dose (30 μM) exerted %17 viability.

Effect of cisplatin on CD133, CD44 and CD90 expressions in HepG2 cells

CIS was administered to HepG2 cells at the doses between 7.1 μM and 22.5 μM for 72 hours and the expressions of the CSC markers were analysed by flow cytometry. After treatment with 30 μM CIS, cells were not enough in number for assessing flow cytometric analysis. Representative dot plots for gating of HepG2 cells and CSC marker expressions are given in Figure 2. Representative overlay histograms and bar graphs demonstrating changes in expressions of CSC markers as fold changes are shown in Figure 3.

CD 44 expression increased in response to the cisplatin doses from 7.1 μM to 22.5 μM following incubation for 72 h. The highest CD44 expression was obtained after treatment with 16.9 μM CIS. A more significant increase in comparison to the control group was observed at 12.7 μM and 16.9 μM CIS treatments than 7.1 μM and 9.5 μM CIS treatments.

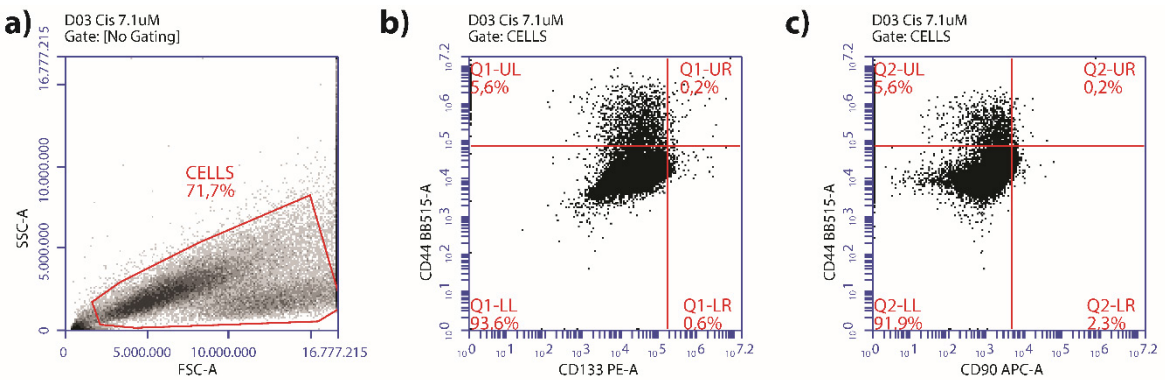

Fig. 2. Representative dot plots illustrate the gating of HepG2 cells (a) and expression of CSC markers (b and c).

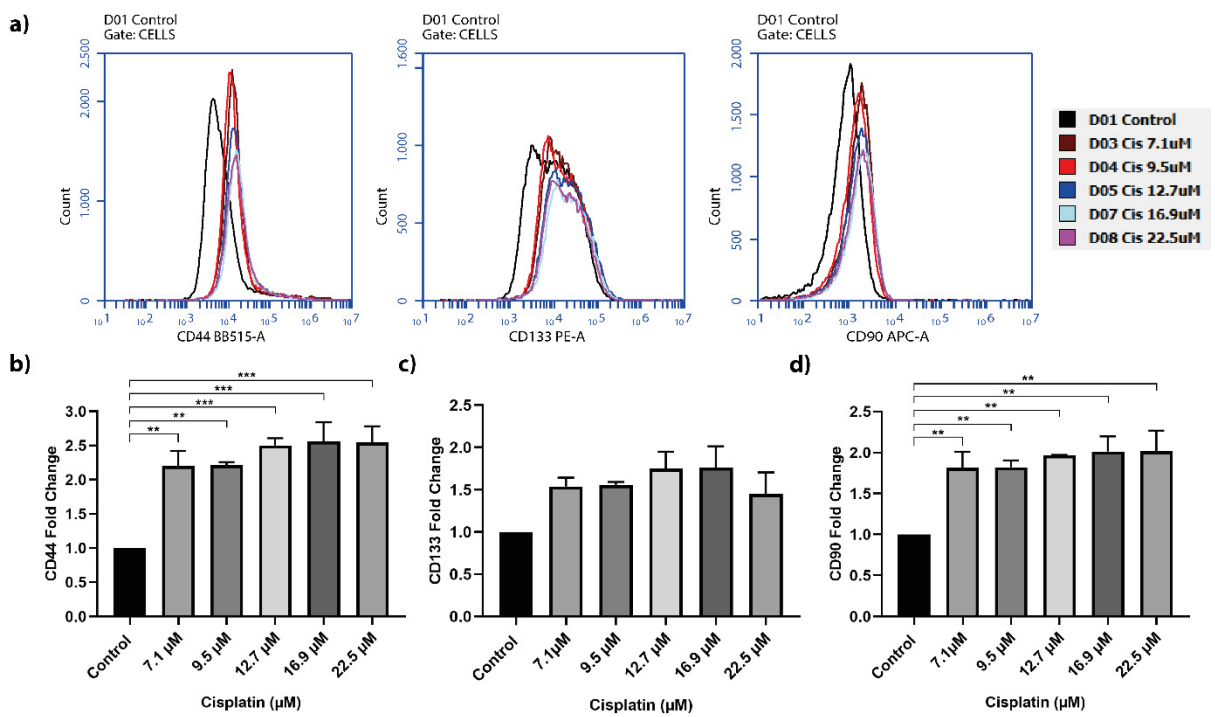

Fig. 3. Representative overlay histograms (a) and bar graphs (b-d) demonstrate the changes in the expressions of CSC markers. Median fluorescence values were obtained with flow cytometric analysis and fold changes were calculated. Statistical evaluation was carried out by one-way ANOVA and post-hoc Tukey analyses were performed. *P<0.05; **P<0.01; ***P<0.001, ****P< 0.0001 compared with control group.

CD 90 expression significantly increased after treatment with CIS at various doses. The groups did not differ significantly and the increase was not dose-dependent.

Unlike CD44 and CD90, the expression of CD133 was comparable with the control group. Although there was an increase in CD133 treatment after CIS treatment, the change was not significant. The effects of the varying doses were comparable with each other.

Discussion

In our study, CIS decreased proliferation in a dose-dependent manner and induced the expressions of CD44 and CD90 but not that of CD133 significantly in hepatocellular cancer cells. These results support that CSCs may play a role in the viability of HCC cells after CIS treatment. CIS was previously shown to increase the fraction of CSCs in head and neck cancer and the researchers suggested the emergence of CSCs as the underlying mechanism for CIS resistance.[13] In hepatoblastoma, a childhood liver cancer, it was clinically shown that increased expression of CSC markers CD44, CD90, and CD133 contributes to reduced survival.[14]

HCC cells demonstrated increased expression of CD44 in response to CIS in a dose-dependent manner. In the lung cancer cells, CIS resistance was decreased with the CD44 knockdown approach.[15] With a parallel aspect, Yin et al. reported that downregulation of CD44 inhibits lung cancer cells and the inhibition is more pronounced when combined with CIS.[16] In another study on HCC cell line, Huh7, the CD44 knockout cells demonstrated that CD44 is involved in the maintenance of CSCs. CD44 seems to be a possible target to overcome CIS resistance also in HCC.[9]

CIS treatment at different doses led to an increase in CD90 expression independent of the dose. Within many CSC markers, CD90 is pronounced as the liver stem cell marker.[10] Wang et al. obtained chemoresistant cancer cells by applying a variety of drugs including cisplatin as single agents or in combination. The researchers showed that CD90 expression increases with drug resistance in PLC, another hepatocellular cancer cell line.[17] Clinically, CD90 expression is significantly associated with rapid recurrence and poor survival in HCC.[18] Moreover, poor response to sorafenib is associated with CD90 overexpression in HCC patients.[12] CD90 is suggested to be a predictor biomarker for therapy.

CD133 expression did not increase significantly following the CIS administration. On the other hand, Zhang et al. induced ALDH1 and CD133 expressions in HepG2 cells using 0-5 μg/mL CIS.[19] Although we observed an increase in CD90 expression, in our study this increase was not found significant. In gastric cancer stem cells, CD133 was found to induce CIS resistance by increasing cell proliferation, anti-apoptosis, and autophagy abilities.[11] In laryngeal cancer, CD133 suppression with curcumin induces CIS sensitivity.[20] Suetsugu et al. examined CSC markers in three hepatocellular cell lines. While HepG2 or Hc cell lines were not stained with anti-CD133 antibody, expression was detected in Huh-7 cells.[21] Our results show the tendency for a change in CD133 expression, yet this trend did not reach statistical significance.

Subpopulations that correspond to CSCs were shown in HCC cell lines including Huh7 and PLC/PRF/5 cells.[22] Cells with CSC properties were detected in several cisplatin resistant cell lines.[23,24] The present study presents increased expression for CSC markers CD44 and CD90 after treatment with cisplatin. Studies on cultured cells isolated from primary tumors would be beneficial to further clarify the role of CSCs in tumorigenicity.

Conclusion

Accumulating evidence prompts the use of the CSCs as an important therapeutic target in HCC. Drug resistance is an important obstacle in pharmacotherapy especially in HCC and exploring drug-resistance related to CSC may lead to new targets. Highlighting the underlying mechanisms is beneficial for the development of novel therapies and might provide a strategy to predict the drug response and overcome the non-response cisplatin treatment.

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ORIGINAL PAPER

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Histological changes following the administration of two different chondroitin sulfate products in experimental osteoarthritis models in rats

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ABSTRACT

Introduction. Osteoarthritis (OA) is generally a progressive disease that affects synovial joints, resulting in abnormalities to articular cartilage subchondral bone, synovium, and adjacent soft tissues.

Aim. The purpose of this work was to examine the histological changes in knee cartilage and bone following the administration of two different chondroitin sulfate products in two experimental OA models in rats.

Material and methods. OA was induced in rats by either a single injection of mono-iodoacetate or four once-weekly injections of dexamethasone. 70 adult rats were included: 30 received mono-iodoacetate, 30 received dexamethasone and the 10 remaining controls received no injection. Samples of knee bone and cartilage were then analyzed histologically.

Results. Animals with OA that received CS had significantly less inflammation, improved motor activity, and better analgesia compared with those that did not receive CS, with little difference between products. Histologically, both products reduced the signs of OA and resulted in the activation of regenerative processes of cartilage and bone and stimulation of proliferation and formation of amorphous material.

Conclusion. These results substantiate the importance of using high-quality pharmaceutical-grade CS to ensure optimal efficacy and safety of the final product in patients with OA.

Keywords. chondroitin sulfate, chondroprotection, osteoarthritis

Introduction

Osteoarthritis (OA) is generally a progressive disease that affects synovial joints, resulting in abnormalities to articular cartilage, subchondral bone, synovium, and adjacent soft tissues.[1,2] It has been estimated to affect over 40 million people in Europe, resulting in reduced quality of life and significant healthcare costs.[3] Treatment options include non-pharmacological interventions (e.g. exercise, weight loss) and pharmacological treatments.[4,5] Guidelines from the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO) for patients with knee osteoarthritis suggest the

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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following – escalating – pharmaceutical options: Symptomatic Slow-Acting Drugs in Osteoarthritis (SYSADOAs), e.g. glucosamine sulfate or chondroitin sulfate (CS); paracetamol; non-steroidal anti-inflammatory drugs; intra articular injections hyaluronic acid and/or corticosteroids; and opioids.[5]

CS, which is a component of cartilage and bone, has been widely tested as a treatment for osteoarthritis.[2] As recently reviewed by Hochberg et al., various *in vitro* and *in vivo* animal studies have shown that CS has anti-inflammatory and anti-apoptotic effects; exerts a beneficial effect on the metabolism of chondrocytes and subchondral bone cells; and reduces cartilage destruction.[2] Meta-analyses of clinical studies have also shown that CS can result in a reduction in joint space width decline and pain.[6,7] However, as recently reviewed by Martel-Pelletier et al., not all CS products are equivalent. CS is a complex, heterogeneous polysaccharide that is extracted from the cartilage of various animals using a variety of extraction processes.[8] As such, different CS products can have different CS content, structure, and molecular weight.[9] This variability could compromise the efficacy and safety of the final product. For this reason, it is very important that patients use a high-quality, pharmaceutical-grade CS as has been used in clinical trials in patients with osteoarthritis, and which has been demonstrated to be effective and safe.[8,10,11]

Aim

The purpose of this work was to examine the histological changes in knee cartilage and bone following the administration of two different CS products in two experimental osteoarthritis models in rats.

Material and methods

Design

A total of 70 healthy adult mongrel white rats (aged 10-12 weeks; weight 180-230 g) that passed acclimatization for 10 days were randomized (on Day 0) into seven groups of 10 animals (5 male and 5 female). There was one control group and six experimental groups, as listed in Fig. 1.

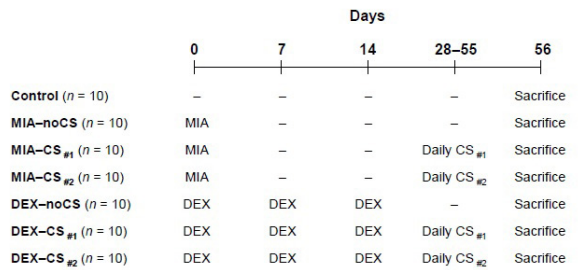

Fig. 1. Study design – $CS_{\#1}$ chondroitin sulfate (Artedja Injections), $CS_{\#2}$ chondroitin sulfate (Mukosat neo), *DEX* dexamethasone, *MIA* mono-iodoacetate

Ethics approval

The Committee on Bioethics of the SI "Dnipropetrovsk Medical Academy of the Ministry of Health of Ukraine" approved the study protocol and all procedures related to the maintenance of the animals, their humane treatment, and their use in the experiments. These also complied with Good Laboratory Practice requirements and the European Convention for the Protection of vertebrate animals used for experimental and other scientific purposes.

Osteoarthritis models

Three groups of rats had osteoarthritis induced by mono-iodoacetate (MIA), and three groups using systemic dexamethasone (DEX) suppression (Fig. 1). The MIA osteoarthritis model involved a single MIA injection (3 mg in 50 µL of sterile saline) into the right hind leg knee joint, as described by Guingamp et al. on Day 0.[12,13] The DEX suppression osteoarthritis model involved three intramuscular injections of DEX solution (7 mg/kg) into the femoral muscle on Days 0, 7 and 14 (Fig. 1).

Chondroitin sulfate administration

$CS_{\#1}$ was Artedja Injections (PRJSC "Fitofarm", Ukraine), whose raw material is CS produced by Bioibérica S.A.U. (Barcelona, Spain). $CS_{\#2}$ was Mukosat neo (RUE Belmedpreparaty, Republic of Belarus). Both products contain chondroitin-4-sulfate and chondroitin-6-sulfate of bovine origin. $CS_{\#1}$ is highly purified (99.9%) and has an average molecular weight of 15.1 kDa. This product has been approved as a prescription treatment for OA in many European countries. $CS_{\#2}$ has a purity of 99.4% and a lower molecular weight (10.3 kDa). Characteristics of both raw materials (for the specific batches used in this study) are detailed in Table 1.

Table 1. Chondroitin sulfate characteristics

Characteristic	$CS_{\#1}$	$CS_{\#2}$
Species	Bovine	Bovine[a]
CS content (%)	99.9	99.4
Molecular weight (kDa)	15.1	10.3
Intrinsic viscosity (m^3/kg)	0.040	0.051
Chlorides (%)	0.34	0.0167
Free sulfates (%)	0.14	0.035
Oxalate (%)	0.01	0.0040
ΔDisaccharide 0-S (%)	5.7	5.5
ΔDisaccharide 4-S (%)	62.8	58.4
ΔDisaccharide 6-S (%)	31.5	28.8

CS chondroitin sulfate, $CS_{\#1}$ chondroitin sulfate (Artedja Injections), $CS_{\#2}$ chondroitin sulfate (Mukosat neo).
[a] Source assumed to be bovine based on disaccharide composition.

Both products were solutions for injection in 2 mL ampoules containing 200 mg CS (100 mg/mL). Animals in the relevant groups (see Fig. 1) were injected intramuscularly with one of the CS products (35 mg/kg/day) during Days 28-56 (Fig. 1). This dose was based on the experience of the team and recommendations from the Center for Drug Evaluation and Research Ministry of Health of Ukraine.[14-18]

Physical parameters

Four rats per group were assessed for the influences of $CS_{\#1}$ and $CS_{\#2}$ on knee size (MIA model only). The knees were measured (largest circumference) at baseline and Days 28 and 56 using micrometer engineering. Four rats per group were assessed for the influences of $CS_{\#1}$ and $CS_{\#2}$ on activity and their analgesic effect (MIA and DEX models). Activity was assessed on Days 28 and 56 by placing the rats into a 1 m×1 m area that had been divided into 16 squares, each with a 3-cm diameter hole. The following parameters were assessed during 2 minutes: the number of borders crossed (horizontal motor activity), the number of hind-leg rises (vertical motor activity), the number of burrows (i.e. looks into the holes; research activity), the number of defecation acts (emotional activity), and the number of grooming acts. Analgesia was assessed on Day 56 by immersing the tails 3 cm into hot water (50°C) and measuring the time to tail flick.

Sectioning and Histology

All animals were killed accordind to Ethical Approvement on Day 57 by intraperitoneal administration of a thiopental sodium solution (40 mg/kg body weight) and samples of bone and cartilage from the right knees were taken. Tissue samples were fixated using 10 % neutral formalin for 5-7 days. They were then decalcified using 10 % nitric acid and embedded in celloidin-paraffin. A microtome was used to prepare thin slices (6-8 μm), which were stained using hematoxylin–eosin. Microphotography was performed using a Ulab XY-B2T microscope.

Statistical analysis

Physical parameters are reported as means ± errors. Depending on the normality of the distribution (as assessed using the Shapiro-Wilk test) and the groups being compared, the Student's t-test, the paired t-test, the Mann-Whitney U-test, or the paired Wilcoxon test were generally used. For knee circumference, a one-way dispersion analysis and Duncan's test were used. The level for significance was taken to be $P<0.05$. Statistical processing was performed using STATISTICA 6.1 software product provided (StatSoft Inc., serial No AGAR909E-415822FA).

Results

Physical parameters

Mean knee circumference increased by 37% from baseline to Day 28 in the MIA–noCS group (P<0.05). On Day 56, rats in the MIA–$CS_{\#1}$ and MIA–$CS_{\#2}$ groups had smaller knees than those in the MIA–noCS group (–22% and –18%, respectively; both P<0.05) (Table 2), showing an anti-inflammatory effect of both CS products.

On Days 28 and 56, motor activity was reduced in the MIA–noCS and DEX–noCS groups compared to Control rats (–18 to –55%; P<0.05) (Table 2). On Day 56, rats in the MIA–$CS_{\#1}$ and MIA–$CS_{\#2}$ groups had better motor activity than those in the MIA–noCS group (25–35% improvement; P<0.05); those in the DEX–$CS_{\#1}$ and DEX–$CS_{\#2}$ groups had only slightly bet-

Table 2. Knee circumference, motor activity (squares visited and hind-leg stands), research activity (burrows), emotional activity (defecation acts), grooming acts, and analgesia (time to tail flick after immersion in hot water) on Day 56

	Control 1 (n = 4)	MIA–noCS (n = 4)	MIA–$CS_{\#1}$ (n = 4)	MIA–$CS_{\#2}$ (n = 4)	Control 2 (n = 4)	DEX–noCS (n = 4)	DEX–$CS_{\#1}$ (n = 4)	DEX–$CS_{\#2}$ (n = 4)
Knee circumference (mm)	23.8±0.4[a]	34.5±0.8*	26.8±0.6**	28.2±1.1**	NA	NA	NA	NA
Borders crossed (n)	14.9±0.5	9.6±0.5*	12.9±0.6**	12.7±0.6**	19.2±0.3	14.3±0.5†	15.9±0.8	15.8±0.8
Hind-leg stands (n)	6.5±0.5	2.9±0.3*	3.7±0.2**	3.9±0.4**	7.9±0.4	6.2±0.6†	6.9±0.4	7.0±0.5
Burrows (n)	1.1±0.9	1.0±0.7	1.1±0.8	1.2±1.0	1.3±1.1	1.1±0.7	1.2±0.9	1.2±1.1
Defecation acts (n)	1.8±1.2	1.4±1.0	1.7±1.1	1.6±1.2	2.3±1.3	1.9±1.4	2.2±1.6	2.1±1.2
Grooming acts (n)	12.4±3.9	11.3±3.6	12.6±4.2	13.1±3.8	12.4±3.9	11.3±3.6	12.6±4.2	13.1±3.8
Time to tail flick (s)	113.5±0.6	91.7±0.5*	106.0±1.5**	105.0±1.5**	102.9±0.7	87.6±1.0†	102.0±0.7††	105.4±1.2††

Data are mean ± error. *$CS_{\#1}$* chondroitin sulfate (Artedja Injections), *$CS_{\#2}$* chondroitin sulfate (Mukosat neo), *MIA* monoiodoacetate, *NA* not available, *noCS* no chondroitin sulfate administered

[a]This value was a baseline measurement in the MIA–noCS group; all other values in the Control 1 column are at Day 56 in the Control 1 group.

*P<0.05 versus Control 1 (or baseline MIA–noCS for knee circumference)

**P<0.05 versus MIA–noCS

† P<0.05 versus Control 2

†† P<0.05 versus DEX–noCS

ter motor activity than those in the DEX–noCS group (8–11% improvement; NS) (Table 2). There were no significant differences in research activity, emotional activity, or grooming between groups at either time point (Table 2).

At Day 56, time to tail flick after hot water immersion was significantly reduced in the MIA–noCS and DEX–noCS groups compared to Control rats (–15% to –19%; P<0.05). Rats in the MIA–$CS_{\#1}$, MIA–$CS_{\#2}$, DEX–$CS_{\#1}$, and DEX–$CS_{\#2}$ had significantly longer times compared to the MIA–noCS and DEX–noCS groups, respectively (15–17% improvement; P<0.05) (Table 2). This indicates that both CS products had an analgesic effect.[17,18]

Histological results

Control group

In histological slides of Control rat knees, the perichondrium, cartilage, and subchondral bone are well visualized (Fig. 2).

The perichondrium, which is moderately oxyphilic, consists of two layers – superficial and deep (cellular and fibrous) – which together form a thin layer around the cartilage. In the deep layer of the perichondrium, there are small, nuclear-type, moderately basophilic cells. The surface layer of the cartilage contains many cells, often arranged in pairs, with intensely basophilic nuclei. The cartilage has is an even, weakly oxyphilic color without areas of hyperchromia. The deeper cartilage is mainly composed of an amorphous substance, with widely spaced groups of cells that have weakly basophilic nuclei, similar in color to that of the amorphous substance. The cartilage contains small groups of 4-10 cells (Fig. 2a), some of which have intensely basophilic nuclei. The boundary between cartilage and bone tissue is clearly visible. The bone trabeculae are moderately oxyphilic and plates of bone tissue have orderly architecture. The osteocytes in the bone tissue have basophilic nuclei. Bone marrow sites and vessels are visible between the trabeculae.

Mono-iodoacetate osteoarthritis model

No chondroitin sulfate

Among rats in the MIA–noCS group, the synovium was thickened, the structure was loose and heterogeneous, and there were areas of degradation (Fig. 3a).

The subchondral bone tissue had altered chromophility, with an increased degree of basophility of some trabeculae. In some parts of the surface, there were visible areas of bone destruction (arrow in Fig. 3a). The surface of the perichondrium had an uneven edge, sometimes vacuolated (* in Fig. 3b), and some layers of the perichondrium had been destroyed due to swelling. The amorphous substances of the deep zones have a hyperchromatic basophilic color. The deep zones also have areas of cell destruction, which are hyperchromic, their structure is not clearly visible (arrows in Fig. 3b). Superficial areas of the basic substance are lighter in color due to swelling of the amorphous substance.

Compared with Control rats, MIA–noCS rats had signs of inflammation of the synovial membrane, disruption of the structure of the perichondrium with swelling, disturbance of trophism of the deep layers of cartilage, destruction of a number of chondrocytes, and changes of the histochemical properties of amorphous materials. Marked destruction of bone tissue was observed and there were isolated pockets of violation of the architecture of the bone trabeculae.[17]

Chondroitin sulfate #1

In the MIA–$CS_{\#1}$ group, the outer contour of the perichondrium was uneven, but without areas of vacuolation (Fig. 3c). No signs of tissue swelling or opening were observed. The density of cells in the germ layer was comparable to that in Control animals. The cartilage contained groups of 4–8 slightly hypertrophied cells,

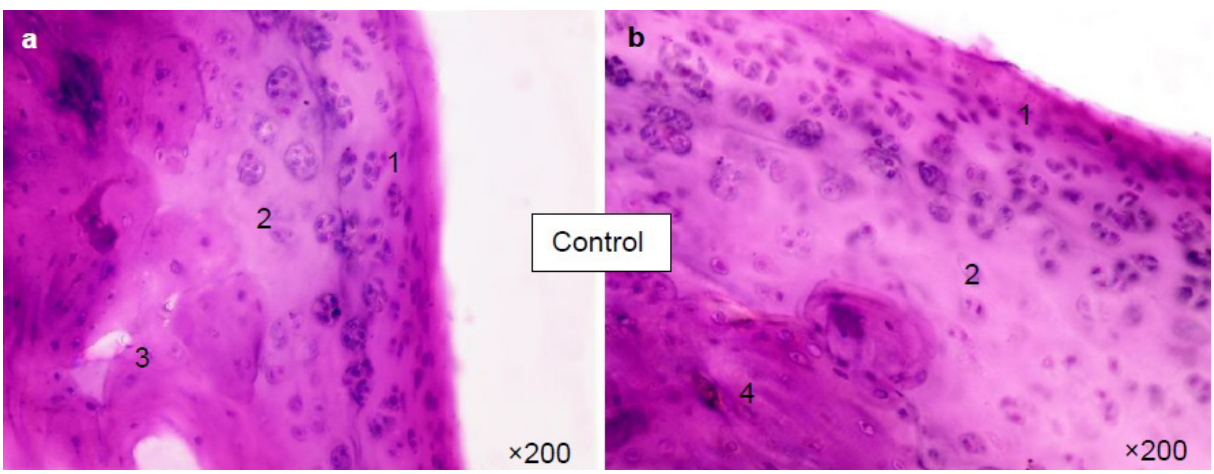

Fig. 2. Knee articular cartilage from Control animals. These images are representative of all Control animals. *1* perichondrium, *2* cartilage, *3* bone trabecula, *4* bone tissue

and the density of the cells in the surface layer was ≥1.5 times higher than in the MIA–noCS group. Unlike in MIA–noCS rats, hyperchromic areas were not observed in MIA–$CS_{\#1}$ rats. The transition zone in the subchondral bone had an irregular contour. There were visible sites of implantation of the bone tissue into the cartilage, indicating active bone regeneration. There were also visible areas of neovasculogenesis (arrows in Fig. 3d). The synovium had a structure comparable to that of Control animals, except for residual signs of inflammation in the joint capsule. Areas of subchondral bone bordering the cartilage had no pronounced structure, indicating that this was newly formed tissue.

Thus, while rats in the MIA–noCS group expressed signs of osteoarthritis, those in the MIA–$CS_{\#1}$ group did not. Cartilage and bone tissue samples from the MIA–$CS_{\#1}$ group were largely comparable to those from the Control group, but with active regeneration of cartilage and bone tissue, with areas of neovascularity. However, $CS_{\#1}$ did not result in recovery of the pronounced inflammation of the joint capsule induced by MIA.

Chondroitin sulfate #2

Cartilage from the knees of rats in the MIA–$CS_{\#1}$ group had a deformed contour (arrows in Fig. 3e). The perichondrium was not expressed and the distribution of layers (fibrous and deep, germ) was violated. Zones of active proliferation were observed in the cartilage and perichon-

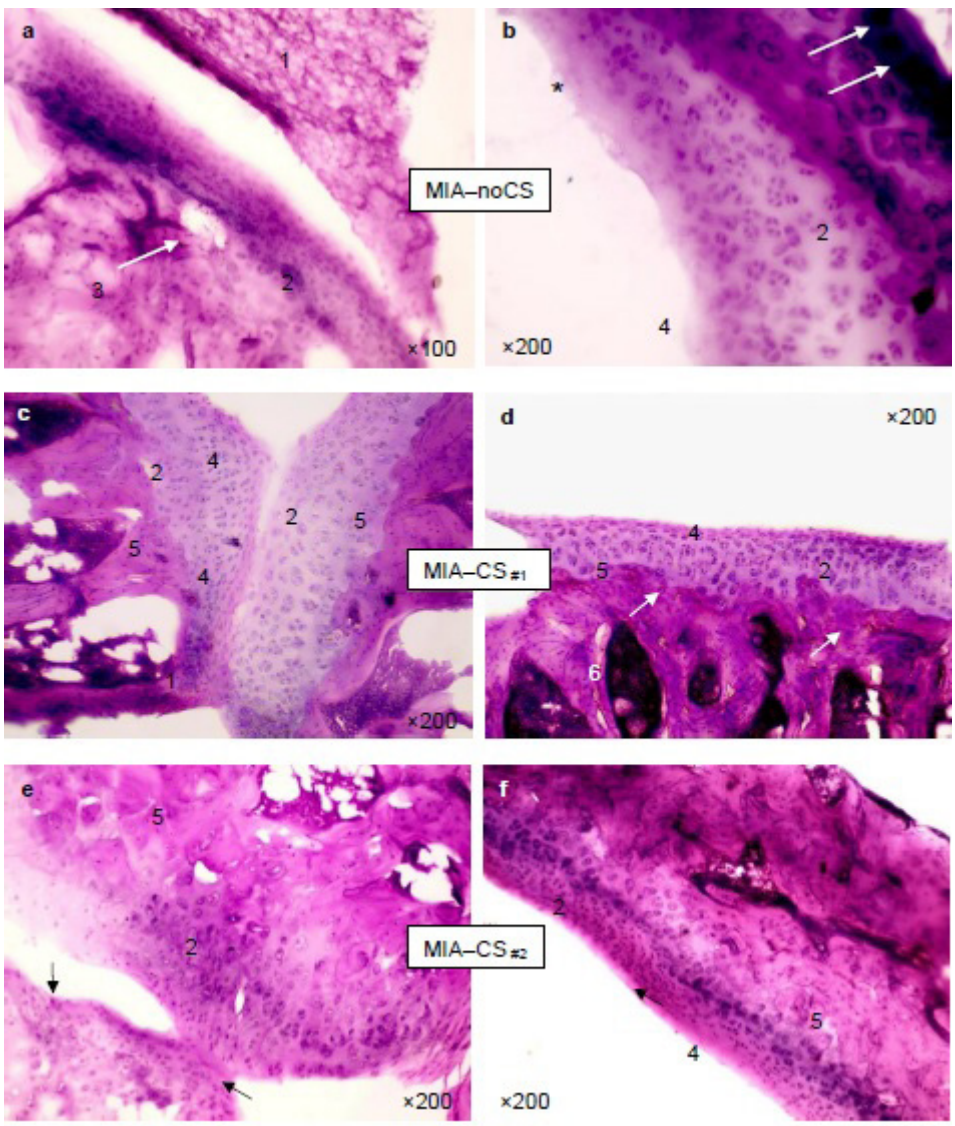

Fig. 3. Articular cartilage of the knee joints of rats in the **a** and **b** MIA–noCS, **c** and **d** MIA–$CS_{\#1}$, and **e** and **f** MIA–$CS_{\#2}$ groups. These images are representative of all animals in the respective groups. *1* synovial capsule, *2* cartilage, *3* bone trabeculae, *4* perichondrium, *5* subchondral bone, *6* bone marrow, * vacuolated perichondrium, *MIA–$CS_{\#1}$* rats with mono-iodoacetate-induced osteoarthritis given chondroitin sulfate (Artedja Injections), *MIA–$CS_{\#2}$* rats with mono- iodoacetate-induced osteoarthritis given chondroitin sulfate (Mukosat neo), *MIA–noCS* rats with mono-iodoacetate-induced osteoarthritis given no chondroitin sulfate. Arrows show: **a**, **b** bone destruction, **d** neovasculogenesis, **e** deformed contour, **f** active proliferation.

drium, defined by a high cell density (arrow in Fig. 3f). However, the chromophilic properties of the amorphous substance and cells were dissimilar to those in Control rats. The structure of the connective tissue (forming the joint capsule) was loose, with signs of swelling. The trabeculae were moderately oxyphilic and consisted of unstructured amorphous material and randomly distributed osteocytes, suggesting that it was "young" tissue. The boundary between cartilage tissue and bone had become blurred.

Compared with the MIA–noCS group, those in the MIA–$CS_{\#2}$ group had stimulated proliferation and formation of amorphous substance in both cartilage and bone tissue. However, samples were not fully comparable with Control animals – there were residual effects of osteoarthritis in the form of modified histochemical properties of amorphous substances and swelling of the connective tissue in the joint capsule. Like $CS_{\#1}$, $CS_{\#2}$ also did not result in recovery of the pronounced inflammation of the joint capsule induced by MIA.

Dexamethasone osteoarthritis model

No chondroitin sulfate

The perichondrium from rats in the DEX–noCS group had a reduced oxyphilic color and consisted of two layers – deep germ and superficial fibrous. The perichondrium was, on average, four times thicker than in animals from the Control group due to swelling (Fig. 4a and 4b).

The cells in the cartilage were randomly placed and

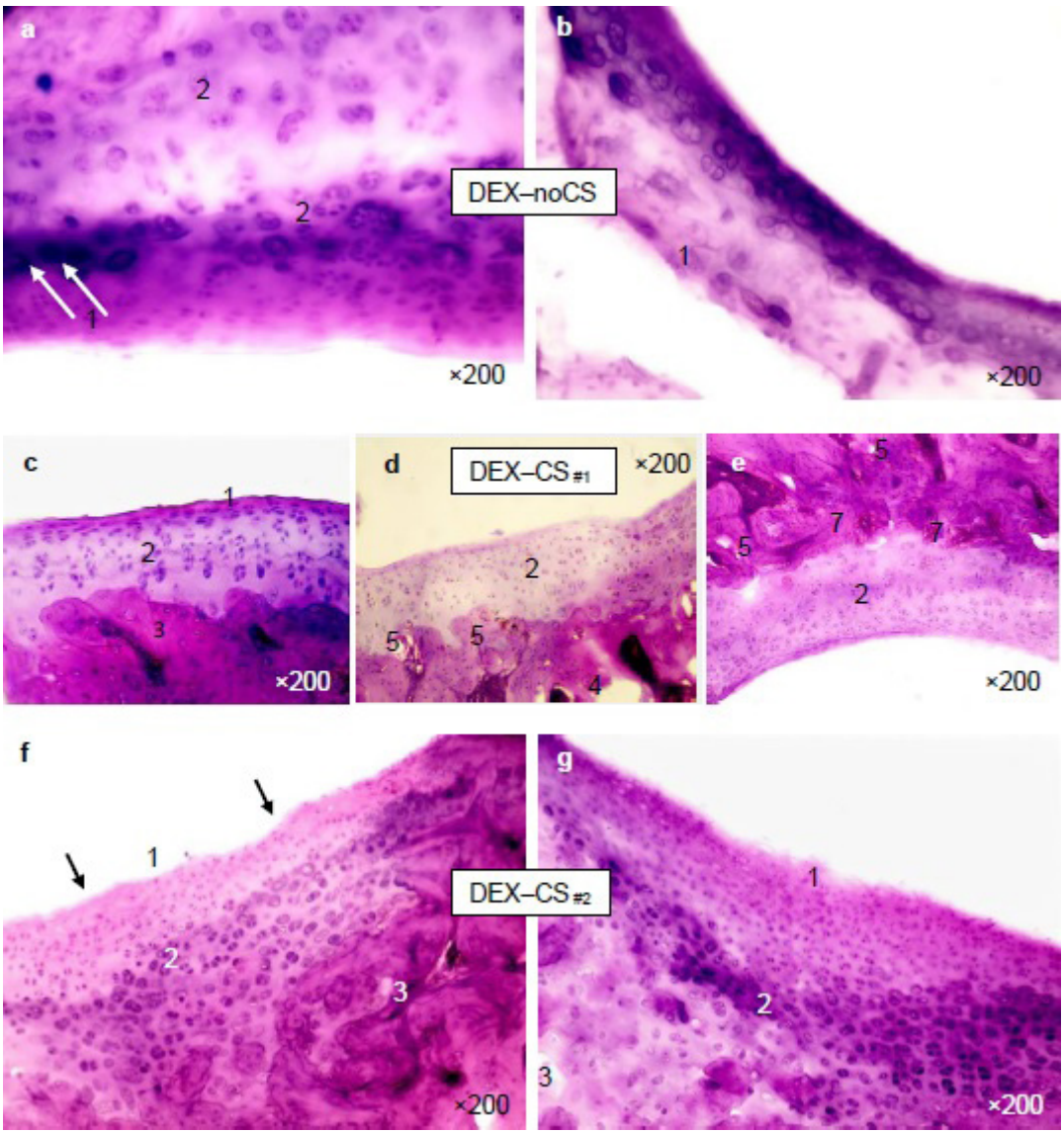

Fig. 4. Articular cartilage of the knee joints of rats in the a and b DEX–noCS, c–e DEX–$CS_{\#1}$, and f and g DEX–$CS_{\#2}$ groups. These images are representative of all animals in the respective groups. *1* perichondrium, *2* cartilage, *3* subchondral bone, *4* area of destroyed bone, *5* zone of active osteogenesis, *6* bony trabeculae, *7* vessels, *DEX–$CS_{\#1}$* rats with dexamethasone-induced osteoarthritis given chondroitin sulfate (Artedja Injections), *DEX–$CS_{\#2}$* rats with dexamethasone-induced osteoarthritis given chondroitin sulfate (Mukosat neo), *DEX–noCS* rats with dexamethasone-induced osteoarthritis given no chondroitin sulfate. Arrows show: a apoptosis, f tissue swelling.

had a weakly basophilic cytoplasm and intensely colored nuclei. One third of the cells had a hyperchromatic nucleus. In the area of cartilage close to the perichondrium, there were hyperchromatic cells undergoing apoptosis (arrows in Fig. 4a). The outer contour of the cells had an intense basophilic color: some were fully "defined" in this way, others, only partly, which we believe represents a different stage of apoptosis. In deeper sites, there were other hyperchromatic cells, but in smaller amounts than in the surface layers (ratio 1:10). Bone tissue showed areas of thinning of the bone trabeculae and areas of violated integrity (destruction), with small cavities inside the trabeculae.

Compared with Control rats, those in the DEX–noCS group had signs of inflammation (swelling) of the cartilage, activated cell death, and changes in the histochemical properties of the amorphous substances. In addition, the bone had signs of destruction. These signs indicate that the DEX induced osteoarthritis in the rats.

Chondroitin sulfate #1

The cartilage from rats in the DEX–$CS_{\#1}$ group had a thin perichondrium with an intensive oxyphilic color and chondroblasts in the deep layer (Fig. 4c). The density of chondroblasts was higher than in Control rats (Fig. 4c vs Fig. 2b). While rats in the DEX–noCS group had hyperchromic areas, these were not observed in DEX–$CS_{\#1}$ animals. The main substance of the cartilage was generally poorly basophilic (Fig. 4d) and consisted of an amorphous substance and groups of cells. The density of the cells in the deep cartilage was slightly higher than in the DEX–noCS group. The contact zone of the cartilage and bone tissue was well visualized. The bone trabeculae had a large area and were moderately oxyphilic, sometimes basophilic. There were signs of active regenerative processes: no areas of destruction in the newly formed bone, a disordered architecture of the trabeculae, and active vasculogenesis on the cartilage–bone border (Figs. 4d and 4e).

Compared to DEX–noCS rats, those in the DEX–$CS_{\#1}$ group had no signs of inflammation or destruction of bone tissue (i.e. osteoarthritis). An active recovery process (regeneration) with the activation of proliferative potential of cartilage and bone tissue had taken place.

Chondroitin sulfate #2

In DEX–$CS_{\#2}$ rats, the perichondrium had a moderately oxyphilic color and a clear boundary with the cartilage (Fig. 4f). The density of cells in the perichondrium was higher than in Control animals and it was several times thicker. The outer contour of the cartilage was uneven and there were small areas of tissue swelling (arrows in Fig. 4f). The cartilage was characterized by high-density groups of chondrocytes, some with an intensively basophilic color. Areas of hyperchromia were observed (Fig. 4g), as were seen in DEX–noCS rats. The boundary between cartilage and subchondral bone was blurred. There were signs of regeneration of bone tissue, but without obvious neovascularity. The amorphous substance of the bone tissue was intensively oxyphilic and the bone trabeculae were not ordered.

Compared with DEX–noCS rats, those in the DEX–$CS_{\#2}$ group had active regeneration of cartilage and bone tissue. However, there were still residual signs of osteoarthritis.

Discussion

Physically, rats in the MIA–$CS_{\#1}$ and MIA–$CS_{\#2}$ groups had significantly reduced knee swelling, improved motor activity, and analgesia compared with those in the MIA–noCS group after 4 weeks of CS injections. Histologically, animals in the MIA–$CS_{\#1}$ and DEX–$CS_{\#1}$ groups had regeneration of bone and cartilage, resulting in tissue structures similar to those in Control rats. Although $CS_{\#2}$ stimulated regeneration of bone and cartilage tissue, it was less effective than $CS_{\#1}$, and some morphological parameters were different from the Control group. Overall, in both the MIA and DEX osteoarthritis models, $CS_{\#1}$ had a more pronounced beneficial effect than $CS_{\#2}$.

These differences could be at least partly explained by the varying properties of the two CS products. Further, the source and structure of CS can result in differences in bioavailability and pharmacokinetic variables.[19] The number and positions of sulfate groups generally differ in CS extracted from different animal sources.[9] In this study, as indicated in Table 1, both CS products are from bovine origin according to the disaccharide composition identified. However, one parameter that is very different between the two compounds is the percentage of free sulfates (0.14 for $CS_{\#1}$; 0.035 for $CS_{\#2}$). Thus, although further studies are required to establish a definite correlation between chemical structure and activity, the difference in free sulfates might contribute to the differential effects of the two tested CS products.

Such discrepancies between CS products is not a new phenomenon. A review by Martel-Pelletier et al. highlighted the differences in purity, composition, chemical properties, and *in vitro* effects between different CS products.[8] Recently, Li et al. tested 15 different CS products: three commercially available CSs (from shark [Yantai Dongcheng Co., Ltd., Yantai, China], porcine [Huamao Shuanghui Co., Ltd., Luohe, China], and bovine [Shandong Kangping Bio Technology Co., Ltd., Linyi, China] cartilage) and 12 low-molecular-weight CSs that they had produced by degradation (four different methods) of the three CS products.[20] *In vitro* testing was used to ascertain which CSs had the best and worst anti-complement activity. These CSs were then given orally at doses of 50, 150, or 450 mg/kg ("best

CS") or 150 mg/kg ("worst CS") to mice in which osteoarthritis had been induced by surgical destabilization of the medial meniscus. The two highest doses of the "best CS" significantly attenuated articular cartilage erosion, while the "worst CS" had a small, insignificant effect.[20] These and our results highlight the differences in effects between CS products and the importance of using high-quality CS.

A number of other histological studies have also examined the effects of CS, but CS sources, doses, durations and routes of administration, animals, and osteoarthritis models have varied widely (Table 3).[20-28]

We chose a dose of 35 mg/kg/day for 4 weeks, but other studies have used doses as low as 500 mg/kg/month or as high as 450 mg/kg/day for durations ranging from just 12 days to nearly 1.5 years.[20,22,24,25] Two of the studies showed that higher doses of CS were more beneficial – Li et al., (as discussed above) and Campo et al.[20,21] The latter induced arthritis in mice via an intradermal injection of bovine type II collagen in complete Freund's adjuvant at the tail base and then administered intraperitoneal CS (Sigma–Aldrich Srl, Milan, Italy) at doses of 30, 60, and 120 mg/kg for 25 days.[21] They found that CS dose-dependently reduced cartilage erosion, proteoglycan depletion, and inflammation; as well as the incidence and severity of arthritis. Regarding length of treatment, Taniguchi et al.[22] studied the effects of CS or glucosamine in Hartley guinea pigs (bred to develop spontaneous osteoarthritis). Oral CS (Seikagaku Co., Tokyo, Japan) 200 mg/kg/day – administered from age 3 weeks to 8, 12, or 18 months – reduced cartilage degeneration at each time point, with better results at 12 and 18 versus 8 months.

Another important factor to consider is the route of administration. In the current study, both products were administrated via intramuscular injection, ensuring higher bioavailability compared to oral treatment. The other studies detailed in Table 2 administered CS orallyor by injection (intraperitoneal, subcutaneous or into the knee joint).[20-28] Although the kinetics of CS are still not well understood, studies performed by Conte et al. suggest that the absolute bioavailability of orally administered CS is 13.2% in humans.[29] Therefore, parenteral intramuscular administration could be a useful approach for CS therapy. In animal *in vivo* experiments, it has been demonstrated that CS administered to rats by intramuscular injection results in very rapid increases in plasma concentrations, with distribution to the liver, cartilage and kidneys.[30] Since the absolute bioavailability of orally administered CS is 13.2%, the bioavailability by intramuscular injection is more than in seven times that of oral administration.[29-31] Hence, the intramuscular route becomes an interesting choice for patients with osteoarthritis.

We studied two different osteoarthritis models – MIA and DEX – both in rats. MIA, at the dose used in this study, has been shown to have a destructive effect on the osteochondral structures of the knee joint, quick-

Table 3. Summary of histological studies that included a "CS alone" arm in animal models of osteoarthritis

Study	CS source(s)	Dose	Route	Duration (weeks)	Animal	Osteoarthritis model	Histological outcome
Current study	PRJSC Fitofarm ($CS_{#1}$) and RUE Belmedpreparaty ($CS_{#2}$)	35 mg/kg/day	IM	4	Rats	MIA or DEX	$CS_{#1}$ more chondroprotective than $CS_{#2}$
Li et al.[20]	Various[a]	50, 150, 450 mg/kg/day	PO	12	Mice	Surgical	"Best CS" attenuated osteoarthritis via the complement system
Campo et al.[21]	Sigma–Aldrich Srl	30, 60, 120 mg/kg/day	IP	4	Mice	Bovine type II collagen	Dose-dependent inflammation; cartilage erosion; apoptosis activation inhibited
Taniguchi et al.[22]	Seikagaku Co.	200 mg/kg/day	PO	32, 49, 75	Hartley guinea pigs	Spontaneous	Cartilage degeneration
Largo et al.[23]	Bioibérica S.A.U.	100 mg/kg/day	IP	5	Rabbits	Ovalbumin	Inflammation; synovial lesions
Xiao et al.[24]	NR	500 mg/kg/month	PO	4, 9, 13, 17, 22	Hartley guinea pigs	Spontaneous	Pathological lesions delayed
Caraglia et al.[25]	NR	0.3 mg/day	PO	2	C57 Black 6N mice	Spontaneous	Histological features of chondrodegeneration; apoptosis
Permuy et al.[26]	Bioibérica S.A.U.	11.5 mL/kg/day	IP	8	Rabbits	Surgery	Cartilage swelling; no effect on cartilage surface, synovial membrane, subchondral bone
Torelli et al.[27]	NR	1 mL of 12%/week	SC	12	Rabbits	Immobilization	Not effective
Chen et al.[28]	DongCheng Biochemicals Co., Ltd.	0.3 mL/week	Knee injections	5	Rabbits	Papain	Degenerative changes not significantly improved vs control

CS chondroitin sulfate; *DEX* dexamethasone, *IM* intramuscular, *IP* intraperitoneal; *MIA* mono-iodoacetate, *PO* per os, *NR* not reported
[a]Yantai Dongcheng Co., Ltd., Huamao Shuanghui Co., Ltd., Shandong Kangping Bio Technology Co., Ltd., and 12 degradation products

ly resulting in osteoarthritis-like lesions and function impairment.[12] Chronic exposure (once per week for 3 weeks) of high-dose DEX (7 mg/kg) has been shown to result in the apoptotic death of 50–70% of rat articular cartilage cells.[15] Other studies, however, have used different osteoarthritis models – spontaneous or induced surgically; by immobilization; or using ovalbumin, papain, or bovine type II collagen in guinea pigs, mice, or rabbits (Table 3).[20-28]

Seven of the nine studies listed in Table 3 reported at least some beneficial effect of CS on histological parameters, three of which have been discussed above.[20-22] In addition, Largo et al. induced inflammatory arthritis and atherosclerosis in rabbits by intraarticular injections of ovalbumin and a hypercholesterolemic diet.[23] Compared with control rabbits, intraperitoneal CS (Bioibérica S.A.U.) 100 mg/kg/day for 5 weeks reduced signs of synovitis and partially prevented inflammatory cell infiltration and intimal layer proliferation in the synovial membrane. Xiao et al. studied the effects of glucosamine (1000 mg/kg) and/or CS (500 mg/kg) monthly for 5 months in Hartley guinea pigs.[24] Pathological lesions developed in the articular cartilage after 1 month in untreated animals, but not until after 3 or 4 months in glucosamine- or CS-treated animals, respectively. Guinea pigs given glucosamine plus CS had virtually no pathological changes by study end.[24] Caraglia et al. tested CS (0.3 mg/day for 12 days) and/or "earth elements" mud therapy (once daily for 12 days) in a spontaneous osteoarthritis mouse model.[25] They found that CS had a beneficial effect on apoptosis and chondrodegeneration, which was further improved by the addition of mud therapy.[25] Permuy et al. tested CS against a range of other SYSADOAs in rabbits with surgically induced osteoarthritis.[26] The SYSADOAs were administered for 8 weeks, starting 3 weeks after surgery. Although intraperitoneal CS (Bioibérica S.A.U.) 11.5 mL/kg/day prevented cartilage swelling, similarly to the other SYSADOAs tested, it had no effect on the cartilage surface, synovial membrane, or subchondral bone. However, the dose of CS in this study is unclear as the strength of the CS solution was not reported.

Two of the studies in Table 3 reported a lack of effect of CS. Torelli et al. induced osteoarthritis in rabbits by immobilization of one knee for 12 weeks. Subcutaneous CS (1 mL of a 12% solution), administered weekly for 12 weeks, did not reduce the histological changes induced by this osteoarthritis model.[27] However, the source of CS was not reported, and CS was only administered once each week, which may explain the lack of efficacy. Chen et al. induced osteoarthritis in rabbits by injecting papain into both knees.[28] CS (prepared from CS [DongCheng Biochemicals Co., Ltd., Yantai, Shandong, China] that had been boiled for 30 min and filtered) and/or hyaluronic acid were injected into the knees once weekly for 5 weeks. Histological studies showed that intra articular hyaluronic acid had a chondroprotective effect, but that oral CS alone had no significant benefit over control, and hyaluronic acid plus CS introduced intra articular was not significantly better than hyaluronic acid alone.[32-34] However, it is unclear what dose of CS was given, or what effect boiling the CS could have had.

Overall, the current study and prior histological animal studies indicate that CS is likely to have a chondroprotective effect, but that factors such as the CS product used and its dose, route of administration, and duration of dosing can affect efficacy.

Conclusions

In two rat osteoarthritis models – in which osteoarthritis was induced by MIA or DEX – both CS products tested resulted in activation of the regenerative processes in cartilage and bone tissue. However, $CS_{\#1}$ had a stronger chondroprotective effect on cartilaginous tissue than $CS_{\#2}$ in both models. These results, along with those from various other studies, highlight the importance of using a high-quality pharmaceutical-grade active principle ingredient of CS in order to ensure optimal efficacy and safety of the final product in patients with osteoarthritis.

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Changes in haematological parameters and serum beta-2-microglobulin levels in CD4⁺ T-cells-stratified Nigerian HIV patients

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ABSTRACT

Introduction. Reports have shown that there is a rise in beta-2-microglobulin (β2M) concentration in patients with HIV infection and that the degree of elevation correlates well with the extent of disease burden and could be an independent prognostic marker for death. However, there is the dearth of information on the interplay between alteration in haematological profile, a common cause of morbidity and mortality in HIV, and β2M.

Aim. Changes in selected haematological parameters and β2M in Nigerian HIV patients stratified based on CD4⁺ T-cells counts were thus assessed in this study.

Material and methods. Forty-eight asymptomatic, drug naïve HIV patients were enrolled into this cross-sectional study. Haemoglobin concentration (Hb), packed cell volume (PCV), total and differential white blood cell count, platelet count and CD4⁺ T-cells count were determined using standard methods while serum levels of β2M were determined using ELISA. Thereafter, the patients were stratified into three groups based on the CD4⁺ T-cells count.

Results. Hb and lymphocyte counts increased with increasing CD4⁺ T-cells count. In contrast, neutrophils percentage, MCV and MCH reduced with increasing CD4⁺ T-cells count. The mean lymphocytes percentage was significantly higher while the mean neutrophils percentage was significantly lower in patients with CD4⁺ T-cells count of 500–800 cells/μl compared with the patients with CD4⁺ T-cells count <200 cells/μl. Similarly, the mean MCV was significantly lower in patients with CD4⁺ T-cells count of 500–800 cells/μl compared with patients with CD4⁺ T-cells count of 200–499 cells/μl and patients with CD4⁺ T-cells count <200 cells/μl. β2M had significant positive correlation with WBC and neutrophils percentage but had a significant negative correlation with lymphocytes percentage and MCH in patients with CD4⁺ T-cells count <200 cells/μl. However, β2M had sig-

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nificant positive correlation with PCV, Hb, monocytes and morphology in patients with CD4+ T-cells count of 500–800 cells/µl. **Conclusion.** It could be concluded from this study that HIV infection is associated with alteration in haematological profile and the alteration is CD4+ T-cells count-dependent. Also, elevation in β2M concentration appears to be a marker of lymphopaenia in patients with low CD4+ T-cells count.

Keywords. beta-2-microglobulin, CD4+ T-cells count, haemocytometry, HIV, lymphopaenia

Introduction

Human immunodeficiency virus (HIV) is a pandemic affecting more than 35 million people worldwide.[1] Infection with HIV is associated with myriads of disorders including haematological complications which are considered the second most common cause of morbidity and mortality in HIV patients.[2] HIV replicates in CD4+ T-lymphocytes, macrophages and dendritic cells resulting in immune system depression and consequently, progression to life threatening opportunistic infections.[3-5]

The most common haematological abnormalities associated with HIV are anaemia and neutropaenia and both are associated with disease progression.[6,7] The anaemia, which ranges from mild-to severe, is significantly associated with reduced survival.[8] Neutropenia is observed in advanced stages of HIV infection after development of AIDS and has been associated with certain types of anti-retroviral medication. In fact, anaemia and leukopenia in ART naïve patients have been documented to result in poor ART-treatment outcome and otherwise, strongly predict mortality.[9] These haematological abnormalities are consequent to HIV mediated bone marrow myelosuppression caused by abnormal inflammatory cytokine expression and alteration of bone marrow micro-environment.[10]

The severity of anaemia in HIV infected persons is associated with CD4+ lymphocyte depletion and progression to AIDS and this serves as one of the strongest predictors of HIV mortality as well as poor responses to anti-retroviral therapy.[2] However, CD4+ T-cells counts have been reported not to always correlate with clinical outcome, possibly because they are not key players at all stages of infection and because CD4+ T-cells count do not reflect the totality of T cells functions.[11,12] In addition, technological problems are associated with lymphocyte phenotyping in CD4+ T-cells count and supplementary methods to improve the predictive information of CD4+ T-cells count are still required.[12,13] Therefore, the need for investigation of immunochemical markers such as beta-2-microglobulin (β2M) for their health monitoring potentials in HIV cannot be overemphasized.

Beta-2-microglobulin (β2M) is a component of the human major histocompatibility complex (HLA) class I molecule. It is coded on chromosome 17and expressed by nearly all nucleated cells.[14] Although β2M is expressed at a constant level in many cells, its formation is enhanced in the presence of IFN-α.[15] β2M has also been shown to induce cellular expression of interleukins 6, 8 and 10, regulate the expression of hormone/growth factor, and coordinate the interaction between cytokines and their receptors.[16-18] These activities could explain its rise in concentration in infections, malignancies and other pathological conditions.[19,20] Moodley and colleagues reported that HIV infected infants had elevated β2M levels with significant elevation at 1 month and 12 months compared with the uninfected infants.[21] Also, Chitra et al. reported elevated level of β2M during infections including cytomegalovirus and HIV.[22]

It has also been reported that the degree of elevation of serum β2M correlates well with the extent of disease burden i.e. ≥5µg/L, as against the normal level of 1.8 µg/L.[23] In addition, Mocroft et al. reported that β2M is an independent prognostic marker for death in patients infected with HIV.[24]

Aim

Although it has been reported that levels of some biomolecules are altered with changes in CD4+ T-cells count, there is still the dearth of information on the interplay between severity of HIV infection, changes in haematological parameters and β2M, this study was thus designed to assess changes in selected haematological parameters and β2M in Nigerian HIV patients stratified based on CD4+ T-cells count.[25]

Material and methods

Study Area

The study was conducted at Adeoyo Maternity Teaching Hospital in Ibadan North East Local Government Area, Ibadan, Oyo State, Nigeria.

Ethical consideration

Before the commencement of the study, the study was approved by the Oyo State Ministry of Health, Ibadan (AD13/479/468). Also, informed consent was obtained from each study participant.

Study Participants

Forty-eight asymptomatic, drug naïve HIV patients attending the HIV-PEPFAR Clinic at Adeoyo Maternity Teaching Hospital, Ibadan, Oyo State, Nigeria were enrolled into this cross-sectional study. Details about the age, gender and stages of HIV infection of the study participants have earlier been reported.[26] Patients with other viral co-infection and metabolic diseases such as diabetes were excluded from the study.

Sociodemographic data collection

A semi-structured questionnaire was used to obtain socio-demographic information as well as medical history of the patients.

Blood sample collection and laboratory analysis

Venous blood (5 ml) was obtained from each participant and dispensed into EDTA containing bottles. A complete blood count (CBC); consisting of total and differential white blood cell count and platelet count along with mean platelet volume (MPV), haemoglobin concentration (Hb), packed cell volume (PCV), Red cell indices including Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and red cell distribution width (RDW); were determined using Swelabalfa 3 part haematology analyzer (9 version 2.1, series no SE12613, Boule medicals AB, Sweden).

$CD4^+$ T-cells count was done using flow cytometer (PartecCyFlow Counter®, Partec, Münster, Germany). About 20 ul of whole blood was dispensed into Partec test tube (Rohren tube). Then, 20 µl of $CD4^+$ T-cell antibody was added into the tube. The contents were mixed and incubated in the dark for 15 minutes at room temperature. After incubation, 800 µl of $CD4^+$ T-cells buffer was gently added to the mixture and mixed gently. The Partec tube was then plugged on the Cyflow counter and the $CD4^+$ T-cells were displayed as peaks and interpreted as figures.

Whole blood remaining after haematological analysis and $CD4^+$ T-cells count was centrifuged and plasma obtained was stored at -20^{0}C until analysed for β2M using a non-competitive (sandwich) enzyme linked immunosorbent assay (ELISA) kit (GenWay Biotech Inc., USA).

HIV Staging

The World Health Organisation staging criteria were adopted.[27]

Statistical analysis

Data analysis was carried out using the Statistical Package for Social Sciences (SPSS Inc., Chicago) version 16.0. Differences in mean of variables in different groups were determined using ANOVA followed by post-hoc and results presented as mean ± standard deviation. Pearson correlation was used to determine the correlations between variables while the association between the parameters and HIV stage was determined using the Chi-square test. *P*-values less than 0.05 were considered as statistically significant.

Results

As shown in Table 1, there was significant progressive rise in Hb count, lymphocyte count and morphology as the $CD4^+$ T-cells count increases. In contrast, neutrophils percentage, MCV and MCH reduced progressively as the $CD4^+$ T-cells count increases. Also, in Table 1, the mean lymphocytes percentage was significantly higher in patients with $CD4^+$ T-cells count of 500–800 cells/µl compared with the patients with <200 cells/µl. In contrast, the mean neutrophils percentage in patients with $CD4^+$ T-cells count of 500–800 cells/µl was significantly lower compared with the patients with $CD4^+$ T-cells count <200 cells/µl. Similarly, the mean MCV was significantly lower in patients with $CD4^+$ T-cells count of 500–800 cells/µl compared with patients with $CD4^+$ T-cells count of 200–499 cells/µl and patients with $CD4^+$ T-cells count <200 cells/µl.

Considering the proportion of patients with altered levels of the haematological parameters, it was observed that anaemia is significantly associated with

Table 1. Haematological parameters and beta-2-microglobulin in HIV patients grouped by CD4+ T-cells count

Parameters	CD4 < 200(cells/µl) (n = 10)	CD4 200-499(cells/µl) (n = 13)	CD4 500-800(cells/µl) (n = 17)	*P*-value
PCV (%)	32.14±5.13	32.87±5.60	35.29±7.36	0.084
Hb (g/l)	9.55±1.69	9.96±1.69	10.66±2.30	0.047*
WBC (x10^3)	6.28±3.77	5.33±2.19	6.70±2.25	0.101
Neutrophils (%)	55.36±15.72	48.21±11.89	44.00±12.03^a	0.002*
Lymphocyte(%)	34.68±15.82	42.04±12.08	45.14±11.63^a	0.003*
Monocytes (%)	9.95±3.88	9.58±3.33	10.86±3.35	0.319
Platelets (x10^3)	226.45±95.40	241.17±72.56	263.36±100.36	0.228
MCV (FL)	87.71±7.52	86.33±5.22	82.36±5.45^{a,b}	0.002*
MCH (pg)	26.26±2.88	25.92±2.18	24.71±2.23	0.036*
MCHC (g/dl`)	29.81±1.27	30.13±1.10	29.93±0.90	0.409
Morph. (x10^{12}/L)	3.76±0.73	3.84±0.61	4.31±0.84	0.004*
β2M (µg/mL)	13816.28±19584.08	12034.74±10148.51	13871.19±15819.20	0.914
CD4 (cells/µl)	116.95±59.50	333.20±81.49^a	669.79±124.8^{a,b}	0.000*

*Significant at p<0.05, acompared with CD4 <200 (cells/µl), bcompared with CD4 200 – 499 (cells/µl)

CD4⁺ T-cells count as the proportion of patients with low Hb was significantly lower in patients with CD4⁺ T-cells count above 500 cells/μl compared with patients with CD4⁺ T-cells count <500 cells/μl and <200 cells/μl (Table 2). Furthermore, the proportion of patients with neutrophilia and elevated MCV was significantly higher in patients with CD4⁺ T-cells count <200 cells/μl compared with patients with ≥200 cells/μl. In contrast, the proportion of patients with lymphocytosis was significantly higher in patients with CD4⁺ T-cells ≥500 cells/

Table 2. Proportions of HIV patients with haematological parameters within and outside reference interval in various CD4+ T-cells count groups

Parameters		CD4<200 cells/μl	CD4 200-499 cells/μl	CD4 500-800 cells/μl	χ2	*P*-value
PCV	Normal	15.0	26.3	40.0	4.571	0.102
	<Normal	85.0	73.7	60.0		
Hb	Normal	15.0	15.0	40.0	6.188	0.045*
	<Normal	85.0	85.0	60.0		
WBC	Normal	72.7	91.6	78.6	4.703	0.095
	<Normal	9.1	4.2	0.0		
	>Normal	18.2	4.2	21.4		
Neutrophil	Normal	81.8	87.5	64.3	7.773	0.021*
	<Normal	13.6	12.5	35.7		
	>Normal	4.6	0.0	0.0		
Lymphocytes	Normal	68.2	54.2	42.9	10.229	0.006*
	<Normal	9.1	4.2	0.0		
	>Normal	22.7	41.7	57.1		
Monocytes	Normal	68.2	83.3	64.3	0.612	0.736
	<Normal	4.5	0.0	7.1		
	>Normal	27.3	16.7	28.6		
Platelets	Normal	77.3	79.2	71.4	4.962	0.084
	<Normal	9.1	0.0	0.0		
	>Normal	13.6	20.8	28.6		
MCV	Normal	85.7	95.8	71.4	9.969	0.007*
	<Normal	9.5	4.2	28.6		
	>Normal	4.8	0.0	0.0		
MCH	Normal	42.9	41.7	35.7	1.334	0.513
	<Normal	52.4	58.3	64.3		
	>Normal	4.7	0.0	0.0		
MCHC	Normal	4.8	12.5	7.1	1.798	0.407
	<Normal	95.2	87.5	92.9		

*Significant at p<0.05

Table 3. Correlation between β2M and haematological parameters in HIV patients grouped by CD4⁺ T-cells counts

β2M	CD4<200 cells/μl	CD4 200-499 cells/μl	CD4 500-800 cells/μl
	r-value, *P*-value	r-value, *P*-value	r-value, *P*-value
PCV	-0.132, 0.457	0.231, 0.328	0.903, 0.000*
Hb	-0.199, 0.258	0.183, 0.440	0.898, 0.000*
WBC	0.416, 0.015*	0.396, 0.084	0.392, 0.166
Neutrophils	0.427, 0.012*	0.276, 0.239	0.119, 0.685
Lymphocytes	-0.478, 0.004*	-0.031, 0.896	-0.331, 0.248
Monocytes	0.134, 0.450	-0.504, 0.024*	0.660, 0.010*
Platelets	0.321, 0.065	0.348, 0.132	-0.159, 0.586
MCV(FL)	-0.250, 0.168	0.103, 0.667	0.210, 0.471
MCH (pg)	-0.349, 0.049*	-0.034, 0.887	0.230, 0.429
MCHC (g/dL)	-0.342, 0.056	-0.245. 0.298	-0.116, 0.693
Morphology	-0.012, 0.945	0.134, 0.573	0.668, 0.010*

*Significant at p<0.05

μl compared with patients with CD4+ T-cells <500 cells/μl (Table 2).

As shown in Table 3, β2M had significant positive correlation with WBC and neutrophils percentage but had a significant negative correlation with lymphocytes percentage and MCH in patients with CD4+ T-cells count <200 cells/μl. In patients with CD4+ T-cells count of 200–499 cells/μl, β2M had significant negative correlation with monocytes percentage. However, β2M had significant positive correlation with PCV, Hb, monocytes and morphology in patients with CD4+ T-cells count of 500–800 cells/μl (Table 3).

Discussion

Haematologic abnormalities are the most common complications of HIV infection and are strong independent predictors of morbidity and mortality in infected individuals.[28,29] These abnormalities are associated with a number of complex and multi-factorial factors including infection of the multipotent haematopoietic progenitor cells by HIV, establishment of latent cellular reservoirs, activation of the reticulo-endothelial system resulting in increased blood cell destruction, and disturbance in the bone marrow microenvironment causing immune dysregulation with its associated cytokine imbalances and disruption of factors essential for normal haematopoiesis.[30] Anaemia is one of the most frequent haematological abnormalities in individuals with HIV infection including those on highly active antiretroviral therapy (HAART).[6,31] The observed progressive rise in Hb with increasing CD4+ T-cells count and the low prevalence of anaemia in patients with CD4+ T-cells count of ≥500 cells/μl indicate that anaemia is associated with CD4+ T-cells depletion and that the anaemia resolves with improvement in CD4+ T-cells count. This observation supports the report of Mata-Marín et al. which showed that anaemia is associated with CD4+ T-cells depletion and progression to AIDS.[32] Anaemia in HIV infection is caused by inadequate blood cell production due to bone marrow suppression by HIV infection mediated by abnormal cytokine expression and alteration of the bone marrow microenvironment.[33,34]

Neutrophils, the most abundant cell type in human blood, play important effector roles in immune response to pathogens.[35] It has been shown that there is decreased peripheral blood neutrophil counts and reduced activities, including chemotaxis, phagocytosis, bactericidal activity, and oxidative burst abilities in individuals with HIV infection.[36] The observed reduction in neutrophils percentage with increasing CD4+ T-cells count is in contrast to the report of De Santis et al. which showed that the mean neutrophil count was lower in HIV patients with low CD4+ T-cells count.[31] Observation from this study could indicate that increase in CD4+ T-cells count resulted in enhanced activities of the adaptive arm of the immune system facilitating protection against opportunistic infections which would result in down regulation of neutrophil activities. Our observation is buttressed by the observed significant reduction in neutrophils percentage in patients with CD4+ T-cells count of 500–800 cells/μl compared with those with <200 cells/μl, the observed higher proportion of patients with neutrophilia in patients with CD4+ T-cells count <200 cells/μl compared with patients with ≥200 cells/μl. Observations from this study suggest that observation of neutrophilia in patients with HIV is a possibility especially in those with low CD4+ T-cells count. Musubire et al. reported elevated neutrophil counts in HIV-infected patients with cryptococcal meningitis and the elevation was associated with mortality.[37]

T lymphocytes are white blood cells with important roles in adaptive immunity. The observed progressive rise in lymphocyte count as the CD4+ T-cells count increases and the elevated lymphocyte count in patients with CD4+ T-cells count of 500–800 cells/μl compared with those with <200 cells/μl corroborate the report of Bhardwaj et al.[29] These observations are not surprising as CD4+ T-cells count are lymphocytes. Obirikorang et al. reported positive correlation between TLC and CD4+ T-cells count and that total lymphocyte count can serve as a surrogate marker for CD4+ T-cells count in drug naïve HIV patients.[38]

Although low haemoglobin level symbolizes anaemia, other indices of red blood cell including mean corpuscular volume (MCV) provide information on the aetiology of the anaemia which could be morphologic (normocytic, microcytic or macrocytic) or pathophysiologic (excessive destruction, loss or diminished production).[39] The observed progressive reduction in MCV and mean corpuscular haemoglobin (MCH) concentration as the CD4+ T-cells count increases indicates that low CD4+ T-cells count is associated with hypochromic microcytic anaemia. This observation is alluded to by the observed improvement in Hb concentration with increasing CD4 count and the significantly lower MCV in patients with CD4+ T-cells count of 500–800 cells/μl compared with patients with CD4+ T-cells count <500 cells/μl. Our observation is indicative of progressive reduction in the degree of inflammation and better utilization of iron in the attempt to correct co-existing anaemia of chronic disease at CD4+ T-cells count of 500–800 cells/μl.

In addition, there was significant association between MCV and low CD4+ T-cells count. MCV, the average size of the circulatory erythrocyte, is an index for the differential diagnosis of anaemia and has been associated with mortality in many diseases.[40,41] On the other hand, MCH is the average concentration of Hb in erythrocytes and anaemia is the most common cause of its low concentration.

Elevated level of β2M has been reported in patients with HIV.[22] The observed inverse relationship between β2M and lymphocytes indicates that elevated level of β2M could suggest lymphopaenia in HIV patients with low $CD4^+$ T-cells count. This observation is in line with previous reports showing that the degree of elevation of serum β2M correlates well with the extent of disease burden.[23] Similarly, the observed inverse relationship between β2M and MCH suggests that elevated level of β2M could be an indication of microcytic anaemia in HIV patients with low $CD4^+$ T-cells count. This observation corroborates the earlier observed low MCH concentration in the group with low $CD4^+$ T-cells count.

Improved $CD4^+$ T-cells count is the hallmark of favourable response to anti-retroviral therapy.[42] The observed positive correlation between β2M and PCV, Hb, monocytes and morphology in patients with $CD4^+$ T-cells count of 500–800 cells/μl suggests that increase in β2M level might indicate improvement or restoration of HIV-induced alteration in haematological profile as the $CD4^+$ T-cells count improves. It has been shown that β2M plays important roles in expression of hormones/growth factors which coordinate various cellular activities.[16-18]

It could be concluded from this study that HIV infection is associated with alteration in haematological profile and the alteration is $CD4^+$ T-cells count-dependent. Also, elevation in β2M concentration appears to be a marker of lymphopaenia in patients with low $CD4^+$ T-cells count but a marker of normal haematocrit level in patients with optimal $CD4^+$ T-cells count.

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ORIGINAL PAPERR

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Analysis of the bacterial biofilm formation in different models of the *in vitro* culture

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ABSTRACT

Introduction. Microtiter plate assay (MPA) remains one of workhorses of *in vitro* biofilm research but it requires optimization of experimental conditions to fulfill the biofilm formation requirements of different bacterial pathogens.

Aim. The aim was to determine the effect of TSB and RPMI1640 culture media and selected culture variables (O_2 vs. 5% CO_2, extended incubation time) on the biofilm production by bacteria commonly involved in biofilm-related infections: *Enterococcus faecalis* (EF), *Escherichia coli* (EC), *Staphylococcus aureus* (SA), *Pseudomonas aeruginosa* (PA), *Klebsiella pneumoniae* (KP).

Material and methods. The investigation was performed using the MPA with crystal violet.

Results. Statistically significant ($p<0.05$) increase in biofilm production between 24h and 72h time points was observed for EF (TSB o_2, RPMIo_2 and RPMIco_2), EC (TSBo_2), SA (TSBo_2, TSBco_2), KP (TSBo_2, TSBco_2), PA (RPMIco_2, TSBco_2). The TSB caused a significantly greater stimulation of biofilm production compared to RPM1640. It outcompeted RPMI1640 irrespective of the atmospheric conditions for SA and KP and under aerobic conditions for EF.

Conclusion. Although the TSB provided the most optimal conditions for biofilm production, the process was influenced by the strain type, atmospheric conditions and period of cultivation which limits the ability to design a single universal model of the *in vitro* biofilm investigation.

Keywords. *in vitro* biofilm, microtiter plate assay, RPMI 1640, tryptic soy broth

Introduction

The ubiquitous ability of potentially pathogenic microorganisms to live attached to biotic (tissues) and abiotic (medical implants) surfaces as sessile communities known as biofilms accompanied by their inherent tolerance to innate and adaptive host defences and antibiotic therapies have brought the biofilm-related infections to the forefront the most significant concerns of modern medicine.[1-3]

A microbial biofilm is defined as a "structured consortium of microbial cells surrounded by a self-produced polymer matrix".[3,4] The matrix is thought to play a key role in the protection of the biofilm-embedded bacteria from host defences and is partially involved in the restricted diffusion of antimicrobial agents into the biofilm.[1]

Moreover, biofilms are characterized by physiological and biochemical gradients. Consumption of oxygen and glucose originating in the surface layers of biofilms,

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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leads to anaerobic nutrition-depleted niches with restricted metabolic activity in the depths of the aggregated structure which corresponds to a top-to-bottom gradient of decreasing antibiotic susceptibility. Antibiotic tolerance is also mediated by accumulation of metabolic waste products and extracellular signalling molecules due to a high cell density in the biofilm depths whereas horizontal resistance gene transfer within and beyond species borders facilitates the spread of antibiotic resistance. Finally, bacteria growing in biofilms can actively adapt to stress by turning on the stress-response genes antagonizing the deleterious effects of antibiotics and the host immune system.[1,3]

Much of the current knowledge about the biofilm-related infections is a result of investigation of surface-associated biofilms produced *in vitro*. The microtiter plate assay (MPA) remains one of the workhorses of the *in vitro* biofilm research. This method is a low-cost, high-throughput biofilm screening approach for the investigation of surface-attached biomass production in liquid media.[5,6] Although the biofilm production is involved in the majority of bacterial infections, it is influenced by different external parameters which still remain to be fully elucidated. Moreover, most of the studies, have investigated the biofilm-producing capabilities of single or mixed bacterial and fungal species.[2,7-10]

Aim

The aim of our study was to determine the effect of two different culture media (TSB and RPMI 1640) and selected culture variables (degree of aeration, incubation period) on the development of biofilms produced by five reference strains of bacterial species commonly involved in the biofilm-related infections. The studied microorganisms included *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*.

Material and methods

Bacterial strains and growth conditions

Five reference bacterial strains: *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* ATCC 700603 used in this study were obtained from the American Type Culture Collection (ATCC). All strains were cultivated on trypticasein soy infusion (TSI, Biocorp, Poland) or trypticasein soy agar (TSA, Biocorp, Poland) at 37°C.

Biofilm formation

The assay of biofilm formation was performed as published previously[11] with some modifications. Formation of biofilms was carried out in 96-well microtiter plates (NUNC, Thermo Fisher Scientific Inc, Denmark). An overnight culture of bacteria (ca. 4 in McFarland standards) was diluted 1:100 in TSB additionally supplemented with 1% D-(+)-glucose and RPMI 1640 medium (Sigma Aldrich). Aliquots (200 µl) of diluted culture were inoculated into five wells each of the 96-well ster-

Table 1. The mean OD values for each of the tested reference strains, at individual time points of incubation, considering different culture conditions

Incubation time point	O_2											
	TBS						RPMI 1640					
	C Mean (SD)	EF Mean (SD)	EC Mean (SD)	SA Mean (SD)	PA Mean (SD)	KP Mean (SD)	C Mean (SD)	EF Mean (SD)	EC Mean (SD)	SA Mean (SD)	PA Mean (SD)	KP Mean (SD)
24 h	0.1375 (0.027)	0.7873 (0.061)	0.6454 (0.108)	0.4265 (0.211)	1.4340 (0.322)	0.9272 (0.373)	0.1372 (0.023)	0.2364 (0.079)	0.1674 (0.029)	0.3613 (0.201)	0.7701 (0.358)	0.2566 (0.062)
48 h	0.1383 (0.018)	1.3589 (0.605)	1.3312 (0.778)	1.5994 (0.519)	1.7791 (0.484)	2.1143 (0.304)	0.1596 (0.003)	0.4866 (0.117)	0.3294 (0.078)	0.494 (0.062)	1.2215 (0.533)	0.3631 (0.095)
72 h	0.1417 (0.018)	2.4769 (0.017)	2.0129 (0.519)	2.0194 (0.452)	2.0064 (0.453)	2.0249 (0.509)	0.2157 (0.062)	0.7054 (0.182)	0.7431 (0.216)	0.5078 (0.678)	1.0431 (0.545)	0.7461 (0.228)
Incubation time point	**CO_2**											
	TBS						**RPMI 1640**					
	C Mean (SD)	**EF Mean (SD)**	**EC Mean (SD)**	**SA Mean (SD)**	**PA Mean (SD)**	**KP Mean (SD)**	**C Mean (SD)**	**EF Mean (SD)**	**EC Mean (SD)**	**SA Mean (SD)**	**PA Mean (SD)**	**KP Mean (SD)**
24 h	0.1167 (0.015)	1.066 (0.545)	0.3244 (0.058)	0.6189 (0.172)	0.8665 (0.344)	1.2056 (0.788)	0.1396 (0.024)	0.237 (0.052)	0.1761 (0.040)	0.3844 (0.189)	1.7814 (0.282)	0.2162 (0.043)
48 h	0.1379 (0.004)	1.1395 0.261)	2.4619 (0.011)	2.4596 (1.131)	1.5764 (0.264)	2.3245 (0.242)	0.1482 (0.001)	0.2631 (0.059)	0.2793 (0.058)	0.3779 (0.125)	2.2371 (0.178)	0.2897 (0.099)
72 h	0.1487 (0.008)	0.9620 (0.218)	1.31 (0.049)	2.2261 (0.758)	1.8382 (0.455)	2.0418 (0.589)	0.1792 (0.010)	0.9963 (0.466)	0.662 (0.323)	0.5078 (0.226)	2.4252 (0.049)	0.4112 (0.267)

SD – standard deviation; C – control; EF – *Enterococcus faecalis*; EC – *Escherichia coli*; SA – *Staphylococcus aureus*; PA – *Pseudomonas aeruginosa*; KP – *Klebsiella pneumoniae*;

ile microtiter plate. The TSB and RPMI 1640 broths (200 µl) were used as negative controls (K). Biofilms were grown statically for 24, 48 and 72 h at 37°C in aerobic conditions as well as in the presence of 5% CO_2. The media were replenished after every 24h of growth. Following incubation, the wells were carefully washed twice with 0.9% NaCl, and dried for 1 h at 50°C. Biofilms in wells were stained with 0.1% crystal violet (CV; 200 µl) for 15 min in order to determine total biofilm biomass. After staining, the wells were washed by flushing the plate three times with 200 ml of distilled water to remove unbound CV and air-dried. The biofilm-bound dye was extracted with 200 µl of 70% (v/v) ethanol. The optical density (OD) was then determined at 570 nm using the microplate reader. Each experiment was performed in triplicate.

Statistical analysis

Statistical analyses were performed using 2-tailed unpaired t-test (2 groups) or one-way ANOVA followed by Tukey's multiple comparisons post test. $P<0.05$ was considered statistically significant. All data are described as mean ± SD in the text.

Results

All tested bacterial strains adhered and developed into biofilms on the wells of the microtiter plates in both culture media (TSB and RPMI 1640) used in the study. However, the degree of the biofilm development reflected by the measurement of the OD was dependent on the type of the culture medium, incubation period and atmospheric conditions: aerobic vs. the increased CO_2 concentration.

It was noted that the biofilm formation progressed for 72 h and its maximum yield was found at this time point for the majority of strains. Nevertheless, we also observed a slight decrease (not reaching statistical significance) in the OD was observed at the 72 h compared to the 48 h time point in six models of the biofilm culture (Table 1).

Statistically significant ($p<0.05$) increase in the OD of the biofilm produced between the 24 h and 72 h time points was observed for *E. faecalis* incubated in TSB O_2, RPMI 1640 O_2 and RPMI 1640 CO_2, *E. coli* incubated in TSB O_2, *S. aureus* incubated in TSB O_2 and TSB CO_2, *K. pneumoniae* incubated in TSB O_2 and TSB CO_2, and for *P. aeruginosa* incubated in RPMI 1640 CO_2 and TSB CO_2 (Fig. 1).

Significant increase in the mean OD between the 24 h and 48 h time points was observed for *E. coli* incubated in TSB CO_2, and for *S. aureus* and *K. pneumoniae* incubated in TSB O_2 and TSB CO_2. Significant increase in the mean OD between the 48 h and 72 h time points was observed for *E. faecalis* incubated in TSB O_2 only.

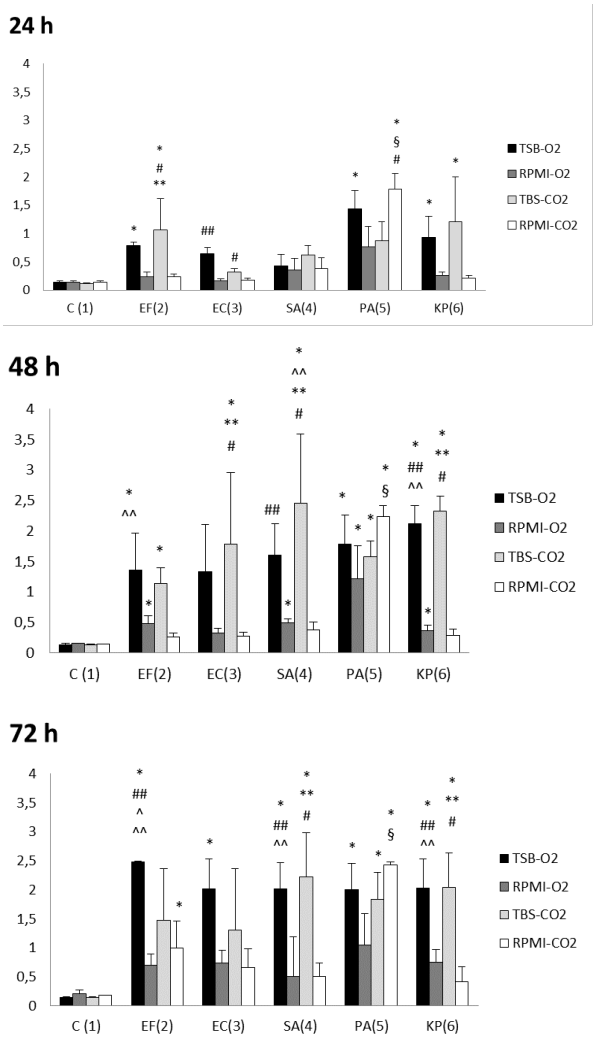

Fig. 1. Bacterial biofilms grown under different culture media and different oxygenic conditions, evaluated at 24 h, 48 h and 72 h time points. Data are mean values performed in triplicate in the same culture conditions. Significant differences ($p<0.05$) between different culture conditions within each tested bacterial species are indicated as follows:

*control vs bacterial strain;

** RPMI-O2 vs TSB-CO2

TSB-CO2 vs RPMI-CO2

TSB-O2 vs RPMI-O2

^ TSB-O2 vs TSB-CO2

^^ TSB-O2 vs RPMI-CO2

§ RPMI-O2 vs RPMI-CO2

The above results indicate that the extended (72 h) incubation results in the formation of the mature, well-established *in vitro* biofilm. On the other hand, as noted earlier, the production of the biofilm did not reach a steady increase throughout the extended incubation period for all tested strains, under all applied incubation conditions. This notion was striking for four out of the five tested strains incubated in TSB CO_2 whose biofilm OD decreased after the 48 h time point.

These observations lead to the conclusion that aerobic incubation stimulates the biofilm formation to the greatest extent. The majority of the strains (4 out of 5) reached the maximal biofilm producing capability at the 72 h time point under aerobic conditions in both culture media (Table 1).

On the other hand, all tested strains cultured in RPMI 1640 at the increased CO_2 reached the maximum biofilm yield (Table 1).

It should be noted, however, that the RPMI 1640 medium was inferior to the TSB in terms of the degree of the biofilm production reflected by its OD. The significantly greater ($p<0.05$) mean OD values at the 72 h time point (Fig. 1) were observed when the two media and the accompanying degree of aeration during culture were compared in the following bacterial species:

- TSB O_2 vs RPMI 1640 O_2 for *E. faecalis*, *S. aureus*, and *K. pneumoniae*
- TSB CO_2 vs RPMI 1640 CO_2 for *S. aureus* and *K. pneumoniae*
- TSB CO_2 vs RPMI 1640 O_2 for *S. aureus* and *K. pneumoniae*
- TSB O_2 vs RPMI 1640 CO_2 for *E. faecalis*, *S. aureus,* and *K. pneumoniae*

The obtained results indicate that for *S. aureus* and *K. pneumoniae* the TSB medium outcompeted RPMI 1640 irrespective of the atmospheric incubation conditions whereas for *E. faecalis* the TSB stimulated a greater biofilm production under aerobic conditions.

An interesting observation was made for *P. aeruginosa* which produced the most profuse biofilm during incubation in RPMI CO_2 (Table 1, Fig. 1). Nevertheless, this strain produced the biofilm under all applied conditions with the only statistical significance noted when the RPMI CO_2 incubation was compared to the RPMI O_2 (OD=2.42 vs. OD=1.04, respectively, at the 72h time point). This medium did not gain a significant advantage over the TSB.

Discussion

The principle of the MPA is to yield the biofilm growth on abiotic surfaces submerged in media and exposed to fluid dynamics of varying degrees. Although it is impossible to indiscriminately extrapolate conclusions drawn from the MPA research to the pathogenesis of the biofilm-related *in vivo* infections due to different growth environments for bacteria provided by urine, blood or other body fluids, missing immune response and inability to reflect complex oxygen and nutrient gradients found in infections compared to the *in vitro* studies, the MPA and other *in vitro* assays allow for the investigation of the *in vitro* biofilm with a stringent control of experimental parameters and simultaneous ability to change single variables.[2,3,4,6]

It has been known that environmental conditions including culture media and available nutrients can modulate microbial biofilm production and its function. They have a major impact on the biofilm growth and development and the metabolic activity of cells in maturing biofilms.[7,9] It has even been suggested that composition of the medium is the most important factor influencing the ability of bacteria to produce biofilm under *in vitro* conditions.[5]

The TSB medium is a commonly used enrichment medium containing enzymatic digests of casein and soybean (providing amino acids and other complex nitrogenous substances), glucose (as an energy source), sodium chloride (which maintains the osmotic equilibrium) and dibasic potassium phosphate (as a buffer to control pH). This medium in routinely used for the cultivation of a wide variety of microorganisms but it has also been frequently used in the biofilm investigation[5]. The RPMI 1640 medium, in turn, mimics the composition of human body fluids as it contains high concentration of amino acids, vitamins and inorganic salts[10]. It is commonly used in cell and tissue culture for growing of a variety of mammalian cell lines.

The application of the two media in our study revealed that TSB stimulated a significantly more profuse biofilm production compared to the RPMI 1640 for the strains tested as evidenced by the increase in the mean OD. We also observed a species/strain dependent advantage of the TSB over the RPMI 1640 at the maximum (72 h) time point. In case of *S. aureus* and *K. pneumoniae* the TSB medium outcompeted RPMI 1640 irrespective of the atmospheric incubation conditions whereas for *E. faecalis* the TSB stimulated a greater biofilm production following incubation under aerobic conditions.

P. aeruginosa, in turn, produced the most profuse biofilm following incubation in RPMI 1640 CO_2 ($p<0.05$ vs. RPMI 1640 O_2) as evidenced by the highest increase in the mean OD among the media and accompanying culture conditions used for this strain. However, this medium did not gain a statistically significant advantage over the TSB. The obtained result pointing at the unexpected most profuse biofilm production in RPMI CO_2 can be explained by the fact that *P. aeruginosa* is a versatile microorganism. It is able to grow both in oxic and hypoxic environments and to use both oxygen and nitrate as an electron acceptor for its heterotrophic respiration.[12]

The only bacterial strain for which none of the culture variables gained the upper hand was *E. coli*. This strain demonstrated the greatest biofilm OD following incubation in TSB O_2 but the difference between this value and other obtained results did not reach a statistical significance.

Our results are generally in line with previous literature data reporting that TSB, especially after supplementation with glucose[7,13] enhances and supports the biofilm formation. Some authors, however, noted a greater bio-

film-promoting ability of the brain-heart infusion (BHI) broth compared to the TSB for the clinical strains of *S. aureus*.[8] The authors noted that BHI is the source of proteins rich in leucine, proline, serine, and aspartic acid which may be essential for the production of bacterial adhesins such as staphylococcal fibronectin-binding protein and clumping factor.[8]

The RMPI 1640 was also examined for the biofilm production in several previous studies which brought conflicting results. Tan et al. revealed that the development of mixed biofilms with *Candida* species and *S. epidermidis* yielded the lowest biofilm formation when grown in RPMI 1640 medium compared to the other two media used in their study, namely TSB and BHI.[9] These authors also reported that the metabolic activity of biofilms produced by mixed biofilms of three *Candida* species and *S. epidermidis* was significantly reduced in RPMI 1640 compared to the TSB and BHI media. Wijesinghe et al. in turn, noted that the adherence of both *P. aeruginosa* and *S. aureus*, either in mono- or coculture, was optimal in RPMI 1640.[10] On the other hand, however, this medium performed worst in terms of support for growth. In their study, BHI medium was the one which fostered the maximal biofilm growth. The authors attempted to explain this observation taking into account the chemical composition of this medium and the ability of bacteria to metabolize different nutrients. Although RPMI is a rich medium containing high concentration of amino acids, vitamins and inorganic salts (which may initially induce the production of extracellular surface components promoting bacterial adhesion), its amino acid composition is higher than that of carbohydrates. During the period of rapid bacterial growth, carbohydrates are quickly used as the primary source of carbon followed by peptides, amino acids, nucleic acids, nucleotides and fatty acids. As the cells enter the stationary phase of growth, amino acid catabolism becomes predominant. As a result, ammonia is released into the medium and causes it to become basic. In order to maintain pH homeostasis in the cytoplasm bacteria must actively acquire protons from the basic medium environment which requires a high energy expenditure. In the next period, readily available nutrients are exhausted and bacteria must obtain nutrients from the dead bacteria which imposes another energy cost on the cell due to the necessity of conversion of nutrients originating from the bacterial debris into their constituent parts. The authors concluded that stress responses associated with the appearance of macromolecular agents in the medium could affect the viable cell mass and lead to its reduction in RPMI medium.[10]

The present data support the notion that growth media significantly influence the ability of bacteria to form biofilms. However, biofilm formation and adherence, as Hancock et al. noted following investigation of *E. coli* and *K. pneumoniae* biofilm formation, are not accomplished by the same mechanisms in different media.[2] The authors examined biofilm formation in two different minimal lab media (ABTG and MOPS), pooled human urine and LB medium. Their study revealed that production of a good biofilm in one medium does not predict an equally good biofilm forming ability in another growth medium, and strains that outperformed others in one medium do not necessarily do so in another growth medium. It is therefore conceivable, that available environmental resources influence the expression of different biofilm-promoting genes and utilization of different strategies involved in adherence (including surface proteins, adhesive factors, cell surface hydrophobicity) by microorganisms. Similarily, Hood and Zottola who studied four foodborne microorganisms (*Salmonella typhimurium, Listeria monocytogenes, E. coli* O157:H7, *Pseudomonas fragi* and *P. fluorescens*) concluded that the medium which produced the highest level of adherent cells was different for each microorganism.[14] Moreover, it was reported that some microorganisms may demonstrate enhanced adhesive abilities when the nutrients are lacking, while others can exhibit high adhesion rates even under basic growth conditions.[15]

According to the available data, incubation time also plays a crucial role in the biofilm development. It promotes the accumulation of greater amounts of the extracellular matrix substances. Most studies have used an incubation period limited to 24-48 hours which may not reflect actual kinetics of the biofilm growth and maturation.[8,9] In our study, a significant increase in the mean OD between the 24 h and 48 h time points was observed in only certain strains and was associated with the growth medium and atmospheric conditions during incubation. This increase was noted for *E. coli* incubated in TSB CO_2, and for *S. aureus* and *K. pneumoniae* incubated in TSB O_2 and TSB CO_2 which, again, indicates the supporting role of TSB for the production of bacterial biofilm under *in vitro* conditions.

Tan et al. in turn, observed increased biofilm biomass from 24 h to 48 h for all tested strains (the mixtures of *Candida* spp. and *S. epidermidis*) cultured in RPMI, TSB and BHI and additionally concluded that changing the culture media after 24 h of growth (which was also done in our study) had a positive effect on the increase in the biofilm biomass.[9]

Senevirante et al. who investigated the effect of culture media and nutrients on the biofilm production by laboratory and clinical strains of *E. faecalis* reported that 72 h of growth is required to achieve robust, mature biofilms, evidenced by *in vitro* and microscopic observation of analysed enterococcal strains. This tendency was observed for all the strains studied and was irrelevant of the cultured medium used (BHI, TSB and "Pg broth").[7]

Our study has brought more diverse results. It was noted that the biofilm formation progressed for 72 h and its maximum yield was observed at this time point for the majority of strains. The TSB O_2 incubation provided conditions that led to the significant increase in the OD of the biofilm between the 24 h and 72 h time points for all tested bacterial species with the exception of *P. aeruginosa*. The significant increase in the biofilm OD biofilm produced by this strain was noted following incubation under increased CO_2 concentration only, irrespective of the medium used. It could also be observed that the kinetics of the biofilm biomass formation between the 48 h and 72 h time points occurred more slowly compared to the increase between the 24 h and 48 h time points. This increase reached statistical significance only for *E. faecalis* incubated in TSB O_2 whereas significant increase in the OD was noted for *E. coli* (incubated in TSB CO_2), *S. aureus* (incubated in TSB O_2 and TSB CO_2), and *K. pneumoniae* (incubated in TSB O_2 and TSB CO_2) when the 24 h and 48 h time points were compared. Moreover, some strains demonstrated a slight decrease in the biofilm OD between the 48 h and 72 h time points with the most striking example of TSB CO_2 incubation which was associated with this decline in four out of five strains.

The obtained results indicate that the *in vitro* biofilm formation is dependent on the experimental design to a significant extent. Although the TSB medium provides the most optimal conditions for the biofilm production, this process is additionally influenced by atmospheric conditions during incubation and the period of the cultivation. The study also revealed the *in vitro* biofilm production capabilities are not only dependent on the external culture conditions but they are also influenced by the type of the bacterial strain tested. This, in turn, in spite of all experimental advantages of the MPA assay, limits the ability to design a single universal model of the *in vitro* biofilm investigation.

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ORIGINAL PAPER

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Neonatal circumcision: profile of neonates with complications resulting from the use of plastibell

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ABSTRACT

Introduction. Circumcision is one of the most performed surgical procedures in neonates.

Aim. The aim of this study was to evaluate our experience with neonates who developed complications following the use of plastibell for circumcision.

Material and methods. This was a retrospective study of male neonates who were managed for complications resulting from circumcision (performed with plastibell) over a 5-year period at the pediatric surgery unit of a teaching hospital in Enugu, Nigeria. Ethical approval was obtained from the ethics and research committee.

Results. Out of the 1794 neonatal circumcisions (using plastibell) performed during the study period, 134 (7.5%) neonates had complications. Sixty percent (1074) of the circumcisions were performed in the teaching hospital while 40% were referred cases**.** The ages of the patients ranged from 7 to 27 days with a median of 10 days and their mean weight was 2.5 kilograms. Majority of the plastibell circumcisions that developed complications was performed by unregistered (auxiliary) nurses. Retained plastibell was the most common complication and its removal was the most performed procedure. No mortality was recorded.

Conclusion. Complications following circumcision with plastibell vary widely. Retained plastibell was the most common in the present study. The most complications occurred when the circumcision was performed by auxiliary (unregistered) nurses.

Keywords. circumcision, complications, neonatal, plastibell

Introduction

Circumcision, a cultural and religious practice, has well documented risks and benefits.[1] The removal of the prepuce to expose the glans penis (circumcision) has been performed for more than 5000 years.[2] It is one of the oldest and most controversial surgical procedures performed globally.[3] In 1971, the American Academy of Pediatrics (AAP) Task Force on circumcision considered circumcision unnecessary. However, in 1999, AAP revisited the issue stating that circumcision has potential benefits. However, AAP did not recommend routine circumcision.[4] Historically, the oldest documented evidence of circumcision dates back to 2345-2181 BC in tomb artworks in Egypt.[4] Male mummies in Egypt were found to be circumcised.[5] The book of Jeremiah, written in the 6th century BC listed Egyptians, Edomites, Ammonites and Moabites as circumcising nations.[4] Lots of controversies surround the issue of circumcision. Cir-

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cumcision is one of the most commonly performed procedures in Africa and about one third of the world male population is circumcised.[6] Worldwide, people continue to circumcise their sons for hygienic, cultural and religious reasons.[7] In Judaism, circumcision is considered a commandment from God and is performed without anesthesia on the 8th day of life.[8] The 3 most common devices used for circumcision include the Gomco clamp, plastibell device and Mogen clamp.[9] The plastibell circumcision device which was invented by Hollister in 1950 is a clear plastic ring with a handle and a circumferential deep groove for tying the suture. Although plastibell is simple device, complications can results from lack of aseptic techniques, use of wrong sized plastibell, loosely tied sutures and lack of follow up.[10] Several studies on circumcision have documented the benefits of circumcision. Such benefits include reduction of penile/cervical cancer, urinary tract infection and sexually transmitted diseases.[11] Some researches on the use of plastibell in childhood circumcision have reported complications whereas other studies documented that complication from plastibell are rare.[12,13]

Aim

The aim of this study was to evaluate our experience with neonates who developed complications following the use of plastibell for circumcision.

Material and methods

This was a retrospective study of male neonates who were managed for complications resulting from circumcision (performed with plastibell) between January 2015 and December 2019 at the pediatric surgery unit of Enugu State University Teaching Hospital (ESUTH) Enugu, Nigeria. ESUTH is a tertiary hospital located in Enugu, South East Nigeria. The hospital serves the whole of Enugu State, which according to the 2016 estimates of the National Population Commission and Nigerian National Bureau of Statistics, has a population of about 4 million people and a population density of 616.0/km^2. The hospital also receives referrals from its neighboring states. Patients with incomplete medical records and those older than 28 days of age were excluded from the study. Neonates who had no complications and those that had other methods of circumcision were also excluded. Information was extracted from the case notes, operation notes, operation register and admission-discharge records. The information extracted included age at circumcision, weight, cadre of health worker who performed the circumcision, type of complication, day post circumcision at which complication occurred, treatment offered, duration of hospital stay and outcome of treatment. Ethical approval was obtained from the ethics and research committee of ESUTH. Statistical Package for Social Science (SPSS) version 21, manufactured by IBM Cooperation Chicago Illinois, was used for data entry and analysis. Data were expressed as percentage, median, mean and range.

Results

Patients' demographics

A total of 1794 neonatal plastibell circumcisions were performed during the study period. Out of this number, 134 neonates had complications and form the basis of this report. The remaining 1660 (1794 minus 134) neonates had no complications. The 1794 neonates had only plastibell method of circumcision; those that had other methods of circumcision were not part of the study. Considering the number of neonates that had complications from plastibell in relation to the total number of plastibell circumcisions actually done, a complication rate of 7.5% (134/1794) was gotten. Sixty percent (1074) of the circumcisions were performed in the teaching hospital while 40% (720) were referred cases. Referred cases were circumcisions performed outside the teaching hospital but were referred to the teaching hospital on account of complications. Details of the demographics are shown in Table 1.

Table 1. Demographic features of the patients that had complications (n=134)

Median age of the patients	10 days (range 7-27)
Mean duration of hospital stay	8 hours (range 3-36)
Mean weight of the patients	2.5 kilograms (range 2.0-4)
Day post circumcision at which complication occurred (mean)	6 days (range 1-10)

Cadre of health worker who performed the circumcision

The level of health worker who performed the circumcision and the specific complication rates are depicted in Table 2.

Type of complications

Delayed separation of the plastibell (retained plastibell) was the most frequent complication. Other complications are shown in Table 3 and figure 1-3.

Treatment rendered

Treatment of complications resulting from plastibell circumcision is dependent on the type of complication (Table 3). For instance, delayed separation of the plastibell (retained plastibell) requires removal of the plastibell to avoid glanular constriction and possible necrosis.

General outcome of treatment

All the patients recovered and were discharged home. There was no mortality.

Discussion

The complication rate of 7.5% recorded in the present study is comparable to the report of other authors.[7,8]

Table 2. Cadre of the health workers and specific complication rates

Cadre	Complication	Number of circumcisions	Number with Complication (%)
Pediatric surgeon		206	7 (3.4)
	Retained plastibell		4 (1.9)
	Bleeding		3 (1.5)
Resident doctor		422	21 (5.0)
	Retained plastibell		5 (1.2)
	Bleeding		5 (1.2)
	Redundant prepuce		10 (2.4)
	Wound infection		1 (0.2)
Registered nurse		446	32 (7.2)
	Retained plastibell		10 (2.2)
	Denudation of penile skin		11 (2.5)
	Skin bridges		8 (1.8)
	Wound infection		3 (0.7)
Traditional birth attendant		464	34 (7.3)
	Retained plastibell		10 (2.2)
	Wound infection		8 (1.7)
	Bleeding		14 (3.1)
	Skin bridges		1 (0.2)
	Urethrocutanous fistula		1 (0.2)
Unregistered nurse		256	40 (15.6)
	Retained plastibell		15 (5.9)
	Bleeding		15 (5.9)
	Wound infection		7 (2.6)
	Urethrocutanous fistula		2 (0.8)
	Glans necrosis		01 (0.4)

Table 3. Complications arising from plastibell circumcision and treatment offered (n=134)

Type of complication	Treatment rendered	No of patients (%)	Result of treatment
Retained plastibell	Removal of plastibell ring	44 (32.8)	Good healing
Bleeding	Pressure and vessel ligation	37 (27.6)	Bleeding controlled
Wound infection	Antibiotics and dressing	19 (14.3)	Infection cleared
Denudation of penile skin	Dressing	11 (8.2)	Good healing
Redundant prepuce	Refashioning	10 (7.5)	Good appearance
Skin bridges	Bridge release	9 (6.7)	No recurrence
Urethrocutanous fistula	Fistula repair	3 (2.2)	Good urinary stream
Glans necrosis	Glans refashioning	1 (0.7)	Conical glans penis

However, Al-Marhoon reported a complication rate of 2.3%.[14] The complication rate following plastibell circumcision may depend on the experience of the person performing the circumcision and on the age of the child. Mousavi and Salehifar in their study concluded that plastibell circumcision should be used in neonates with thin prepuce.[8] The median age of our patients is similar to report of Razzaq et al.[15] The cultural and religious practices in different settings may explain the peak age at circumcision. Plastibell method of circumcision usually does not require hospital admission. However, when there are complications such as bleeding, the neonate is admitted for observation in the hospital. As recorded in the present study, one-fourth of the patients were observed overnight in the hospital due to bleeding problems. The mean age of our patients is in agreement with the result of one study from Iran.[8] The size of the plastibell device corresponding to the diameter of the glans penis should be used for plastibell circumcision. The retained plastibell in our patients may be attributable to the plastibell size. Abdullah et al and Nasir et al reported the importance of choosing the right size of plastibell for circumcision.[16,17] The time of occurrence of complication following plastibell circumcision has to do with the type of complication. For instance, bleeding is more likely to occur before wound infection. In the present study, bleeding occurred within 24 hours of the circumcision and the neonates were observed in hospital. Hammed et al also reported bleeding in their patients and their patients were admitted for observation in the hospital.[18] Plastibell circumcision is usually a day case (outpatient) procedure. The length of time a

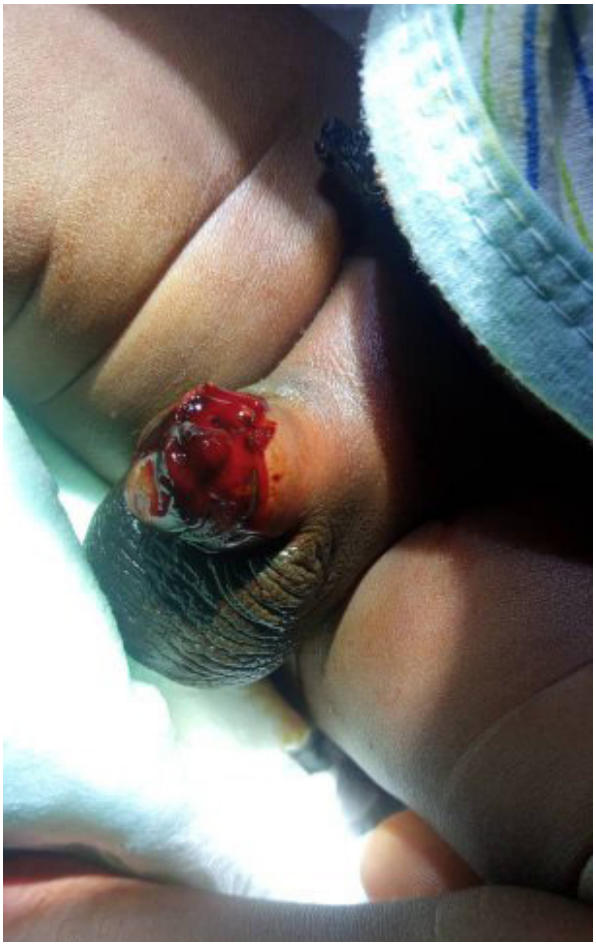

Fig. 1. Bleeding from plastibell circumcision

Fig. 2. Retained plastibell

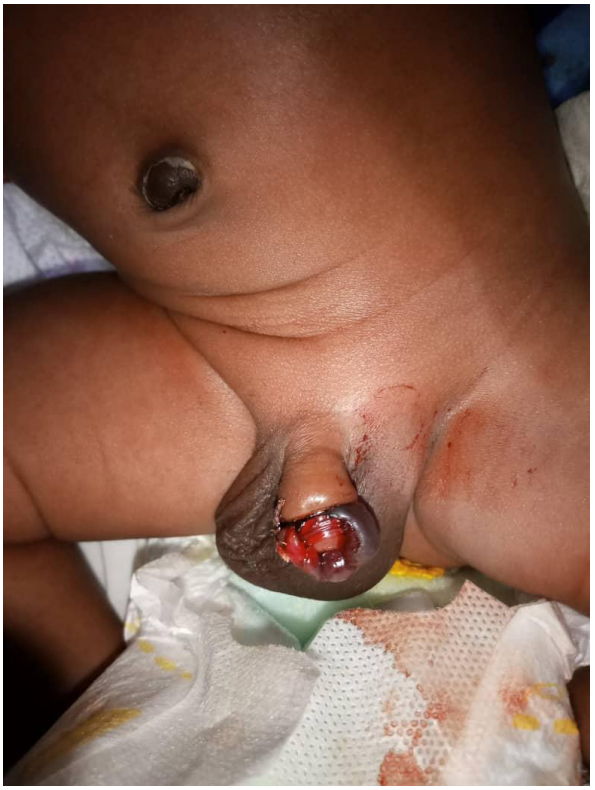

Fig. 3. Redundant prepuce from poorly performed plastibell circumcision

neonate stays in the hospital after circumcision may depend on the presence or absence complications at the time of the circumcision. Neonates who are bleeding or who are unable to pass urine may stay longer in the hospital. However, none of our patients had difficulty passing urine.

In the index study, most complications were seen in circumcisions performed by unregistered and unqualified nurses working in maternity homes. In developing countries, maternity homes are quite common. One study from Bradford hospital in United Kingdom reported higher complications in plastibell circumcision performed by nurses.[19] Another study from Oman reported that the risk of complications with plastibell complication is increased eight-fold when performed by nurses compared to surgeons.[14]

Plastibell is widely used for circumcision due to its versatility; it is even used by non-qualified people because of its ease of use.[17] Plastibell circumcision is fraught with complications and the complication may be life threatening.[14] Retained (impacted) plastibell was the most frequent complication recorded in the current study. Other series also reported retained plastibell as the most common complication.[20,21] Howbeit, other researchers reported bleeding and surgical site infection as the most frequent complication.[12,16] The reason for these differences in complication is not known. Retained plastibell may not reflect an intra-procedural technical

problem but a pre-procedural problem in the choice of right size of plastibell for a particular patient and this may cause complications. There are seven sizes of plastibell device and retained plastibell may result from the use of inappropriate plastibell size. Retained plastibell causes glanular constriction and possible proximal plastibell ring migration. Bleeding following circumcision with plastibell may result from tearing of the frenulum during insertion of the plastibell ring or from too long dorsal slit of the foreskin such that part of the slit is not secured with the tie around the ring.[18]

Treatment of complications resulting from circumcision with plastibell varies with the specific complication. Early complication such as hemorrhage required pressure application and ligation of bleeding vessels. Diluted adrenaline (1: 200,000 dilutions) was applied topically in recalcitrant oozing of blood. Redundant prepuce does not require immediate treatment. An interval of 6 months post circumcision was required for scar tissue maturation, subsequently excess skin was excised taking care not to damage the urethra ventrally. Retained plastibell required immediate removal of the plastibell. The plastibell was cut with Mayor's scissor. Smith et al reported the novel use of orthopedic ring cutter for removal of retained plastibell without any sequelae.[7] Infected circumcision wounds required repeated wound dressing using normal saline and appropriate antibiotics, based on the culture and sensitivity results. Skin bridges (penoglanular adhesions) required taking down the adhesion and separating the raw areas using non-adherent dressings to avoid recurrence. In glans necrosis, time is given for the proper separation of necrotic tissues from viable tissues. The remnant viable glans tissue was fashioned into a conical glans.

Urethrocutanous fistula due to plastibell circumcision occurred at a mean period of 9 days, post plastibell insertion. The fistulous communication resulted from pressure necrosis of the intervening tissues between the plastibell and urethra due to tight plastibell that stayed longer than necessary. Treatment of urethrocutanous fistula entailed repair of the fistula with or without a urethroplasty.

Although no mortality was recorded in the present study, one study from Benin, Nigeria reported a mortality of 1.2%.[22] Death from plastibell circumcision results from uncontrolled blood loss.[23]

Conclusion

Circumcision with the use of plastibell may be safe in trained hands. However, a wide range of complications can occur if proper care is not taken. The most complications occurred when the circumcision was performed by auxiliary (unregistered) nurses; these circumcisions were performed outside the teaching hospital. Retained plastibell and bleeding were the most common complications in the present study.

Recommendations and limitations of study

Circumcision in the hospital by experts, creation of parental awareness and early referrals of children with complications are advocated.

This was a retrospective study and a single institution experience which may not be generalizable.

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REVIEW PAPER

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Socio-economic status, iron deficiency anemia and COVID-19 disease burden – an appraisal

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ABSTRACT

Introduction. Severe Acute Respiratory Syndrome-2, possesses varying degrees of susceptibility and lethality worldwide and WHO declared this as a pandemic of this century.

Aim. In this background, the aim of this present narrative is to provide a complementary overview of how low iron stores and mild anemia offers protection from infectious diseases like COVID-19 by restricting the viral replication and also to suggest some potential adjuvant therapeutic interventions.

Material and methods. Therefore, we performed a literature search reviewing pertinent articles and documents. PubMed, Google Scholar, Chemrxiv, MedRxiv, BioRxiv, Preprints and ResearchGate were investigated.

Analysis of the literature. Recent studies reported drastic systemic events taking place that contribute to the severe clinical outcomes such as decreased hemoglobin indicating anemia, hypoxia, altered iron metabolism, hypercoagulability, oxidative stress, cytokine storm, hyper-ferritinemia and thus Multi Organ Failure, reportedly hailed as the hallmark of the COVID-19 hyper-inflammatory state. Interestingly it is globally observed that, countries with higher Socio-economic status (SES) have considerably lower prevalence of Iron Deficiency Anemia (IDA) but higher Case Fatality Rate (CFR) rate due to COVID-19 while, low SES countries characterized by the higher prevalence of IDA, are less affected to COVID-19 infection and found to have less CFR, which is almost half to that of the higher SES counterpart.

Conclusion. Present review presumed that,low iron stores and mild anemia may play a beneficial role in some cases by offering protection from infectious diseases as low iron restricts the viral replication.Thus, suggested iron chelation or iron sequestration as an alternative beneficial adjuvant in treating COVID-19 infection.

Keywords. COVID-19, iron deficiency anemia, socio-economic status

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Introduction

COVID-19 is a novel infectious disease, caused by SARS-CoV-2 which belongs to the family Coronaviride.[1] SARS-CoV-2, is a severe, complex, and multifactorial diseaseand driven by a combination of genetic and epigenetic factors.[2]This disease endangers disproportionately the elderly especially those with pre-existing co-morbidities.[3]From the large family of Coronaviruses, three have been known to cause severe pneumonia such as Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and recently recognized Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), possesses varying degrees of susceptibility and lethality worldwide.[4-7] Researches demonstrated that SARS-CoV-2 enters the human body through Angiotensin Converting Enzyme 2 (ACE2) receptor, present in the lung epithelial cell and develops a typical form of the acute respiratory distress syndrome (ARDS).[8,9] Current management system of COVID-19 is aptly focused on the fact that ARDS is the leading cause of fatalities.[10] Nevertheless with massive global research efforts and contemporary data and perceptive, continues to generate surge of information on COVID-19 pathogenesis. Consecutive studies reported drastic systemic events taking place that contribute to the severe clinical outcomes such as; decreased hemoglobin i.e. anemia, hypoxia,altered iron metabolism, hypercoagulability, oxidative stress, cytokine storm, hyper-ferritinemia and multi-organ-failure (MOF) which are reportedly hailed as the hallmark of the COVID-19 hyper-inflammatory state.[11-14] Evidences shows that these complications are inevitably associated at a systemic level, and suggests some other pathogenic mechanisms which remains largely un-elucidated.[15]

Data suggests SARS-CoV-2 can form a secreted protein encoded by ORF8 and a novel short protein encoded by ORF3b, which play a role in the viral pathogenicity.[16] Recently, an innovative pathophysiological hypotheses based on *in silico* demonstration proposed that a number of transcribed non-structural proteins (ORF1ab, ORF10, ORF3a and ORF8) coordinately attack the heme on the 1-beta chain of hemoglobin and inhibited the heme anabolic pathway which in turn increases the level of free floating irons.[17] Subsequent in vitro immuno-electron microscopic studies, provided evidences on the possible virus spike (S) protein interaction with hemoglobin (Hb) in red blood cells and with iron metabolism using the CD147 and/or CD26 receptors, other than ACE2.[18,19] Due to the wide expression in erythrocytes these receptors are proven to be deeply implicated in extensive pathologies associated with oxidative hemolysis, like decreased Hb level, hypoxia, thrombosis, objectively related to the clinical symptoms highlighted in course of COVID-19 infection.[20-23] Iron containing protein Hb is a functional unit of Red Blood Cell (RBC). Being an essential component of RBC's Production i.e. erythropoiesis, iron is also important for proper functioning of the host immune system and regulating many physiological process.[24,25] Furthermore, for proper functioning of host immune system body have to maintain iron homeostasis, a balance between iron absorption, transportation, storage and utilization.[26,27] The interaction of the peptide hormone, hepcidin and iron exporter ferroportin, interplays central role in establishing this delicate balance.[28-30] But interruption of this balance results in impaired iron homeostasis, can lead to both iron deficiency and iron excess which have detrimental effects.[31] Hyper-ferritinemia is a response of excess iron load, characterizes with several autoimmune diseases, and evince pathogenic role on the ground of its immunomodulatory properties, which has already been described as a cardinal feature of COVID-19.[12,13,32,33]Low iron concentration on the other hand restricts iron uptake by erythrocyte precursors, limits hemoglobin synthesis and causes anemia in which Hb concentration drops below the normal level (Male: >13.0 f/dl; Female: >12.0 g/dl) and become incapable to meet an individual's physiological requirements.[34,35]Anemia has multiple etiologies including nutrient deficiencies, acute and chronic infections, and genetic hemoglobinopathies.[36,37] Iron deficiency is often considered as the primary and commonest cause of anemia globally.[37]The onset of iron deficiency anemia (IDA) is influenced by various host factors such as age, sex, physiological, pathological, dietary and socio-economic status (SES).[38] Iron exists in twoforms, heme iron (rich in meat) and non-heme iron (rich in whole grains, nuts, seeds, legumes).[39] Although the intake of non-heme iron rich foods did not differ across different SES strata but study reported heme iron uptake increased as household income rose.[40] Studies also reported significant association between low SES and higher prevalence of anemia which in turn related to the severity of several communicable and infectious disease.[44-46] Higher SES (defined by the countries' GDP per capita) seems to have a protective effect on anemia and its related health complications.[40]

But in case of COVID-19, interestingly it is observed from the world COVID-19 tracker that, countries with higher SES (United States, Canada, Europe, Australia) have considerably lower prevalence of IDA, but accounts for higher CFR rate due to COVID-19.[47,48] On the other hand, low SES countries for example Africa (poorest continent of the planet) and Indiacharacterized by the higher prevalence of IDA, are less affected to COVID-19 infection and possesses less CFR due to COVID-19, which is almost half to that of the higher SES counterpart (Fig. 1).[47-49]

Hence, developed countries denoted by higher SES condition and normal hemoglobin level possibly

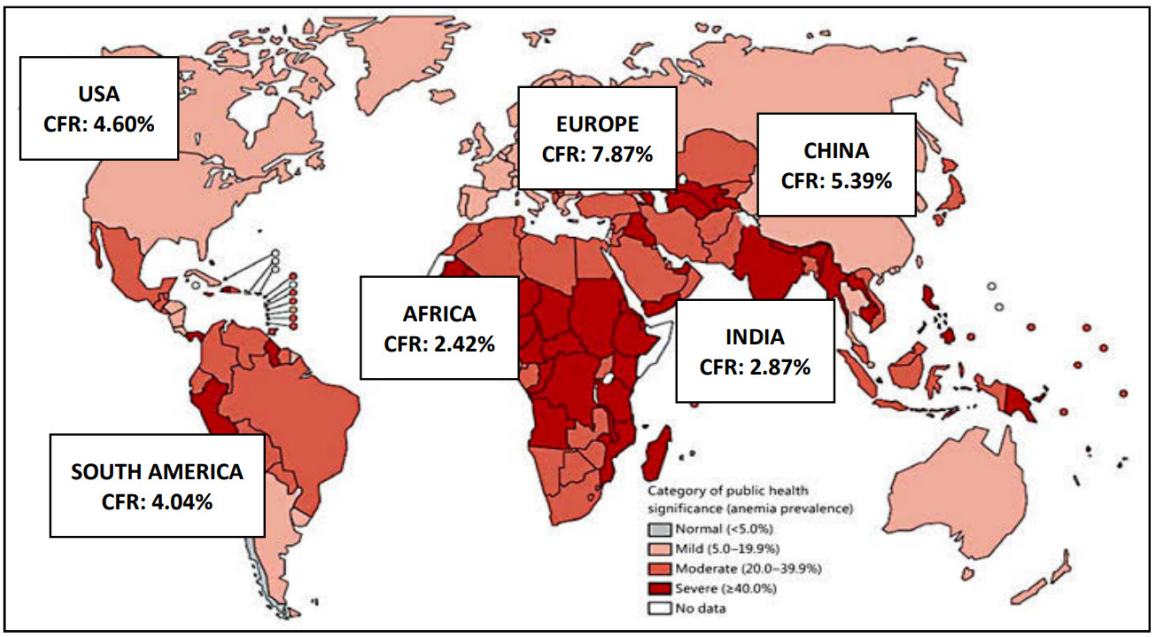

Fig. 1. Iron Deficient Anemia prone regions revealed low CFR by COVID-19, CFR: Case Fatality Rate (July, 2020 COVID tracker) Source: Pandey S, Singh V. Food Fortification to Combat Iron Deficiency Anaemia. International Journal of Advanced Nutritional and Health Science 2013;1(1): 39-47.

possesses greater severity of COVID-19 than the anemia endemic regions. While vaccines are yet to be approved, Remdesivir, an antiviral drug used for treating Ebola, SARS, MERS, has shown efficacy against SARS-CoV-2 infection.[50] Not only that, lopinavir/ritonavir, an approved anti HIV drug also has been recommended for treatment of SARS-CoV2 infection.[51] Most recently, high dose dexamethasone has shown efficacy in patients who are critically ill with COVID-19.[52] Although these drugs are showing a promising efficacy, but indeed, there is no specific drug against SARS-CoV-2. The drugs that are currently used for the treatment of COVID-19 are still assessed in clinical trials.[53] Therefore, it is urgently imperative to find out strategies for prevention and is urgently needed to recognize the possible factors causing variations in severity and fatality of the disease in different human populations.

Aim

In this background, the aim of this present narrative is to provide a complementary overview of how low iron stores and mild anemia offers protection from infectious diseases like COVID-19 by restricting the viral replication and also to suggest some potential adjuvant therapeutic interventions.

Material and methods

Therefore, we performed a literature search reviewing pertinent articles and documents. PubMed, Google Scholar, Chemrxiv, MedRxiv, BioRxiv, Preprints and ResearchGate were investigated, using the following headings and keywords, linked to the words COVID-19 or Sars-CoV-2: hemoglobin, heme, erythrocyte, hematopoiesis, erythroblast, hemolysis, hypoxia, hypoxemia, iron, hepcidin, ferroportin, ferritin, ferroptosis, hemochromatosis, iron chelation, translational medicine, oxidative stress, drugs, nutrition, food supplements, CD147, CD26, thromboembolism.

Analysis of the literature

Although there is fewer information about anemia or iron regulations in SARS-CoV-2 patients, some clues could be observed based on previous viral infections such as SARS,MARS, HIV-1.[54-57] Iron is crucial for both the host and the pathogen.[57,58] For the host, iron is essential for appropriate physiological process.[25]Likewise, several pathogens including virus, bacteria, fungi, and protozoa uses iron (host-cell elements) as niches for their survival.[29]Many Viruses, most likely including HCoVs rely on iron for their protein synthesis and genome replication in host cells. In the context of HIV-1 infection, iron is involved in several key steps of virus replication.[57,59] In the reverse transcription of viral RNA into DNA, the required dNTPs are generated by RNRs whichare an iron-dependent proteins.[54] NF-κB, contributes to the activation of HIV-1 promoter can be activated by iron and IκB kinase activation.[60-62] Nuclear export of new transcribed viral RNA is also iron dependent.[63] Finally, iron-binding ATPase, involved in the assembly of the gag capsid proteins into mature HIV-1 virions.[64] ATP hydrolysis is necessary for the unwinding activity of helicases of SARS-CoV and MERS-CoV during the viral replication.[55,56] Virus also use intracellular iron for their propagation beside heme iron.[57] Increased iron

storage in Macrophages also facilitates its replication which are presumed to be infected by SARS-CoV-2.[8] Thus, it is likely that SARS-CoV-2 requires iron for viral replication and for its functions.

Furthermore, studies reported many virusesincluding SARS-CoV-2disrupts iron homeostasis (induced by hemolysis) and increases the intercellular iron load, leads to the faster viral replication and ultimately the severity of the disease.[18,57,59] This iron overload in turn leads to significantly higher Ferritin level i.e. hyperferritinemia.[65] Ferritin, serves to bind iron molecules and to store iron in a biologically available form for vital cellular processes but moderate levels of hyper-ferritinemia are associated with autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis and antiphospholipid syndrome (APS) whereas, typically elevated levels are associated with other conditions including macrophage activation syndrome (MAS), adult-onset Still's disease (AOSD), catastrophic APS (cAPS) and septic shock.[66,67] Iron dependence of viral replication and modulation of host iron metabolism by viruses, signifies the importance of adequate iron supply for the completion of these replication process.[68] Butgrowing pile of clinical evidences reported that low iron stores and mild anemia may be beneficial in some cases by offering protection from infectious diseases as low iron restricts the viral replication.[29,58,59,65,69-71] Nevertheless, clinical data also indicated that poor prognosis is related to the condition of iron overload observed in patients with infection of hepatitis B/C (HBV/ HCV) virusesand iron depletion have a marked anti-HIV effect.[72-74]

As a consequence of above mentioned pathogenic scenario linking iron, ferritin and infection, it could be presumed that the potential of iron chelation or iron sequestration as an alternative beneficial adjuvant in treating SARS-CoV-2 infectionbecause of its ability to make a complex by binding with the iron and excrete from the circulation without any organ damage (Fig. 2) and also denying iron to invading microorganisms andprotecting the host tissues from hyper-ferretinemia related consequences.[57,65,75,76]

This diagram depicts COVID-19 leads to inflammation and during a heightened inflammatory state, cytokines, particularly IL-6, altered iron homeostasis and stimulate ferritin and hepcidin synthesis. Hepcidin, the key iron regulatory hormone, sequesters iron in the enterocytes and macrophages, leading to intracellular iron overload. Hyper-ferritinemia is associated with a state of iron overload. Excess intracellular iron enhances viral replication, interacts with molecular oxygen, generating reactive oxygen species (ROS) and also results in mitochondria dysfunction, microbiota dysbiosis (lungs and gut) and hyper coagulopathy. But, iron chelators may provide protective effects by inhibiting intracellular iron.

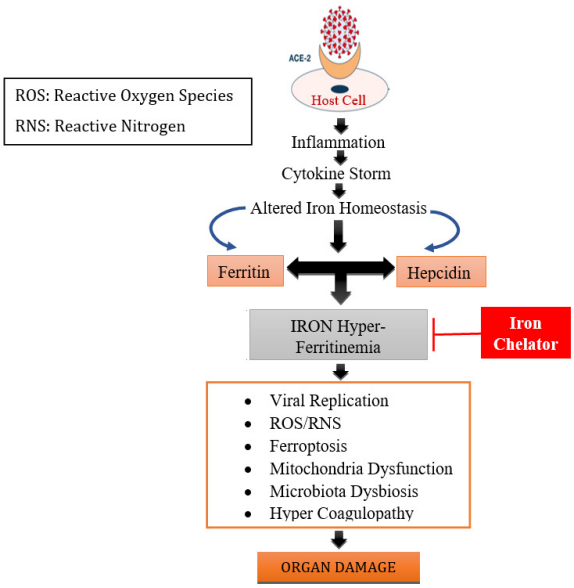

Fig. 2. Cycle of COVID-19 pathogenesis and the role of iron chelation

There are several iron chelators have been designed to excrete tissue iron through urine or feces. Each of these chelators has their own advantages and disadvantages. So while choosing iron chelation therapy one has to select very carefully according to the levels of deposited iron and clinical symptoms of the afflicted patients and the disease itself.[74] Beside this modulation of hepcidin and ferroportin expression during infection and inflammation increases iron metabolism as a host defense mechanism and decreases iron availability to invading pathogens.[29] This lead to the concept of nutritional immunity, as a whole of constitutive and inducible mechanisms that regulate the iron availability to pathogens and thus limit their capacity to infect the hostby disturbing the viral metal dependence which would presumably exhibit antiviral effects.[76,77]

Limitations

This review is mainly based on theoretical modeling, and on limited evidence. A number of scientific researches in this regard are needed in the next future. Data on COVID-19 case fatality rate (CFR) are taken using world COVID-19 tracker which might be biased by testing only symptomatic individuals, and not asymptomatic individuals for some countries. The speculative reasoning provided in this review may contribute to stimulate future studies, to corroborate or disprove our elaboration.

Conclusion

Present review presumed the potential of iron chelation or iron sequestration as an alternative beneficial adjuvant in treating COVID-19 infection due to its dual function of denying iron to invading microorganisms and protecting the host tissues from oxidative stress.

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REVIEW PAPER

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Membrane lipids under norm and pathology

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ABSTRACT

Introduction. Lipid is an essential component of the cell and its organelles membrane. The uniqueness and selectivity of lipids to specific functions and asymmetry of lipid distribution in the organelle's membrane give the cell ability of being highly qualified and specified.

Aim. The paper provides a comprehensive review of membrane lipids in different tissues and organelles of the cell in norm and disease.

Material and methods. The paper analyzed the present literature data on membrane lipids behavior in physiology and pathology.

Analysis of the literature. The major structural and functional lipids of the cell membrane are phosphatidylcholine > phosphatidylethanolamine. The absence/deficiency or augmentation of a specific type of lipid results in serious defects and usually life-threatening with a permanent disability. The observations discussed here suggest, the lipid peroxidation severity depends on the membrane lipid composition of the cell. Some tissue cells can handle lipoperoxidation and protect themselves from the peroxidation damaging products better, while other cells cannot compensate. Therefore, some organs are highly sensitive to peroxidation and irreversible changes occur rapidly.

Conclusion. To sum up, the understanding of lipid's role in norm and disease is clinically crucial to evaluate a novel therapeutic target to treat many metabolic disorders such as metabolic syndrome and some lysosomal storage disorders via targeting specific new signaling pathways, lipid molecules, and enzymes.

Keywords. cholesterol, lipid distress syndrome, membrane lipids, peroxidation, phosphatidylcholine, plasmenylethanolamine

The list of abbreviations:

AD - Alzheimer disease, Akt - protein kinase B, Chl - cholesterol, Co-Q - coenzyme-Q, COX - cyclooxygenase, Hsp70 - heat shock protein 70, IMM - inner mitochondrial membrane, INM - inner nuclear membrane, LOX - lipoxygenase, LT - leukotrienes, MAM - Mitochondria associated Membrane, MDA (MA)- malonyldialdehyde (Malondialdehyde), NE - Nuclear Envelope, OMM - outer mitochondrial membrane, ONM - outer nuclear membrane, PA - phosphatidic acid, PC (PtdCho) - phosphatidylcholine, PE (PtdEtn)- phosphatidylethanolamine, PhA_2 - phospholipase A2, PG - Phosphatidylglycerol, PlsC - plasmenylcholines, PE (PlsEtn)- plasmenylethanolamine, PrP - prion

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protein, PS (PtdSer) - phosphatidylserine, PSD - phosphatidylserine decarboxylase, PUFA - polyunsaturated fatty acid, SL - sphingolipid, SM - sphingomyelin, VSMC - Vascular smooth muscle cells, ARE- antioxidant response element, NFE2- nuclear factor erythroid 2, Nrf2- nuclear related factor 2, GPX4- Glutathione peroxidase 4, ER- endoplasmic reticulum

Introduction

In 1855, at the age of 34, Rudolf Virchow stated his popular aphorism "the whole pathology is the cell pathology".[1] For many years it was believed, all diseases begin on the cell level until recently when well developed the molecular biology field and the appearance of high-resolution images. In fact, the pathological process digs deeper inside the organelles of the cell that can be considered as complete structural and functional units that combined and give rise to this magical machinery unit called a cell. Usually, membrane lipids build in double layers to have more efficacy in their function. Since lipids constitute 40% of the cell and its organelles membrane with their irreplaceable functions, this yielded the importance of studying the lipids structure, function, and their role in norm and pathology.[2] The membrane lipids are the primary units of normal cell physiology and anatomy. The membrane lipids are extremely important because they condition the proper environment for the cellular processes. Physiologically, the membrane lipids function differently from each and are unevenly distributed in different cell compartments according to their task including receptor, signaling pathway as a first and second messenger, protection against prions, regulate permeability and membrane surface charge, and ion supply to the cell. Disturbance to such crucial and complex units in the cell with no doubt results in serious defects (ex. Gaucher disease and Tay-Sachs disease). Lipids consist of oxygen, carbon, and hydrogen; some may have phosphate and nitrogen. In humans, there is approximately a thousand major lipid including phospholipids, triacylglycerols (TAG), and sterols, besides the minor lipids.[3] The organelles lipid bilayer membrane contains variable admixtures of lipid depending on the task assigned to it. For instance, in the mitochondrion, the lipids comprise up to 25% of the inner mitochondrial membrane (IMM).[3] While the endoplasmic reticulum (ER) has the same lipid structure of the outer nuclear membrane (ONM), but in less cholesterol (Chl) concentration due to different functions.[4–6] The lysosome, peroxisome form a single phospholipid layer, and Golgi apparatus membrane lipid consists of phosphatidylserine (PS), sterols, and sphingolipids.[7] (Table 1) The major lipids of outer plasma membrane leaflet are phosphatidylcholine (PC), sphingolipids (SL), and cholesterol while in the cytosolic surface phosphatidylserine and phosphatidylethanolamine (PE). To sum up, the understanding of lipid's role in norm and disease is clinically crucial to evaluate a novel therapeutic target to treat many metabolic disorders such as metabolic syndrome and some lysosomal storage disorders via targeting specific new signaling pathways, lipid molecules, and enzymes. The lipidated proteins contribute to the appearance of a wide range of diseases since lipids build a critical percentage of the cell. The presence of lipids in such an amount determines the function and health of cells therefore revealed the importance of studying the composition and metabolism of membrane lipids in norm and disease besides lipid homeostasis disorders has become an urgent problem in recent decades.

Aim

The study aimed to analyze the literature data on the problem of homeostasis of membrane lipids in various intracellular structures of body tissues in the norm and pathology in addition to the function of each in the pathogenesis of diseases. The review comprehensively will discuss the major membrane lipid types, lipid biosynthesis and degradation, protein lipidation and lipid rafts, and lipid peroxidation.

Material and methods

The paper analyzed the present literature data on membrane lipids behavior in norm and pathology.

Membrane lipid synthesis and conditioning

Every synthesis process begins from the nucleus by stimulating a specific sequence of DNA, which then passes through the central dogma and gives rise to proteins that can serve and do their great duty. However, for the lipids, it is somehow different, since they are taken directly from the food that you intake as a chylomicron which contains TAG, then breaks down into free fatty acid and glycerol (therefore, the researcher believes that obesity is not hereditary, it's just a metabolic disorder, may be explained by the disturbance in the regulation mechanisms of lipid synthesis). Besides, some of the fatty acids are synthesized from Acetyl Co-A and NADPH.[8] What is interesting that the cell uses only 5% of its genome to synthesis all these various types of lipids.[9] Scramblase; present on the cytosolic surface of the ER while the flippases and floppases are present in the cytosolic surface; responsible for picking up the phospholipids and flipping them to the opposite side to balance the absolute number of phospholipids. There are up to 200 different types of phospholipid molecules depending on the need of this particular cell.[10]After the inflows of the food to the cells then they break down by specific enzymes to form free fatty acids and phosphate. Then the fatty acid will bind with the phosphate group through specific enzymes present on the outer surface (cytoplasmic leaflet) of ER, so they will add phospholipids

only to the outer surface of the ER (It's not known how the phosphoglycerolipids across the ER bilayer).[11] Thus, lead to augment the density of the cytosolic leaflet and its bending. This tension and change in physical properties in the membrane are detected by scramblases. Then, scramblases will randomly pick up non-selective phospholipids and flip them to the opposite side (luminal side) to sure the number of phospholipids on both sides is the same. Once the membrane has been made in the ER, it is sent through the cytoskeletal that connects with the Golgi apparatus (lipid trafficking/modifications).

The Golgi apparatus does not synthesis a new phospholipid membrane; it only modifies the lipid according to the necessity in different types of cells. Two basic rules in Golgy work; different locations (nucleus, lysosomes, peroxisome, ER, plasma membrane) within the cells have different needs, therefore they have different chemistry. Asymmetry of the inner and the outer surface working structure of the membrane (leaflet). It is responsible for conditioning the phospholipid bilayer, giving its specificity and selectivity even in the different parts of the same organelle or membrane. The question is how Golgi does that, and how does it know the destination to which it should send.[10,16]

Flippases and floppases present on the cytosolic surface of the Golgi apparatus too, they are specific for every phospholipid molecule (because there different shapes and active site differences). Flippases flip specific phospholipid molecules (maybe all these specific phospholipid molecules or just a percentage) from the outer surface to the inner surface (cytofacial), more exactly the PS and PE. Floppases work oppositely by flipping another specific phospholipids molecules from the cytosolic surface (PC, SL, and Chl) to the outer surface to correct the asymmetry of deposition of the phospholipids which has done by the scramblases. All these processes are against gradient therefore they use ATP. The flip-flop process occurs less than once a month for any individual molecule.[14] On the interior surface of Golgi present specific enzymes that can add sugar groups to the inner surface phosphate heads, later through a specific orientation, these sugar groups will found only on the outer membrane to form glycolipids. Therefore, the Golgi can modify the chemistry of phospholipids directly or indirectly to condition it to the location where it is needed to do its job.[10]

When the lipids have synthesized in the ER, they are sent to their final destination (plasma membrane, nucleus membrane, ER membrane, lysosome membrane, peroxisome membrane, and to the Golgi complex structure membranes) through an elusive non-clear mechanism, suspected to be by one of these pathways; the serum albumin, lipoproteins, vesicle transport, lipid transfer proteins (LTPs), lipid lateral diffusion through membranes, free diffusion through the cytosol, membrane to membrane contact, lipid flip-flop.[3,17–19] Glycerophospholipids undergo base-exchange, methylation, and decarboxylation reactions for interconversion. These reactions and activities of phospholipases A_2, C, and D are involved in the turnover, compositional maintenance, and rearrangements of glycerophospholipids in the membrane.[20]

Membrane lipid degradation

The etiological clues that damage the lipid layer structure of different organelles of the cell are: free radicals; reactive nitrogen species, reactive oxygen species, star-

Table 1. Lipid composition of subcellular fractions of rat and human liver cells, a membrane of human RBC, neuron, and myelin. Data from.[3,12–15] N.D. indicates not detected and blank indicates not analyzeľd. (OMM; outer mitochondrial membrane, IMM; inner mitochondrial membrane, NE; nuclear envelope, ER; endoplasmic reticulum)

	Rat liver cell (mol % of total phospholipids)														
	Mitochondrial Membrane			Endoplasmic reticulum Membrane	Lysosome Membrane	Golgi Membrane	Plasma membrane	NE	Human RBC Membrane	Neurons	Myelin	Mammalian Liver cell	Human ER	Human mitochondria (IMM and OMM)	Lysosome membrane
	Total	OMM	IMM												
PC	44	54	40	48-60	48	51	40	44	20	48	11	45-55	48	38	23
PE	35	29	34	19-23	13-17	21	24	17	18	21	17	15-25	19	29	13
PI	5	13	5	8-10	6	12	8	6	3	7	1	10-15	8	3	6
PS	2	2	3	2-4	0-3	6	9	4	7	5	9	5-10	4	0	-
Cardiolipin	14	<1	18	1	1-5	1	1	1	-	0	-	2-5	-	14	5
Phosphatidic acid	<1	1	-	1	1	<1	1	-	-	-	-	1-2	-	-	-
SM		1		3-5	23-24	8	17	3	18	4	8	5-10	5	0	23
	mol % of total lipids														
Chl	3	N.D.	N.D.	6	14	8	50	10	20	11	28	10-20	6	3	14
Glycolipid		Trace		Trace	-	0	-	Trace	3	3	20	-	Trace	Trace	-
Others		21		10	16	43	-	11	11	1	6	-	10	13	16

vation, ischemia, toxins, high intracellular Ca^{+2} level, some types of snake venom, etc.[21] The process of degradation of the membrane lipid may be enzymatically derived (physiologically) or non-enzymatically (pathologically) such as free radicals results of lipid membrane peroxidation.[22] The chain of damage by free radicals continues if the protecting antioxidant defense system cannot eliminate the free radicals. In compensated state degradation done by the phospholipase group of enzymes PhA_2, the most important one is the Ca^{+2} independent PhA_2 enzyme. When they do so this results in the formation of arachidonic acid, then enzymatically transferred into eicosanoids (leukotrienes, prostacyclins, and prostaglandins). Three main pathways for eicosanoids formation, firstly by cyclooxygenase (COX) enzyme synthesis prostaglandins (PG_s), thromboxane A2 (TXA_2), and prostaglandins I 2 (PGI_2) then via some reactions the PG_s reduces into PGG_2 and PGH_2, these are unstable molecules and short-lived.[23] Their synthesis depends on the expression of specific PG-synthesizing enzymes.[23,24] For instance, the TxA_2 is synthesized in platelets and macrophages, whereas PGI_2 is the dominant COX product of macrovascular endothelial cells.[24,25] Secondly, the lipoxygenase (LOX) enzymes such as 12-lipoxygenase and of particular importance 5-lipoxygenase which responsible for the synthesis of leukotrienes (LT) that contribute to the host defense and immediate-type hypersensitivity reactions.[25,26] Finally, through the cytochrome P450 enzymes can catalyze the arachidonic acid and result in the formation of hydroxy or epoxy derivatives of arachidonic acid as their major products.[27] In addition to the enzymatical pathway, there is a non-enzymatically pathway due to the effect of free radicals, lipid peroxidation, and lipids stress syndrome that can lead to the formation of PG-like compounds called isoprostanes.[28,29]

Protein lipidation and lipid rafts

After lipids uptake by lipophagy, it is stored intracellularly in the form of lipid droplets. Lipidation of protein is a term used to describe a protein conjugated with membrane lipids that physiologically attributes to membrane trafficking, control localization, organelle specificity, and intracellular signaling pathways.[30] In contrast, lipidation contributes to the development of various pathological disorders such as cancer progression and some neurodegenerative prion diseases, particularly via Rab25 gene mutation results in breast and ovarian cancers development, while mutation to palmitoylation of c-Src proto-oncogene tyrosine-protein kinase leads to prostate cancer. Besides, GPI-anchored disorders were found to be correlated with paroxysmal nocturnal hemoglobinuria (PNH).[31–33] The lipid rafts are glycolipoprotein lipid microdomains that range from 10–200 nm in size, present on the plasma membrane; consisting of glycosphingolipids, cholesterol, and protein receptors. The lipid rafts participate in various signaling transduction such as EGF, IgE, T- and B-cell antigen receptor signaling. Also, they serve as a platform for virus entry into the cells.[34]

Membrane lipid alteration under peroxidation

Peroxidation is a broad process that encompasses destroying membrane lipids, membrane proteins, enzymes, receptors, and even the ion channels, therefore in the clinical setting can find elevation in the hydrophobic and hydrophilic products of peroxidation.[35,36] Lipid peroxidation (LP) begins with initiation through the propagation and eliminates with termination.[37–39] LP starts after the abstraction of a hydrogen atom from a methylene group of polyunsaturated fatty acid (PUFA) that results in the formation of unstable carbon-centered free radicals, peroxyl radicals, alkoxyl radicals, and lipid hydroperoxide derived from unsaturated fatty acids; phospholipids; glycolipids; cholesterol esters, and cholesterol itself.[37,40] The later degradation to hydrocarbons, alcohols, ethers, epoxides, F2-isoprostane, and aldehydes.[41] Malondialdehyde (MDA) and the 4-hydroxy-2-nonenal (4-HNE) are the main functioning products of lipid peroxidation that can provoke apoptosis in addition to the inhibition of the gene expression process.[42–44] The aldehydes can cause damage to a far distance from their origin due to their relative long-lived, that promote aldehydes binding to the macromolecules and cause further damage by lipid peroxidation. The endogenous origins of the reactive oxygen species are the mitochondria, ER, plasma membrane, peroxisomes.[45] Melatonin and albumin show to have free radical scavenger and antioxidant effects.[46–48] PhA_2 serves as a secondary antioxidant via the elimination of the products of peroxidized fatty acid and forming a new one. If it is not completely replaced by a new fatty acid, it can act as a detergent and destroy the membrane. Other mechanisms of defense are glutathione peroxidase, particularly the phosphohydrolipid glutathione peroxidase that scavenge the hydroperoxides.[49] GPX4 is the only known enzyme that efficiently reduces lipid-hydroperoxides within biological membranes.[40] The few previous decades findings concluded that nuclear-related factor 2 (Nrf2) induces the detoxification and elimination of exogenous and endogenous chemicals through enhancing drug-metabolized enzyme by antioxidant and electrophiles, while this requires antioxidant response element (ARE) that parallel nuclear factor erythroid 2 (NFE2)-binding motive, culminating in enhancing anti-oxidative stress response.[50] In various tissues and different types of inflammation, the lipid peroxidation effects are variable depends on cell membrane lipid type (hepatocytes, nephrons, lung cells, intestine cells, etc.). The natural detoxification organs of lipid peroxidation products are the lung, liver,

kidney. Interestingly, some researchers go further and make it organ-specific, referred to as organ lipid distress syndrome. For instance, in the lung, higher PhA2 activity and lower lipid peroxidation and oppositely in the liver.[36] The primary indicators of detoxification impairment of peroxidation products are elevation in conjugated dienes, peracids, epoxides, plasma malondialdehyde (MDA), mono-keto/mono-hydroxy(epoxy) ratio, and high activity of PhA2.[36,51,52] Moreover, a decrease in the cell protector antioxidant defense system level such as superoxide dismutase, Catalase in the peroxisome, myeloperoxidase, thioredoxin peroxidase, glutathione peroxidase, urate oxidase, heat shock protein, haptoglobin, ceruloplasmin, transferrin, bilirubin, vitamin E and C, etc.[36,53] Researchers have shown, under the uncompensated lipid peroxidation, the higher the cell content of lipid the more and intensive endotoxemia and damage to the organism, since the damaged membranes lipids become a source of toxins.[36] More importantly in the clinical setting, urine and plasma isoprostane levels have proven to be reliable markers of lipid peroxidation and oxidant stress in vivo.[28,29] The stable lipid peroxidation biomarkers help to measure the level of systematic or tissue-specific oxidative stress.[53] For example, elevated levels of urinary isoprostanes were detected in women with android obesity and in individuals with alcohol-induced liver injury.[54,55] Both conditions are associated with increased oxidant stress and inflammation, as determined by other independent markers. Generally, when present lipid distress syndrome thus leads to elevate destroying of TAG, monoacylglycerol (MG), and diacylglycerol (DG), at the same time increase in the free fatty acid (FFA) level and variable effects on the cholesterol, sphingolipid (SL), and Chl-ester in the cell. The oxidative agent increases the availability of PE in the outer leaflet of the plasma membrane.[56] For eliminating lipid peroxidation and or its products, some studies in vitro have shown that the reduced form of Co-Q (Co-QH_2) can be described as an antioxidant.[57] Co-QH_2 is affecting the initiation process and inhibit the synthesis of lipid peroxyl radicals. Therefore, the researchers suppose it has more efficacy than quenching these radicals by tocopherol.[58,59] In the few previous years, Vlasova in vivo showed that ethoxidol has a similar modification on mild lipid peroxidation products via enhancing the self-antioxidant defense system in a mechanism still not clear, expected to be by inhibiting the formation of free radicals products through its capacity to donate electron and protection the membrane lipid to not be peroxidized.[36] Due to the role which is played by the balance between the saturated and unsaturated fatty acids in the ER lipid membrane, we may control lipid peroxidation through some medication that can minimize and inhibit the lipid peroxidation on the ER level. [60]

The major types of the membrane lipid

Phospholipids are the major structural and functional units of the membrane lipid in the plasma membrane, where they account for 60-75% of total lipids.[16,61] Phospholipids attribute to cell growth, proliferation, and cell permeability regulation depending on the fatty acid tail state. About 65% of the nuclear envelop lipids (NE) are phospholipids.[62] Therefore, disruption of the phospholipid composition is associated with a huge number of diseases.

Plasmalogens comprise about 18% of the total phospholipids mass in humans.[63] Containing two head groups; plasmenylcholines and plasmenylethalomines. About 30-40% of human heart choline glycerophospholipids are plasmalogens.[3] Plasmalogen phospholip-

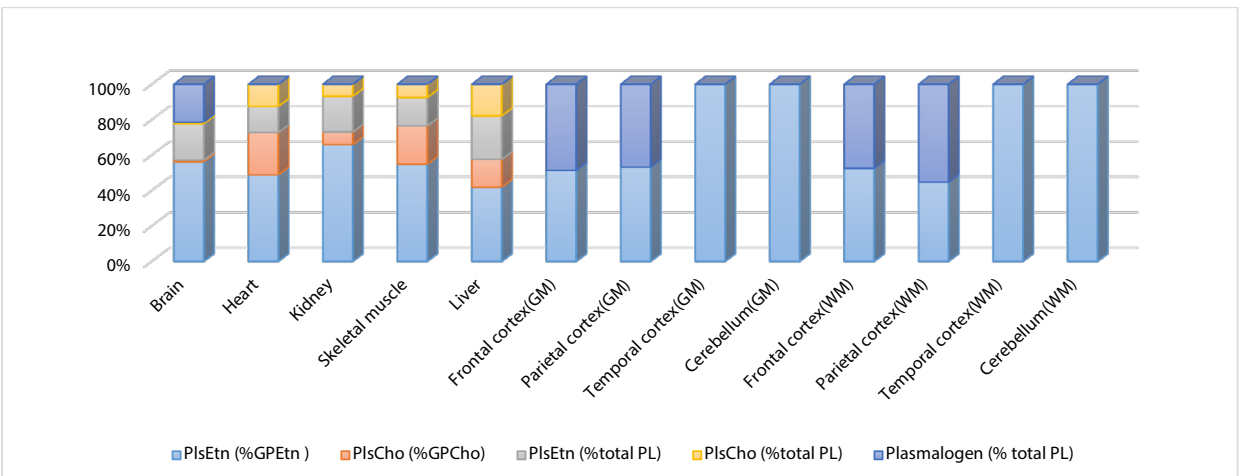

Fig. 1. Plasmalogen content in different human tissue. Abbreviations: GM; gray matter, WM; white matter, GPEtn; glycerophosphoethanolamine, GPCho; glycerophosphocholine, PLsEtn; plasmenylethanolamine, PlsCho; plasmenylcholines. The zero-percent does not necessarily mean absent. Source from.[71–74]

ids are involved in HDL-mediated cholesterol efflux.[64] Plasmenylcholine (PlsC) is known as the necessary storage for arachidonic acid in the heart.[65,66] Myelin sheath of the brain neurons has a high concentration of plasmenylethalomines and polyunsaturated fatty acid (PUFA) (Fig. 1).[4] Researchers believe plasmalogen disruption has been linked to Alzheimer's disease, Down syndrome, molecular signaling abnormalities, and cancer.[67] Plasmalogens synthesized in the ER then transported to the plasma membrane, depends on cellular ATP level. Disturbance of plasmalogen homeostasis impairs cholessterol biosynthesis.[68] Plasmalogens represent a major source of arachidonic acid, an important second messenger; it is believed that plasmalogens have a crucial role in protecting against oxidative damage.[69,70]

Sphingolipids (SL) are majorly founded in the outer leaflet of the plasma membrane, and devoid in the mitochondrial membrane. SL makes up 10% of all lipids in mammalian cells.[16] There are more than 60 different types of sphingolipids that function as a structure of different biological membranes, signal transduction, and biological recognition of these molecules.[3,75–78] Sphingomyelin is one type of SL; present in the outer membrane of the lipid bilayer plasma membrane, mostly founded in the nerve myelin sheath of myelinated neurons, lenses, and outer leaflet of the mammalian cell membrane. New research approved the anti-oxidant effect of SM in the elimination of lipid peroxidation propagation via the formation of an H-bond network within membranes as a biophysical antioxidant.[11,52] The inhibition of sphingolipid synthesis in the neurocytes is correlated with α-synuclein formation. The researchers pointed out that sphingolipids may decline with age in the human brain in PD and possible deficits of SL.

Ceramides are a key structural component of the stratum corneum of the epidermis serves as a skin barrier where they account for 50% of total lipid. Ceramides are a class of potential degradation products of sulfatides with some other molecules that are vital for the normal brain and the whole nervous system development. Ceramide Alteration is believed to be responsible for atopic dermatitis development. Their elevation in the white matter is expected to be responsible for dementia even the very mild dementia and AD.[79–82] Ceramides have a significant and key role in signal transduction in apoptosis, cell differentiation and maturation, regulatory function in the cell cycle, and cell stress.[83,84] Studies have shown that elevation of ceramide level can stimulate apoptosis in purposeless cell growth, and vice versa, the attenuation of ceramide content results in limiting the apoptosis process. For instance, in the endothelial cells and fibroblasts, ceramides regulate differentiation, maturation, and cell cycle arrest.[84–86] Ceramide synthesis inhibition prevents insulin resistance obesity, impairing the fatty acid oxidation, liver steatosis, and regulatory role in the inflammation.[43] Research has shown, ceramide can serve as a second messenger and inhibition for vascular smooth muscle cells (VSMC) division.[87–89] The ceramides are biosynthesized in the ER then transferred to the Golgi apparatus to be converted into complex sphingolipids then packed to their last destination. While GluCer and LacCer are the most common neutral glycosphingolipids in higher organisms. Elevation in their level is used as a marker of Gaucher disease (a rare lysosomal storage disorder).[90,91]

Phosphatidylglycerol is a minor lipid that comprises 1–2 mol% of phospholipids in a mammalian cell, but it has an important role and more abundant (10 mol%) in the lung surfactant. This indicates their significant role in protecting the alveoli from collapse and keep them open during expiration.[12,92] Also, researchers have demonstrated that under acute respiratory distress syndrome (ARDS) develop a depletion of phosphatidylglycerol (PG) and phosphatidylcholine (69 % from surfactant patient's fluid) via the PLA2G2A protein, which is strongly correlated to the high secretory phospholipase A2 (sPhA2) activity besides to extra alterations (elevation) in PI and SM levels in the surfactant fluid due to alteration their synthesis pathway or decreases consumption. Moreover, a novel study revealed the anti-inflammatory role of the PG in pulmonary tissue, in particular, viral infections and skin inflammatory diseases by inhibiting the DAMP. PG synthesized by head group exchange of phosphatidylcholine enriched phospholipid, using the enzyme phospholipase D. The presence of PG in the mitochondria and 20 mol% of CL in the IMM, with a high ratio of phosphatidylcholine (PC)/phosphatidylethanolamine (PE), assures its origin from bacteria.[93–96]

Phosphatidylcholine (PC) is one of the major components of the mammalian cell membrane (80%) and lipoproteins phospholipid, most of it in the outer leaflet (80%), while it accounts for 40-50% of total phosphoglycerolipids and is keenly involved in cell signaling.[9,11,97–99] About 40 mol% of the lipids in eukaryotic cells are phosphatidylcholines.[100] Choline, pyrimidine, and PUFAs are the regulatory precursors for PC synthesis. PC in the brain stimulate novel synapses formation, neurotransmitter formation and releasing, and cognition state of the individual, while thus can be of use in curing of AD. PC plays a key role in the alveoli surfactant, by forming its largest portion about 70-80% of its total lipid (90% lipid and 5-10% protein). PC forming approximately 40% of total lipids in the disks of the outer segment of the rods (light receptors, transfer electromagnetic to electrochemical signals, and has

rhodopsin and phospholipid in ratio 1:70).[101] Scientists have reported that PC microbial catabolite products; choline, trimethylamine oxide, and betaine elevate the atherosclerosis formation risk in mice, while its oral administration is used to improve ulcerative colitis.[102,103] PtdCho is primarily synthesized in the ER via repeated methylation of ethanolamine glycerophospholipids by S-adenosylmethionine (S-AdoMet), in addition to a minor pathway that seems to be similar to PtdEtn and PtdSer glycerophosphatides in the nucleus.[104] The rapid progress now being made in the area of chromatin organization as related to such factors as transcription regulation, RNA splicing, and nuclear transport mechanisms will simplify the role of lipid signaling in these processes.[5,105–107] PC is the major ER membrane bilayer phospholipid. Since the ER responsible for protein folding, therefore whatever discrepancy between its demand and supply for PC results in attenuation of its capacity for protein folding that culminates in unfolded protein response (UPR).[108–110] PC is required to facilitate the translocation of protein chain across the ER membrane due to its fluidity like property, moreover, its deficiency in the ER results in promoting calcium transport.[60]

Phosphatidylinositol (PI) is a major inner leaflet of plasma membrane phospholipid that comprises 10 %.[100] Usually found in brain tissue where it accounts for 10% of total phospholipids, while 98 % of the total in the liver and about 92 % in the brain are PI. PI is mostly founded in the INM and IMM where they are expected to be responsible for maintaining Ca^{2+} homeostasis in the nucleoplasm.[111] There are two important roles played by the plasma membrane PI when phosphorylated, first, they are the site for binding other enzymes, secondly, serve as a substrate for phospholipase C. When PI has been broken down this gives inositol triphosphate and diacylglycerol each of which important for further signaling events. Anderson and his colleagues have shown, a homozygous mutation in LPIAT1$^{-/-}$ (gene coding for variable PI species) reduces the PtdIns and PtdInsP2 content in the brain and liver approximately 26–44%, also PC and PE levels by 47% and 55% respectively, and non-compensable elevation in the less abundant PI species; lyso-PtdIns by 300% and 525% respectively, confirming reacylation disorder.[112] After hydrolyzing PI into DAG and inositol triphosphate, they serve as a second messenger in signal transduction, gene expression, hormone signaling transduction and metabolism, ion channels, pumps, transporters, control both endocytic and exocytic processes, and vesical traffic.[8,113–120] While these events can initiate parallel metabolic cascades that can mobilize intracellular calcium stores, activate protein kinase C, and release arachidonic acid. PI comprises 7-15 % of total phospholipids in the mitochondria where it is synthesized in small quantities. PI biosynthesized in the ER from phosphatidic acid via the intermediate cytidine diphosphate-diacylglycerol (CDP-DAG) derived by the rate-limiting enzyme CDP-diacylglycerol synthase (Fig. 2).[12] Its synthesis rate is regulated by the relative concentrations of the precursors and products. The phosphatidylinositol cycle proteins are responsible for transferring lipids between the ER and plasma membrane in both directions through membrane-associated family (PITPNM or nir2). The dysregulation of PI metabolism and signaling is a factor in many diseases, including cancer. Mutation in the phosphatidylinositol glycan class A (PIGA) gene results in Paroxysmal Nocturnal Hemoglobinuria.[121] The PI widely present in the ER much of it in the cytoplasmic surface, its deficiency results in ER stress that ends with unfolded (misfolded) protein response (UPR) in addition to extra metabolic disorders. While the external supply of PI precursor (myoinositol) could be beneficial for ER by enhancing its function and response to ER stress, insulin resistance (still ambiguous but thought to be through improving the putative inositol-containing mediators signaling pathway), and non-alcoholic liver steatosis. Alongside PI shown benignant effects on the weak spermatogenesis, polycystic ovary syndrome, gestational diabetes, metabolic syndrome, and retinopathy of prematurity.[122]

Phosphatidylserine (PS) is one of the essential components of membrane phospholipids where it accounts for 3-5 mol% of total phospholipids in the mammalian cell and approximately 2-15% of total phosphoglycolipids in the plasma membrane mostly in the inner leaflet (80%).[8,123] PS is most abundant in the brain tissue where it comprises 15% of total lipid.[101] More than 36% of the PS in the gray matter consists of docosahexaenoyl acyl chain, it is believed they responsible for normal brain and visual system functioning and development.[124] PS contributes to the activation of the synaptotagmin, dynamin-1, Annexin V, protein kinase C that regulates PS synthesis by phosphorylation (in vivo), and protein kinase B (Akt).[125–128] PS exposure to the cell surface has a significant role in platelet aggregation as well as in the elimination of apoptotic cells by the macrophages.[129–133] On the contrary, PS exposure to the inner leaflet results in plasma membrane bending and endosome formation.[134] The intracellular function of PS had not been discovered until recently, its ability to target proteins to phagosomes, and enhancing ion channel synthesis in the plasma membrane by binding to the heat shock protein (Hsp70) as well as caveolae formation in the plasma membrane through the signaling events.[135–137] PS comprises 13% of the total phospholipids of the disks of the outer segment of the rods.[99] Study by Zachowski on the erythrocyte membrane indicates that > 96% of PS resides on the inner leaflet of the bilayer lipid membrane.[138] PS lowest concentration in the mitochondria

particularly in the IMM.[139] PS biosynthesized by calcium-dependent base-exchange reactions in the ER where there is liberation for one polar head group choline or ethanolamine from the pre-existing phospholipid by the enzymes PS synthase-1 and PS synthase-2 in the mitochondria-associated membrane (MAM).[140,141] Through direct ER/MAM contact sites PS is transferred to mitochondria for decarboxylation and PE synthesis by phosphatidylserine decarboxylase (PSD).[142] PS is obligatorily required for cell viability, and its synthesis is regulated by PS cellular level. PS synthase-1 mRNA is highly activated in the brain, liver, and kidney.[143] While PS synthase-2 mRNA is expressed in the nurse cells of the testis and less in the liver and the brain.[144–146] Disruption to PS synthase-1 impairs the PS synthesis in 95% and appears to be responsible for Lenz-Majewsky syndrome development.[11,147] The elevation of osteoclast PS level showed to enhance bone formation with no change in the resorption rate.[148–150] Studies have shown that in the first stages of cytotoxic T-cell apoptosis increases the PS as well as PE level on the outer leaflet of the plasma membrane. Other findings indicated that tumor vasculature endothelial cells and cells under irradiation have elevated PE level on the outer leaflet plasma membrane too.[151–153]

Phosphatidylethanolamine (PE) compromises about 20-40 % of total phospholipids, while it accounts for 15-25% of total phospholipids in the mammalian cell.[11,100,154] PE distributed asymmetrically between the inner and outer leaflet plasma membrane (approximately 80% in the inner leaflet).[8,97,123] For instance, 5% out of the total phospholipids in the outer leaflet of the human RBC plasma membrane are PE.[155] Diacyl, alkylacyl, and alkenylacyl are the three main PE subgroups. Alkenylacyl accounts for 0.8% of the hepatocytes, while in the brain plasmenyl PE comprises 70% of total ethanolamine phospholipids, particularly 30% of neurocyte plasma membrane from total phospholipids and 90% from the ethanolamine phospholipids.[67,70] Researchers believe that the senile attenuation in PE level in the brain responsible for PD development via the formation of α-synuclein foci (unfolded or misfolded protein) in the Lewy bodies of the damaged dopaminergic neurons of pars compacta.[156–160] PE is required in the cytokinesis for disassembly of the contractile ring, also to evoke membrane curvature and fusion.[161,162] PE makes about 45% of total phospholipids in the nervous tissue such as the white matter of the brain and spinal cord. PE functions as an endogenous cofactor that by itself can facilitate prion propagation using PrP molecules from multiple animal species and without the assistance of any proteins or nucleic acids.[139,163] PE present in the inner mitochondrial membrane (IMM) in the highest concentration (40% of total phospholipid) than any other intracellular organelles lipid membrane. Moreover, sometimes there is a possibility to develop antiPhosphatidylethanolamine autoantibodies result in phospholipid syndrome.[164] PE accounts for 40% of the total phospholipid in the disks of the outer segment of the rods.[99] PE biosynthesized by four different pathways, one in the mitochondria (PSD pathway contributes in 5%) and the others in the ER (CDP-ethanolamine pathway contributes to 50% of rat hepatocytes PE synthesis, base-exchange pathway contribute to synthesis 8-9% of PE in the rat hepatocytes, and through the acylation of lyso-PE).[165–168] Most likely, it depends on the type of the cell to determine which pathway to use, for instance, fibroblast produces 80% of its PE through the PSD pathway.[169] The de novo pathway is regulated by the NF-Y transcription factor, protein kinase c-mediated phosphorylation.[170,171] PE, phosphatidic acid (PA), phosphatidylglycerol (PG), cardiolipin (CL), and CDP-DAG can be synthesized in the mitochondria as an auxiliary pathway.[172,173] The disruption or decrease of PE synthesis in the mitochondria results in impairment of the mitochondrial respiration activity of proteins of ETC and even mitochondrial morphological alterations and its fragmentation, this endorses the hypothesis that mitochondrial PE synthesized inside of the mitochondria. The mitochondrial malfunction is thought to be correlated with the development of serious defects such as neurodegeneration progression, cardiovascular dysfunction (due to its cardioprotective role against ischemia/reperfusion injury through activating STAT-3 transcription factor).[174–176] Besides, it has been shown that elevation in the PE ratio to the PC in the mitochondria could have opposite effects by stimulating mitochondrial respiration activity of ETC proteins and energy liberation. The PSD-derived PE plays an important role in the autophagy process through binding to LC3 protein and autophagosome formation, therefore PE deficiency leads to impairment autophagy and processing of GPI-Aps.[165,177–179] In addition to its role in the mitochondria, PE has a significant structural and functional role in the ER membrane shows that the misbalance between the PE/PC ratio of 1.3 in the ER membrane can activate the UPR.[60,180,181] And this would explain how the neurodegeneration progress under low PE level in the dopaminergic neuron and how choline could rescue the low content of PE in vitro. PE also contributes to the cannabinoid receptors synthesis in the brain.[182] Recent studies have shown that ferroptosis can oxidize the PE and forming cytotoxic species, which can be prevented by decreasing the content of long polyunsaturated ω^6 fatty acids with no need for Glutathione peroxidase 4 (GPX4). PE of the ER has been associated with the arachidonic acid and adrenic acid (AdA) oxidation in ferroptosis results in oxidized PE hydroperoxides species formation that kill cells.[60,183] Moreover, PE-AA-OOH molecule has been shown to promote ferroptosis,

while vitamin E inhibits oxidation through lipoxygenase (LOX) enzyme, which is considered an effective tool for ferroptosis prevention. It remains to be seen whether a link exists between ferroptosis and pathophysiological events, such as in ischemic-reperfusion injury or neurodegenerative disease. [184,185] PE is a key regulator of membrane fluidity in eukaryotic cells and helps pre-osteoclast fusion to form osteoclasts.[186,187]

Cardiolipin (CL) binding of PG molecules to a PA molecule forms a CL that comprises 20% of total lipids.[21] Tetra-linoleyl-CL (TLCL) is the most abundant species of CL, accounts for 80–85% of total CLs, and is associated as a precursor of signaling molecules. CL is symmetrically founded with all four FA molecules enriched in the inner leaflet of the IMM mostly of the cardiomyocytes, liver, and muscles.[8,133] CL forming the CO-Q also referred to as the third complex in the IMM, which involved in ETC as an electron carrier, extra-mitochondrial electron transport, endogenously synthesized lipid-soluble antioxidant, regulation mitochondrial permeability transition pores, and activation of mitochondrial uncoupling proteins, etc.[57] Also, it contributes to the intrinsic apoptosis, therefore CL responsible for mitochondrial stability and dynamics. The translocation of CL from IMM to the OMM is a sign of cell execution and completion of apoptosis.[133,188] CL interacts with respiratory chain complexes and substrate carrier proteins that are involved in the organization of the element of ETC into a higher assembly. Many enzymes of the respiratory chain are activated by CL and its lack results in serious defects. CL insufficiency is suspected to be responsible for the development of Barth syndrome, probably due to impaired remodeling of its fatty acids.[189] CL expression on the cytoplasmic surface of the mitochondria is a positive signal for autophagy and PhA2 activity to eliminate the damaged mitochondria through the microtubule-associated protein 1 light chain 3 (LC3). CL biosynthesized from PG and cytidine diphosphate diacylglycerol (CDP-DAG) in the inner leaflet of IMM besides some reactions occur in the ER.[190–192]

Phosphatidic acid (PA) is an intermediate in glycerolipid synthesis and cell signaling.[98,193] Studies have shown that the lacking form of PA for one fatty acid moiety (Lyso-PA) is highly involved as a signaling molecule, which contributes to the proliferation, migration, and survival of cells. Lyso-PA belongs to lysophospholipids (LPLs), where they normally provoke cell survival, ply mitogenic/antimitiogenic control of the cell cycle, affect cell motility and shape, control cell specialization, regulate Ca^{+2} homeostasis, lipid second messenger, and regulate the immunological response.[117,118,194] LPLs are shown to be correlated with tumor invasion, angiogenesis, neointima development, heart ventricles development, resistance in radiation and chemotherapies, facial dysmorphism, nociception, and suckling behavior. The PA founded in little quantities in the mitochondria as a minor lipid serves as a precursor of CDP-diacylglycerol for synthesis PI and PG in mitochondria.[12,195] Finally, Lysobisphosphatidic acid (LBPA) is specific for lysosomal membrane and secondary endosomes, where it appears to play an important role in controlling the formation of multivesicular bodies.

Cholesterol (Chl) is a non-polar sterol lipid and a major membrane component; range from 0.1% to 40% depending on the cell species and which subcellular compartment is under consideration.[16] Chl serves as a precursor for all steroid hormones. Approximately 20% of human erythrocyte weight is cholesterol.[100] While trace amount of Chl in IMM present.[3] In the NE can be founded only in the ONM and ganglioside (GM1) founded in the INM of neuronal cells. Chl is embedded in both cell membrane phospholipid bilayer structure, between phospholipids and phospholipid bilayers.[100,196] Cholesterol constitutes about 50% of the total lipid. About 10% (3 that of ER) of the NE lipids are cholesterol; lesser amounts of other neutral lipids (Chl-ester, diacylglycerol (DG), and triacylglycerol (TAG)). Generally, sterols are minor lipid components in the mitochondrial membrane.[3,12,62,197] Cholesterol supports and helps to stabilize the cell membrane and imitates as a fluidity buffer by regulating the permeability during high and low temperature and prevent leakage of small water-soluble molecules.[2,197] Chl organizes clusters of transmembrane proteins into lipid rafts (segregated, ordered domains within the cellular membranes formed by SM and cholesterol 50 mol %) and as a molecular "glue" that holds together membrane lipid rafts.[3,115] Elevation in cholesterol leads to serious pathological consequences such as atherosclerosis. Chl is unevenly disrupted in the ER, Golgi, and endosomes. In spite, Chl synthesized in the ER through the mevalonate pathway but it alternatively has low Chl content (<5 mol %). Cholesterol depletion inactivates Akt and strengthens membrane–cytoskeleton adhesion to be more rigid, while cholesterol incorporation activates Akt.[198] In 1985 the laureates Brown and Goldstein won a Nobel Prize for their discovery "receptor-mediated endocytosis" of the LDL cholesterol which contributes to the building lipids of the cell membrane, that later appeared to play a key pathological role in the familial hypercholesterolemia (FH) development via a mutation of LDL receptors that leads to high LDL plasma level and atherosclerosis formation.[199] Extra investigations should be done to role-out the influence of changes in cellular cholesterol levels and cholesterol distribution among cellular membranes on cell signaling. Chl- ester is not a highly significant structural part of the cell membrane, but it constitutes a huge portion of

the adrenal glands, and they concentrate inside the fatty lesions of atherosclerotic plaques.[200]

Triacylglycerol (TAG) has three free fatty acid chains and a glycerol group. These are the main fuel depot. FFA accounts for 15% of total lipid in the NE.[62] Studies In vitro show, Pcyt2 gene mutation causes TAG and DG accumulation in the hepatocytes, indicating the impairment of DG utilization into PE that leads to fatty liver development.[201,202] In the few past years, scientists indicated that whatever disturbance in the ER membrane saturated FA and/or cholesterol culminates in UPR directly (through the misbalance) or indirectly (due to UPR).[203–207] The PUFA is a precursor in stimulating PC synthesis.

Glycolipids are one of the three major lipids of the plasma membrane and loosely founded in the mitochondrial membrane, comprise of carbohydrate and lipid with sphingosine backbone.[12,14] Glycosphingolipids build about 5-10% of lipids in the outer surface of the plasma membranes. Besides, glycolipids have an important role in intercellular communication and protect in the harsh environment on the surface of the epithelial cells. Alteration to glycolipid was shown to be related to CNS pathologies. Glycolipids are thought to be responsible for the cell recognition process in the cell-cell adhesion process. Gangliosides are the most complicated glycolipids founded in the plasma membrane of the neurocytes, due to their charge gangliosides are believed to be responsible for control membrane potential especially the Ca^{+2} at the membrane surface.[14,208]

Prenol – few data suggested that prenol is an important simple isoprenoid that functions as an antioxidant and precursor of vitamin A.[209] When sugar groups bind directly to the membrane lipid this complex is known as a saccharolipid, which serves as a major structural component of the outer leaflet of the plasma membrane.[61] Polyketides are synthesized by classic enzymes as well as iterative and multimodular enzymes with semiautonomous active sites that share mechanistic features with the fatty acid synthases, including the involvement of specialized acyl carrier proteins; commonly used polyketides or polyketide derivatives as antimicrobial, antiparasitic, and anticancer agents such as erythromycins, tetracyclines, nystatins, avermectins, and antitumor epothilones. Besides some polyketides are potent toxins.[210,211]

Conclusion

Thus, an analysis of the literature data showed that the lipid composition of membrane structures depends on the type and function of organelles and the cell as a whole, as well as the type of tissue. The uniqueness and selectivity of lipids to specific functions and asymmetry of lipid distribution in the organelle's membrane gives power to the cell to be highly qualified and specified. The major structural and functional lipids in the cell membrane are Phosphatidylcholine (PC) > Phosphatidylethanolamine (PE). The absence/deficiency or augmentation of a specific type of lipid results in serious defects usually life-threatening with a permanent disability. The apparent indicator under lipid peroxidation is the dramatic elevation of peroxidation products that smash the membrane lipids, particularly when the protecting antioxidant defense system is impaired and cannot compensate to eliminate the highly reactive species. Further study of lipid homeostasis of cell membranes will reveal new intracellular signaling pathways, functions of lipid molecules, expanding knowledge about their role in normal and pathological cells. To sum up, the understanding of lipid's role in norm and disease is clinically crucial to evaluate a novel therapeutic target to treat many metabolic disorders such as metabolic syndrome and some lysosomal storage disorders via targeting specific new signaling pathways, lipid molecules, and enzymes.

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CASUISTIC PAPER

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Lian Gong for treatment of fibromyalgia – a case study

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ABSTRACT

Introduction. As a Complementary Integrative Practices (CIPs), Lian Gong has been increasingly used in the practice of Physiotherapy.

Aim. This study aims to verify the effects of Lian Gong in a patient with fibromyalgia.

Description of the case. In this case study the patient (one patient is evaluated) was diagnosed with fibromyalgia 29 years ago and sought care following discontent with previous treatments. Pain intensity was assessed with a visual analog scale (VAS), quality of life with SF-36, and the pressure pain threshold algometer (PPT). There were 16 visits with two weekly 60-minute sessions. When the patient was reevaluated, a Global Perception of Change (GPC) scale was added to assess general health. A folder was submitted to the patient for follow-up of exercises at home and asked to return after 4 weeks (follow-up period).

Conclusion. The results indicate improvement in pain, functional capacity and general health. As a first therapy treatment, Lian Gong proved promising results in one case of fibromyalgia. The possible benefits when combined with other forms of care should be explored by clinical trials to expand knowledge of health benefit potential.

Keywords. fibromyalgia, modalities of physiotherapy, Qigong, quality of life, rehabilitation, traditional chinese medicine

Introduction

Fibromyalgia is a chronic, rheumatic disease of undefined cause, likely to occur due to abnormalities in neuroendocrine regulation and as a response to stress, and has a higher prevalence in women between 40 and 60 years of age.[1] Despite the rheumatology approach, there are common neuropathic symptoms (paresthesia, numbness, and thermal sensitivity). Its incidence is 2-4% in the general population, with diffuse muscle pain as its main characteristic.[2] Genetic pre-disposition, emotional and cognitive factors, body-mind relationship, and ability to cope with stressful situations are all characteristics that contribute to the onset of fibromyalgia, but its etiology remains uncertain.[3]

Besides the above, there are other symptoms such as fatigue, sleep disorders, depression, changes such as hyperalgia, allodynia, morning stiffness, and headache (which can be exacerbated by hormones), physical and mental stress, temperature changes, changes in diet and sleep window.[1] The diagnosis is based on a set of symp-

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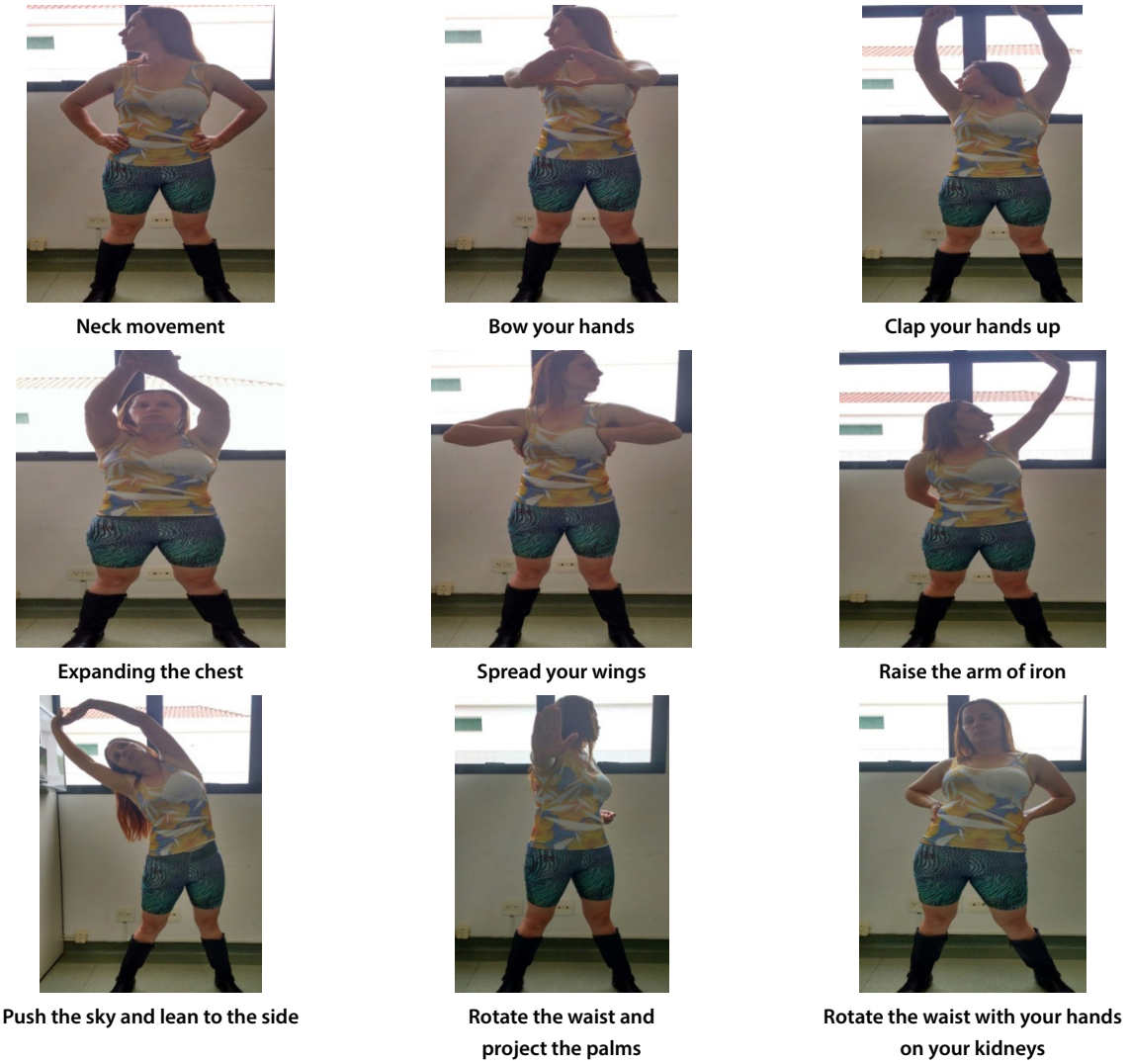

Fig. 1A. Booklet with representation of the exercises to be performed by the participant

toms that need to be present for at least three months, with pain in both hemicorps, tender points in eleven (or more) specific areas, generalized fatigue, depression and anxiety.[3]

As there is no cure for fibromyalgia, the treatment is based on symptom control and improves quality of life with drug management and non-pharmacological therapies. Physical exercise is part of the forms of treatment indicated in the multidisciplinary approach, as well as low power laser, balneotherapy and cognitive--behavioral therapy. Besides the usual practices of aerobic activity, strengthening and stretching, alternative and complementary therapies (Acupuncture, Massage, Meditation, Tai Chi Chuan, Shiatsu, among others) are applied in the management of fibromyalgia. A recent literature review on the use of such therapies in fibromyalgia has pointed to promising results, however, with the exception that these effects were based on small studies, but given the low risk, they could be considered an adjunct form of treatment. Although Lian Gong was briefly mentioned, there were no major considerations on the subject.[1] In recent years, there has been considerable growth in the use of resources beyond conventional Western medicine in rheumatic patients, yet scientific evidence of these practices remains under investigation. Lian Gong is considered one of these mind--body interventions that are part of the list of alternative treatments possibilities, consisting of gentle movements, breathing exercises, and meditation, producing gains in muscular power and endurance, flexibility, balance, and affecting psychological factors such as distress and anxiety.[1,5–8]

Lian Gong is of Chinese origin, has a long history (more than five thousand Years), and is practiced by more than 60 million people in China. Lian Gong can be defined as the ability to release, strengthen, and direct Qi "life energy" through specific exercises, harmonizing breathing, posture, body movements, and mind. Qi flows through meridians and its balance determines good overall health according to Traditional Chinese

Fig. 1B. Booklet with representation of the exercises to be performed by the participant

Medicine (TCM).[8–10] With this line of reasoning, the technique would be in a position to accelerate the self-healing process[6]. They are simple, smooth movements, with a choreographed, rhythmic routine, with respiratory control and can be adapted depending on the needs of the practitioner.[6,7] Although its effectiveness continues to be investigated, it is aimed at improving physical and mental well-being and quality of life.[8]

In Brazil, one of the strategies incorporated for health promotion by the National Policy of Integrative and Complementary Practices (NPICP), better known as "CIPs", published in the form of Ministerial Ordinances No. 971 on May 3, 2006 and No. 1,600 on July 17, 2006.[9] Some Brazilian cities have implemented, within their health policies, the use of Lian Gong for the population.[10,11]

While the effects of Lian gong in the West are still being explored, this resource is indicated in cases of fibromyalgia.[8]

Aim

The objective of this study was to present the effects of the technique, as a single therapy, in a patient with fibromyalgia that had been ongoing for decades.

Description of the case

The present study is characterized as a case study, being previously approved by the Research Ethics Committee of Centro Universitário Lusíada – UNILUS under number 2,243,010. The patient chosen was on the waiting list of the UNILUS Physiotherapy Clinic, with a diagnosis of fibromyalgia.

The patient E.M.L, 46 years, with 1.63 cm height and 67 kg, had been diagnosed at 17 years of age, and had performed physiotherapeutic treatment (infrared, TENS and hydrotherapy) and medications (muscle relaxant and antidepressant) before, but remained dissatisfied with the results.

Before the evaluations began, the procedures were explained to the patient and the informed consent form

Fig. 1C. Booklet with representation of the exercises to be performed by the participant

was signed. We applied the visual analog pain scale (VAS - ranging from 0 (no pain) to 10 (maximum pain possible), SF-36 quality of life questionnaire (0 - 100, which evaluates functional capacity, physical aspects, pain, general health status, vitality, social, emotional and mental health) and the pressure pain threshold (PPT) (Tester® - Kgf) on the 18 points related to the tendon points of individuals with fibromyalgia (adding the maximum pressure supported at all points, to generate a single final value).[5,9,16]

We also analyzed which Lian Gong movements the patient would be able to perform correctly or need only minimal adjustments, and then the treatment plan was constituted. After the second meeting, the sessions lasted 60 minutes, performing exercises that included the axial and appendicular skeleton, respecting the painful limit. It is therefore recommended that the session be made of six exercises in three series, totaling 18 exercises. For the evaluation of the exercises applicable to the patient in question, each movement was harmoniously performed three times, with respiratory control and following the work sequence of the "anterior and posterior part" of Lian gong's manual, totaling about 20 exercises per session.[11] The treatment was performed for eight weeks, twice a week, reaching 16 sessions.

In the reevaluation, in addition to the items mentioned above, the Global Perception of Change (GPC) scale was added (ranging from -5 to + 5, positive values indicate greater satisfaction), helping to analyze how efficient the treatment was.[13] After the reevaluation and the end of the clinic visits, the patient received a booklet with photos (figure 1A–C) showing how to perform the exercises, which she was already familiar with, so that she could continue at home and return for a new reevaluation after four weeks (follow-up). She was also guided to access the following links in case of doubt in the execution: https://www.youtube.com/watch?v=FgQkY-SAmJ6o https://www.youtube.com/watch?v=rbXOUi-VFLDo.

The other instrument used for general pain assessment, VAS, showed a significant pain reduction effect, occurring 50.9% and 58.4% of reduction at completion and follow-up after treatment, respectively. The SF-36 questionnaire recorded increases (Functional Capacity and General Health Status), stabilization (Physical, Social, Emotional and Mental Health Aspects) and decrease (Vitality) of its values (Table 1).

Table 1. Quality of life assessment through SF-36. Higher scores indicate improved quality of life

	Begin	End	Follow-up
Functional capacity	80	80	90
Physical aspects	100	100	100
Improvement of pain	51	51	51
General health status	30	47	57
Vitality	95	90	90
Social aspects	100	100	100
Emotional aspects	100	100	100
Mental health	84	92	84

The evaluation of the pain pressure threshold recorded a progressive increase in the capacity to withstand a pressure of 21.6% at the end of clinic visits and a total of 23.1% when returning for follow-up evaluation. The improvement in pain accompanied by VAS and PPT, probably participation in the positive response, was noted and maintained at the end and the return to the last evaluation of the study, by grading its improvement by +3 on the GPC scale (Table 2).

Table 2. Parameters linked to pain and general improvement*

	Begin	End	Follow-up
VAS	5.3	2.6	2.2
PPT	1315	1600	1620
GPC	*	+3	+3

*VAS – visual analogue scale, PPT – pressure pain threshold, GPC – global pain change

Discussion

Use of Lian Gong has proven useful as an add on therapy in the treatment of fibromyalgia in the case studied. These findings corroborate those found in other studies, which describe positive results with Lian Gong among the possibilities of alternative and complementary therapies when treating fibromyalgia.[14] The fact that it does not prescribe any specific device or tool for its performance, makes its practice valid in any environment and time. Through TCM, Lian Gong would be able to elevate the body's physical energy by promoting the fluidity of Qi and blood circulation, as showed by the increased electrical conductivity of meridians. It has already proven effective in preventing bone mineral loss, reducing oxidative stress, increasing antioxidant enzymes, homeostasis of the autonomic nervous system, activation of immune system cells, such as in reducing pain, improving balance, flexibility, agility, strength, fatigue, quality of life and sleep.[4,5]

This potentially justifies the improvements in overall pain, functional capacity, and general health status of the SF-36. Generalized chronic pain is the main complaint of fibromyalgia patients and is related to decreased quality of life. This association has previously been demonstrated in the comparison between the Fibromyalgia Impact Questionnaire (FIQ) and the VAS.[15] Despite the short intervention period (8 weeks) and follow-up (4 weeks), these were already sufficient for pain to be minimized at half of the initial assessment, corroborated by improvement in the other instruments (PPT and GPC). Since with the severity of the pain, there is a reduction in functional capacity. The painful improvement is assumed to have contributed to the increase in Functional Capacity in the SF-36.

The SF-36 was lower than the initial value, only in the item Vitality, there was improvement in pain measured both by the VAS and the pressure gauge and satisfaction with the proposed treatment via the Global Perception of Change scale. This way, it is believed that this variable did not present much of a change because the data from the other domains initially already presented high values. Since diffuse pain is usually the main complaint of these patients and although it has not been altered via the SF-36, the benefit achieved by other assessment instruments (VAS, PPT and GPC) stands out. This improvement in pain complaint, added to the increase in Functional Capacity and the General Health Status, generates a promising idea of the effects of Lian Gong on fibromyalgia.

Commonly used in association with VAS, the pressure algometer in the comparison of individuals with fibromyalgia to rheumatoid arthritis, dyspareunia and controls, shows lower pressure tolerance values, i.e., they feel more pain with smaller stimuli.[2] The characterization of fibromyalgia is generalized pain in 4 out of 5 regions (4 quadrants and axial).[20] The increase in pain pressure threshold with the practice of Lian Gong generates optimism and the need for studies with a larger number of participants and time, to determine how much improvement is expected to the longevity of a mind-body exercise routine.

GPC is easily and quickly applied, described as clinically relevant in cases of fibromyalgia when related to the patient's general clinical picture and previously used with EVA and body map (18 common tender point sites). The higher the values obtained through the GPC, the better the pain (via VAS and painful points) and all other measures of fibromyalgia severity.[13] What was found was in fact improvement of the painful condition (VAS and PPT) and the GPC.

Since fibromyalgia is not only musculoskeletal issue, there is psychoemotional involvement, Lian Gong fits the demand to be a mind-body therapy.[4] Mental Health of the SF-36 showed this effect during the face-to--face period of the intervention, but it returned to initial values during the four weeks of follow-up. Therefore, the result may have been the engagement of the professional during the sessions and not restricted to the technique. It is noteworthy that there are indications for the exercise to be performed regularly and even daily, in the present study the indicated practice was regular, but with 2 sessions per week.[8]

Since fibromyalgia is not a curable disease, therapies that help control symptoms and improve quality of life are the focus for these patients.[1] The benefits gained from the practice of Lian gong, its adaptability, and low cost, have caused this alternative to spread and conquer followers in Brazil.[10] CIPs such as Lian Gong, act in the promotion and integrality of health, surpassing the rooted curative model as the assertive one. It is common for Lian Gong to be practiced in groups, providing socialization, strengthening of the professional-patient relationship, and amplification of the individual's notion of his or her general state of health through contact with others in similar conditions. The use of Lian Gong in cases of fibromyalgia strengthens the understanding of the need for active participation in the care process adding to medical approaches. Although the publication of the National Policy of Integrative and Complementary Practices in SUS was made in 2006 and Lian Gong is not a new therapy, both the training of professionals focused on these practices and the scientific basis require investment.

The methodology used for monitoring the case reported here, while effective in reflecting symptomatic changes, could have been more complete if it had used specific instruments that evaluated sleep, fatigue, anguish, anxiety, and depression, which are to the painful complaint and the SF-36 items are to be addressed.[1,4,5] In addition to this limitation, it should be taken into consideration that this is a case study and cannot extrapolate the findings to all patients with fibromyalgia. Clinical trials will be more reliable in this response and it is worth noting the need for a control group to evaluate the real effects of this therapy, both its evaluation as an isolated form of treatment, as proposed here, although it is commonly described as adjunct. Progress has been made in proposing a placebo form of Lian gong.[4]

Conclusion

Lian Gong stands out for its adaptability, easy application, low demand for the necessary structure, low cost, and the main one, promising results through fibromyalgia. With the organization of health systems to adhere to self-applicable or simple inclusive care, Lian Gong seems to successfully meet the purpose of CIPs.

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CASUISTIC PAPER

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Persistent hyperthyroidism in a patient after total thyroidectomy: the thyroid anatomy has implications for treatment

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ABSTRACT

Introduction. Grave's disease (GD) can be treated using three modalities: anti-thyroid medications, radioactive iodine therapy (RAI), or surgery. If surgery is selected, total thyroidectomy is the procedure of choice. Patients with hyperthyroidism frequently have an enlarged thyroid gland, occasionally with a pyramidal lobe.

Aim. We point the usefulness of thyroid scintigraphy, which provides valuable information regarding the thyroid anatomy.

Description of the case. The manuscript presents a case report of 43-year-old woman with unstable Grave's disease, who underwent thyroidectomy and developed persistent hyperthyroidism postoperatively. She was referred by an endocrinologist to a nuclear medicine outpatient clinic for RAI therapy. I-iodide scintigraphy revealed two foci with excessive tracer accumulation. One of the foci in the middle of the neck corresponded to the pyramidal lobe.

Conclusion. The thyroid anatomy anomalies can lead to unnecessary implications for treatment. Identifying the pyramidal lobe preoperatively and removing it from patients requiring total thyroidectomy may decrease the recurrence rate of hyperthyroidism. Thyroid scintigraphy is a useful diagnostic tool to visualize the pyramidal lobe.

Keywords. Grave's disease, pyramidal lobe, scintigraphy, thyroidectomy

Introduction

Grave's disease is a kind of hyperthyroidism in which thyroid hyperplasia and toxicosis occur in response to high levels of antibodies to the TSH receptors. GD can be treated using three modalities: anti-thyroid medications, RAI, or surgery. The choice of treatment for GD requires presenting all short- and long-term side effects of the treatment options to the patient.

RAI therapy or total thyroidectomy are options for those resistant to medication or suffer from relapse of symptoms. Approximately one in four patients with GD may undergo total thyroidectomy. Patients with hyperthyroidism frequently have an enlarged thyroid gland, occasionally with a pyramidal lobe.[1] On the other hand detection of pyramidal lobe in patients with hyperthyroidism very often indicates an autoimmune reason of thyrotoxicosis. The pyramidal lobe (PL) is a remnant of the thyroglossal duct that extends superiorly from the isthmus and can reach the level of the hyoid bone. It is more common in men than in women, most commonly

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develops left of the midline, and has multiple anatomical variations. Based on anatomical studies, the frequency of pyramidal lobe development is between 15% and 75%.[2,3] In a study conducted by Wahl et al., pyramidal lobe was observed to have left lobe origin at 53%, right lobe origin at 39% and isthmus at 8%.[4] We report a patient who underwent total thyroidectomy and developed persistent hyperthyroidism postoperatively.

Aim

We point the usefulness of thyroid scintigraphy, which provides valuable information regarding the thyroid anatomy and can be helpful not only for endocrinologists but also for surgeons.

Description of the case

A 43-year-old woman presented to the nuclear medicine outpatient clinic with a few-months history of weakness, increased sweating and emotional lability. She was referred by an endocrinologist to a nuclear medicine outpatient clinic for possible RAI- therapy and it was our first meeting with the patient. The woman underwent total thyroidectomy 10 months prior due to unstable GD for three years (unsuccessfully treated with thyrostatic drugs). Preoperative ultrasound of the thyroid gland revealed a goiter with a volume about 55.5 ml.

Thyroid scintigraphy was not performed before surgery. She was smoking cigarettes. The choice of surgical treatment was associated with a large volume of the thyroid gland , high levels of antibodies (TRAb) and the patient's preferences. Information from the referral also included thyroid ultrasound examination after the surgery and histopathology. Please notice that the thyroid ultrasound showed only *stumps* of the *thyroid* lobes following thyroidectomy. The histopathology report described two lobes and isthmus of the thyroid and confirmed goiter hyperactivity. After surgery she received replacement levothyroxine (LT4) supplementation in a dose of 75 ug per day from a surgeon. Due to first symptoms of hyperthyroidism (six months after surgery) the endocrinologist reduced the dose of LT4 to 25 µg and next stopped the LT4 supplementation. The patient still presented symptoms of hyperthyroidism. Physical examination at the nuclear medicine outpatient clinic revealed inactive mild orbitopathy, increased sweating, regular heart rate of 90 beats/minute, normal blood pressure and an absence of pathology during palpation the neck area.

Ancillary investigations showed: suppressed serum levels of thyrotropin (TSH) 0.001 µIU/mL (0.55–4.78 µIU/mL), free thyroxine (fT4) 1.39 ng/dL (0.89–1.76 ng/dL), free triiodothyronine (fT3) 2.82 pg/ml (1.88-3.18 pg/ml), thyrotropin receptor antibody (TRAb) 34.0 IU/L (<1.5 IU/L). The thyroid ultrasound showed *stumps* of the *thyroid* lobes and the PL was not visualized. ^{131}I-iodide scintigraphy revealed two foci with excessive tracer accumulation. One of the foci in the middle of the neck corresponded to the PL (Fig. 1). The thyroid radioiodine uptake during the 24th hour was 39%. RAI therapy was administered after the patient's agreement. She was treated with 21.6 mCi of I 131 and developed symptoms of hypothyroidism.

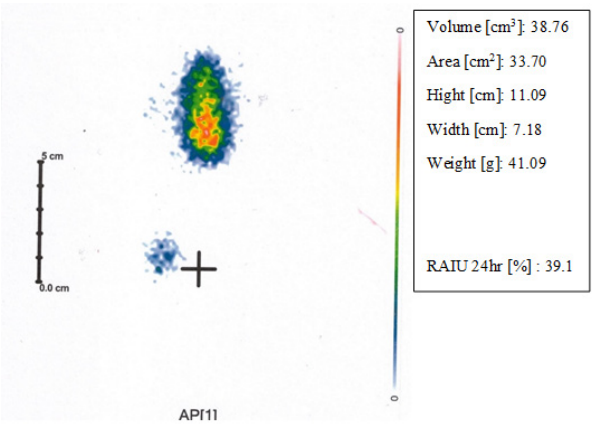

Fig. 1. Thyroid ^{131}I-iodide scintigraphy: foci with excessive accumulation of the tracer. In the middle of the neck, the pyramidal lobe was revealed

Currently, the patient is under observation in an endocrinology outpatient clinic. The patient is in a better condition without symptoms of hyperthyroidism, she takes LT4 (100 and 112 µg every other day, alternately). The thyroid hormone levels are normal.

Discussion

The pyramidal lobe could be a source of pitfalls in thyroidectomy, due to unreliable preoperative diagnostics. Imaging evaluation may influence the conduct and extent of thyroidectomy. The European Thyroid Association guidelines for the Management of Grave's hyperthyroidism recommend that if surgery is selected, total thyroidectomy is the procedure of choice. The initial thyroid imaging study is ultrasonography and should be performed in all patients before surgery. According to these guidelines scintigraphy of the thyroid is suggested when thyroid nodularity coexist with hyperthyroidism, and prior to RAI therapy.[5]

Total thyroidectomy rarely results in the removal of all thyroid tissue. The use of single-photon emission computed tomography to define specific anatomical sites of residual RAI uptake foci after total thyroidectomy shows uptake in 99% of patients, with 46 % in the pyramidal lobe. [6] When performing total thyroidectomy it's very important to look for identify and remove PL. Thyroid cells in the PL can become active after excision of the functioning thyroid tissue, so hyperthyroidism can appear in patients after total thyroidectomy due to GD.[7] The analysis of the available data presented by

Kim et al. revealed the sensitivity of preoperative sonographic detection of thyroid PL about 81%. There was no statistically significant difference in the sonographic detection rate of thyroid PL according to sex but the sonographic detection rate decreased with increasing age. In this study the number of false-negative cases of thyroid sonography were 11.4 %.[8]

Our patient had only undergone thyroid ultrasound examination before the surgery in which PL was not visualized. Undoubtedly, RAI ablation should be considered for hyperthyroidism recurrence after surgery, but the usefulness of thyroid scintigraphy before planning a surgery must be noted.

In many cases scintigraphy provides considerably more functioning and anatomic details than ultrasound.[9] According to the surgeon's guidelines, thyroid scintigraphy is not necessary before surgery.[10] Some reports have indicated that preoperative diagnosis of the PL based on scintigraphic images is unreliable, while other reports have suggested that thyroid scintigraphy is recommended in every patient before surgery.[3,7]

According to reports Braun et al. it is not reliably diagnosed the presence of PL by scintigraphy imaging because it can only give functional information. The authors note that the anterior cervical region has to be investigated very carefully during operation in order not to leave residual thyroid tissue in total thyroidectomy.[3] In a study presented by Cengiz et al. the prevalence of PL visualization using thyroid scintigraphy can reach 18%. PL visualization rate in patients with diffuse goiter was found to be significantly higher compared to other patients.[7]

In thyroid scintigraphy, PL is observed in higher rates in patients with hyperthyroidism and large thyroid gland.[7,11] In the study by Siraj QH et al. a PL was visualized on thyroid scintigraphy in 85 (41%) of the 207 patients.[12] In another study, PL was observed at a rate up to 81% among Graves' patients.[4]

The thyroid scintigraphy is useful for evaluating size, location and ectopia of thyroid tissue. Visualization of radiotracer uptake within a thyroglossal duct remnant and PL is frequently seen in the Graves' disease.[13] Neck CT is also useful for detecting the presence, size, configuration, and location of the PL but due to cost currently is less common.[14]

PL often remains non-visualized during preoperative imaging studies so anterior compartment of the neck should be explored for variations of the PL and completely excised during thyroid surgery.

Conclusion

This case shows that the thyroid anatomy anomalies can lead to unnecessary implications for treatment. The knowledge about the presence of the PL of the thyroid is very important for surgeons to perform better resection of the thyroid tissue (especially in cases of thyroid cancer, but also in cases of GD). Identifying the PL preoperatively and removing it from patients requiring total thyroidectomy may decrease the recurrence rate of hyperthyroidism.

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CASUISTIC PAPER

Piotr Przyczyna [1,2(ABCGF)], Elżbieta Trojnar [1(ABCGF)], Dorota Bartusik-Aebisher [3(DFG)], David Aebisher [4(DFG)]

Metastasis of cancer from Merkel cells to the thyroid gland

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ABSTRACT

Introduction. Merkel cell carcinoma (MCC) is a rare and aggressive neuroendocrine skin cancer.

Aim. Herein described is a case of hypertensive patient, after removal of Merkel cancer of the left gluteus skin (2011), after pulmonary embolism (2013), with degenerative changes of the spine and uterine myoma, chronically treated with Warfarin, because of suspected thyroid cancer.

Description of the case. A 70-year-old woman case after removing Merkel cancer of the left buttock skin (2011), after pulmonary embolism (2013), with degenerative changes of the spine and uterine fibroids treated chronically with Warfarin because of suspected thyroid cancer is described.

Conclusion. Increasing evidence of Merkel cell carcinoma with immunodeficiency and neoplasia, and the management and outcome of these patients requires study.

Keywords. Merkel cells, metastasis of cancer, thyroid gland

Introduction

Merkel cell carcinoma (MCC) is a rare skin cancer that mainly affects older people (median age is 69 years), white skin with light complexion. This tumor tends to resume locally and metastasize to regional lymph nodes.[1,2] The most common is formed in the dermis and infiltrates subcutaneous tissue. The diagnosis of MCC by means of light microscopy is difficult, therefore pathologists support immunohistochemistry and electron microscopy.[3] In the United States, about 1,500 cases are diagnosed. Due to the rarity of this cancer, we do not have fixed schedules. Although several studies conducted since 2000 have used population-based data sources to investigate the epidemiology of MCC, many studies lacked diagnostic and therapeutic data, and therefore the impact on the relapse and survival of

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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MCC could not be investigated to help improve treatment of the disease.[5-8]

Aim

The aim of this work was histopatological study of Merkel cancer cells. We describe a case an 70-year-old woman with hypertension and degenerative changes of the spine and uterine fibroids.

Description of the case

A 70-year-old woman with hypertension admitted to the Department of Internal Diseases was admitted after removing Merkel cancer of the left buttock skin (2011), after pulmonary embolism (2013), with degenerative changes of the spine and uterine fibroids treated chronically with Warfarin because of suspected thyroid cancer. In thyroid biopsy, atypical, probably cancer cells have been described. In the laryngological consultation it was found that there was no mobility of the right half of the larynx. In the CT scan of the neck and chest, in the right thyroid lobe there was a change in TU, modeling neck organs. The change went down behind the sternum, reaching the level of the aortic arch, surrounding the brachiocephalic trunk and its branches, with the narrowing of the right cervical lumen to min. 6 mm; the inner and outer jugular veins on the right side were not contrasted. In abdominal CT, bilateral adrenocortical tumors with high durability were found bilaterally. Due to the necessity of thyroid surgery, the patient was prepared for annihilation urgently and transferred to the Department of Surgery. Operational failure was found to be inoperable. Samples were removed and tracheostomy performed. On the second day after the procedure, the patient was operated on due to dyspnea. Due to the result of histopathological examination – metastasis of cancer from Merkel's cells, the patient was consulted with the Department of Soft Tissue Cancer, Bone and Melanoma in Warsaw. After the oncological consultation, the patient was disqualified from chemo/radiotherapy treatment due to poor general condition. After disqualification, the patient was transferred to the Palliative Department.

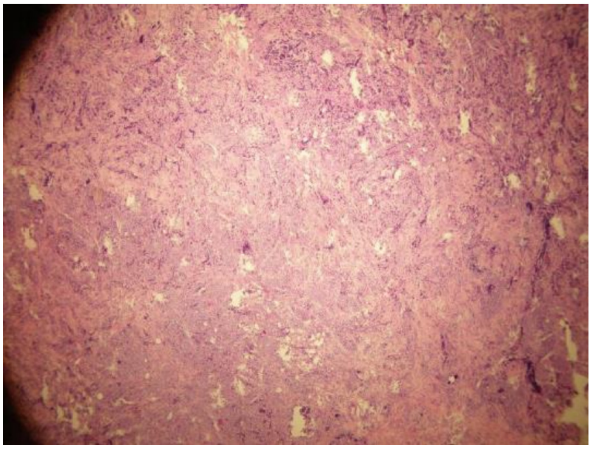

Fig. 1. Infiltrate of signaling cells in metastasis of cancer from Merkel's cells (H&E, 40x)

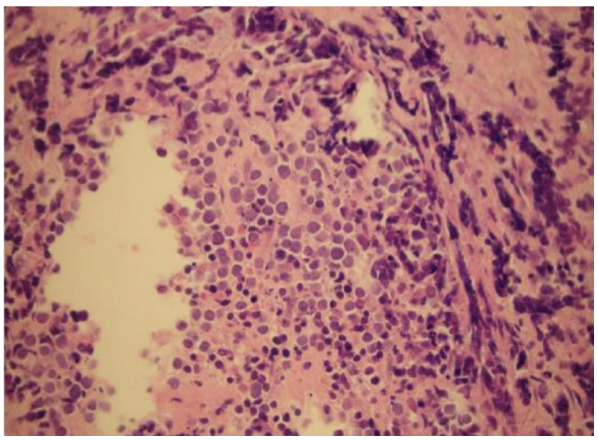

Fig. 2. Infiltrate of signaling cells in metastasis of cancer from Merkel's cells (H&E, 400x)

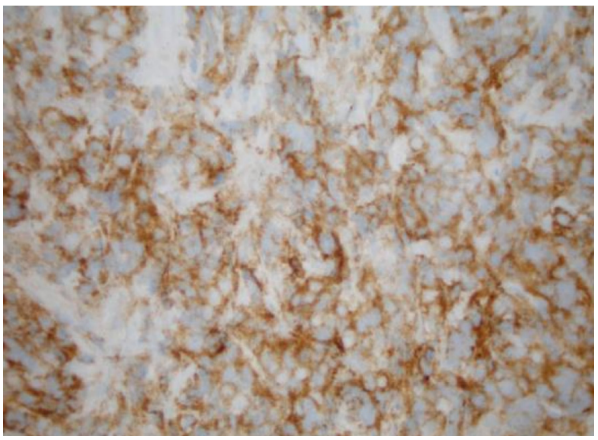

Fig. 3. Infiltrate of signaling cells in metastasis of cancer from Merkel's cells, D56 (40x)

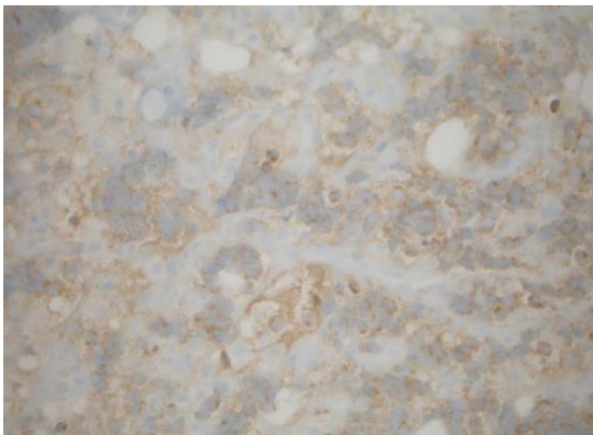

Fig. 4. Infiltrate of signaling cells in metastasis of cancer from Merkel's cells, Synaptophysi (40x)

Despite the fact that the cancer of Merkel cells is a rare cancer, it is an important problem because we do

not really know how to deal with patients. It is a challenge not only for clinicians who have problems with establishing chemo-and radiotherapy, and for histopathologists because of its similarity to other cancers (Figures 1-5).

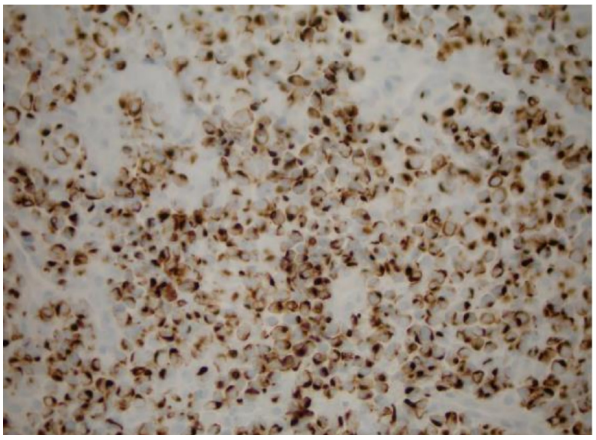

Fig. 5. Infiltrate of signaling cells in metastasis of cancer from Merkel's cells, CK-20 (40x)

Discussion

Recently, there have been studies stating that patients with an unknown primary site have a better prognosis, both relapse and survival.[9,10] MCC rarely affects the thyroid, so far there are only three such cases in the literature.[11] Differential diagnosis covers a wide spectrum of changes. From cancers originally originating from the skin - basal cell carcinoma, generalized diseases - lymphomas, metastases from other organs - small cell lung carcinoma. MCC rarely affects the thyroid, so far there are only three such cases in the literature.[11] Differential diagnosis covers a wide spectrum of changes. From primary cancers - basal cell carcinoma, generalized diseases - lymphomas, metastases from other organs - small cell lung cancer.

Conclusion

The prognosis in this cancer is poor, with a high mortality rate in the case of distant metastases. Metastasis to the thyroid gland is very rare and may present diagnostic difficulties.

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CASUISTIC PAPER

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Urolithiasis due to renal dystopia and vascular anomalies

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ABSTRACT

Introduction. Variations in the urogenital vascular anomalies in the abdomen are common. However, they warrant attention due to their importance in operative, diagnostic, and endovascular procedures.

Aim. The aim of this article is to show an example of a patient with rare kidney and vessels anomalies. We want to prove that those anomalies contributed to development of urolithiasis in this case.

Description of the case. During dissection of abdomen in a female cadaver, unique vascular anomalies and a position disorder of both kidneys were observed.

Conclusion. Vessel abnormalities were congenital and appeared simultaneously with renal dystopia. Both anomalies could contribute to stone formation and nephritis. Understanding of the urogenital anatomical variations and their relations to adjacent structures is significant during surgical and radiological procedures.

Keywords. renal anomalies, urolithiasis, vascular anomalies

Introduction

Congenital abnormalities of kidney and urinary tract occur in 3.3%-11.1% of the population.[1] Renal malrotation is a rare congenital variation of kidneys and hilum position, more common in males, with a prevalence of 1 in 2000 autopsies. The process of nephrogenesis is important to understand since most developmental abnormalities arise during the period of nephron formation. The sensitive exposure window, consisting of prenatal or postnatal periods of structural and functional development of the kidney, during which chemical agents or trauma may lead to kidney damage.[2] Congenital anomalies of kidney and urinary tract occur approximately 20,7 per 10 000 births according to the Polish Registration of Congenital Anomalies.[3]

Renal dystopia is a position disorder of the one or both kidneys, which is associated with disturbances in the process of their ascending, in other words they do

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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not reach their target location. One-sided dislocation is the inhibition of the kidney through its natural path of ascending. Cross displacement is a change in the direction of ascend. The kidney is then situated in an abnormal position outside its natural ascending path.[4] Displaced kidneys are more frequently located on the left side (80% of cases), characterized as hypoplastic and have an imperfect turn. The length of renal vessels and ureters are appropriate to the location of kidneys.[5]

Urolithiasis is a multiple and intersectional disease which results from multiple interactions between geographical, climatic, ethnic and genetic factors. The development of urolithiasis can be also induced by lifestyle factors such as eating habits or decreased intake of fluid. Hormonal or anatomical abnormalities also influence its pathogenesis.[6,7,8]

Aim

The aim of this article is to show an example of a patient with rare kidney and vessels anomalies. We want to prove that those anomalies contributed to development of urolithiasis in this case.

Description of the case

Presented case showing rare kidney and vascular anomalies was found in corpse of the prosectoring collection of the Department of Human Anatomy University of Rzeszow during dissection of 64 years old women. Unfortunately, we were not be able to obtain more information about the history of hospitalization and death of that woman.

Renal dystopia in patient

The observed position of the kidneys indicates a developmental defect - malrotationduring normal fetal development, the kidneys migration from the sacral to the lumbar region, caused by the growth of the posterior abdominal wall is followed by rotation. In this case, the kidneys did not rotate properly by 90 degrees, hence their hila point upwards, not medially.

Morphological changes in the left kidney

Smaller than the right one, with following dimensions - longitudinal dimension: 7 cm, transverse dimension: 3 cm, sagittal dimension: 3.5 cm. The thickness of the parenchyma was smaller than on the right side. We observed dilatation of pyelocalyceal system and abnormal arrangement of vessels in the hilum (extra renal arteries running in front, artery bifurcating in front of the hilum between the pelvis) (Fig 1-3). Comparing to correct and most common variant, the left kidney has a greater longitudinal dimension than the right, it is slightly thicker and lies higher than the right one.

The left ureter run between two branches of the renal artery in the hilum, then descended downwards covered with colonic vessels, on the psoas major muscle, lied laterally from the common iliac vessels, without crossing them. Moreover, the left colon artery run directly on the left ureter, which is laterally displaced from the correct position, and which may have caused additional compression and impeded the urine flow even more (Fig 2). During the widened ureter opening we found a stone. After the kidney was cut transversely and dilated, we saw multiple stones as well.

Fig. 1. Arteries anomaly, *Courtesy of Chair of Anatomy*
1 – right renal artery, 2 – left renal artery, 3 – right common illiac vein, 4 – left ureter, 5 – left common illiac vein, 6 – left renal vein, I – inferior vena cava

Fig. 2. Veins anomaly, *Courtesy of Chair of Anatomy*
1 – vena cava inferior, 2 – right renal vein branch, 3 – right renal vein branch, 4 – right common illiac vein, 5 – left renal vein branch, 6 – left renal vein branch, 7 – left common illiac vein; yellow arrows – ureters (the left – significantly widened along its entire length)

Morphological changes in the right kidney

Bigger than the left one, with dimensions - longitudinal dimension: 8 cm, transverse dimension: 6 cm, sagit-

tal dimension: 4.5 cm. The thickness of the parenchyma was greater than that of the left kidney uneven, lumpy and rough surface indicated chronic inflammation. We noticed also abnormal arrangement of the vessels in the hilum (an extra renal artery that runs to the lower pole of the kidney) (Fig 1-3). Enlargement of the size, greater thickness of the parenchyma and the ureter with a lumen of the correct diameter may indicate that the kidney has compensatively grown and has partially taken over function of the left kidney. Course of the right ureter is placed normally, it exited the kidney cavity rearward from the vein and artery, then run on the psoas major muscle, forward from the common iliac vessels.

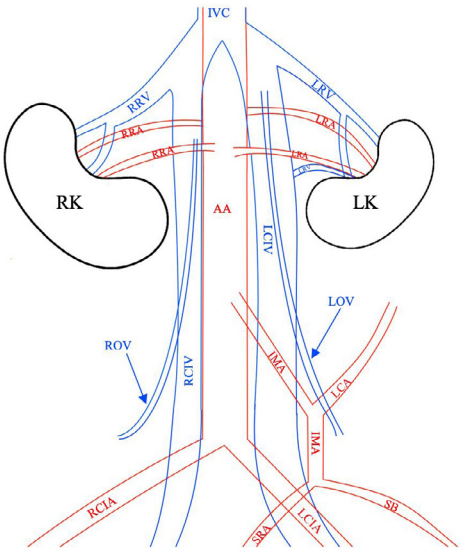

Fig. 3. Diagram of vessel anomaly (picture does not reveal correct proportions; some irrelevant vessels were omitted; author: Anna Pliszka)
RK – right kidney, LK – left kidney, AA – abdominal aorta, RRA – right renal artery, LRA – left renal artery, IMA – inferior mesenteric artery, LCA – left colic artery, SB – sigmoid branches, SRA – superior rectal artery, RCIA – right common iliac artery, LCIA – left common iliac artery, IVC – inferior vena cava, RRV – right renal vein, LRV – left renal vein, RCIV – right common iliac vein, LCIV – left common iliac vein, ROV – right ovarian vein, LOV – left ovarian vein

Vascular anomalies

We found additional renal arteries and veins, high joint of common iliac veins, and longer common iliac arteries (Fig. 1-2). The kidney was primarily vascularized by the branches of the common iliac artery when entering the lumbar region, it obtained vascularization from the abdominal aorta, while the branches originating from the common iliac artery disappeared.[9] Each kidney has an additional renal artery that extends from the abdominal aorta. We also observed long common iliac veins (the right common iliac vein is 20 cm long, while the left - 28 cm) joined into a short inferior vena cava above the renal arteries (at the level of the first lumbar vertebra L1). The left renal vein entered the left common iliac vein. The dissection also showed that the renal veins entered the inferior vena cava at an acute angle, so they do not form a typical „cross pattern". When it comes to longer common iliac arteries in this case -the length of the right and left arteries is 10 cm (to compare, the average length of the common iliac arteries is 5 cm).[10] Survived and accessory vessels pressed on the ureter and impeded the flow of urine from the kidney pelvis.

Discussion

The renal anatomy and its development are complex. This complexity is associated with numerous variations. Each renal variation has its own clinical and surgical importance

Frequently, malrotation of the kidney with vascular aberrations predisposes patients to renal or ureteral obstruction, with consequent upper tract infections, stone formation, and abdominal pain; other common symptoms are hypertension and renal disorders, or even kidney failure. Urine in some cases may flow backward from the bladder to the kidney, leading to vesicoureteral reflux and kidney scarring.

Although renal dystopia is not the most common anomaly to deal with, urologists and surgeons should be aware of its sometimes even unexpected occurrence during their practice. More frequent pathology is horseshoe kidney (incidence of renal stones about 20% - 40%) and ectopic kidney – ectopic pelvic, crossed ectopic fused or separate (about 37% has insignificant residual fragments while normal kidneys - 18,5%).[11,12] Such cases need special approach in treatment. According to the algorithm the management depends on the stone(s) size, density in Hounsfield Units (HU), occurrence and type of vessels anomalies, kidneys number and location and urinary tract drainage. The final surgical procedures are percutaneous nephrolithotomy (PCNL), retrograde intrarenal surgery (RIRS), extracorporeal shock wave lithotripsy (ESWL), laparoscopic pyelolithotomy (LP).[11]

More complicated cases might need more modern procedures using robots such as robotic pyelolithotomy.[13] We have to be aware of possibility of rare kidney anomalies occurrence such as kidney malformations in our practice and detect them to provide complications during surgery and other following diseases. There are modern and precise procedures which we should use in such rare and complicated cases to decrease the risk of complications related to abnormal kidney location or vessel anomalies.

Kidneys rotation and vessel formation occur in the same time during embriological development.[14,15] Vessel abnormalities were congenital and appeared simulta-

neously with renal dystopia. Concluding, both anomalies contribute to kidney or ureteral obstruction, with consequent upper tract infections, stone formation and nephritis.[16–18] Abnormal position of kidneys and abdominal vessel anomalies represent indirectly a risk factor for urinary tract diseases. The diseases are recurrent urinary tract infections and secondary development of urolithiasis due to the abnormal passage of the upper urinary tract.[11,19,20]

Conclusion

The nephrolithiasis could appear due to incorrect position of the kidney associated with presence of additional blood vessels impeded urine flow to the bladder and caused urine retention in renal pelvis. This process not only contributed to upper urinary tract distension, but also to the formation of stones. Uneven, rough surface, size enlargement and parenchyma thickening are obvious post-mortem evidence that patient underwent nephritis; we can conclude that it was caused by nephrolithiasis due to the dystopia.

On second thought, vascular anomalies could simultaneously be a risk factor for urolithiasis. The abnormally located vessels pressed on the ureter and led to urine flow perturbation. That process caused urine retention, which leads to urolithiasis. Moreover, in that variant of the disease's etiology, retention should have been also in renal pelvis so that fact explains its distension, kidney stones and nephritis.

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CASUISTIC PAPER

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Crohn's disease – a case study

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ABSTRACT

Introduction. Surgical treatment of patients with Crohn's disease can be a big challenge, due to the high risk of complications that is associated with chronic inflammatory process, immunosuppressive, biological treatment, malnutrition, patient's wasting or prone to recurrence of inflammatory changes.

Aim. The aim of this work ist o present the case of patient underwent surgery, resection of the terminal ileum, right hemicolectomy and segmental jejunum resection.

Description of the case. In this case patients with Crohn's disease were accompanied by progressive nutritional deficiencies and cachexia.

Conclusion. Crohn's disease can lead to very severe abdominal and septic complications that require long-term treatment, repeated surgery, and open belly therapy with the use of vacuum therapy

Keywords. biological treatment, Crohn's disease, inflammatory process

Introduction

Crohn's disease is a chronic, non-specific inflammatory process in which changes can affect any part of the gastrointestinal tract from the mouth to the anus, the changes are segmental (healthy sections occur between the diseased sections), asymmetrical and full-walled. Despite the advances in conservative and pharmacological treatment, most patients require surgery at some point during the disease.[1-3] The need for surgery is 10-14% during the year and 18-35% during the 35 years of the disease.

Indications for surgical treatment can be divided into:

1. urgent (intestinal obstruction, perforation of the intestine with peritonitis, bleeding, fulminant disease not subject to pharmacological treatment, intra-abdominal or perianal abscess causing sepsis)

2. planned (symptoms of chronic intestinal obstruction, chronic disease symptoms causing disability, intra-abdominal abscesses and fistulas resulting in malabsorption syndrome, intestinal epithelial dysplasia or cancer).

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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From 5-15% of patients have entero-enteral fistulas. Operations of patients with Leśniowski- Crohn's disease are at increased risk of complications and relapses. Surgical treatment in Crohn's disease can be a big technical challenge for a surgeon due to chronic inflammation, biological treatment, immunosuppression, cachexia, patient malnutrition, or a tendency to relapse inflammation.[4-7] Leakage, anastomosis dehiscence, abscess or fistula are life-threatening complications requiring reoperation and intensive treatment in the Intensive Care Clinic. Open abdomen treatment is a method of treating patients with severe septic peritonitis and the risk of abdominal compartment syndrome. Providing patients with OA is a challenge, it is associated with massive loss of fluid, electrolytes and protein, a high risk of infection, bleeding, obstruction or perforation of the intestine, as well as frequent multi-organ failure and high mortality.[8-12] Abdominal decompression can cause peritoneal adhesions, fistulae formation, fascia contraction and large postoperative hernias requiring abdominal wall reconstruction. It is rational to treat the open abdominal cavity as an open abscess. There are various ways of temporary abdominal closure. Initially, their task was to cover the internal organs (closing the skin itself, "Bogota bag", sewing the zipper), now, in addition to visceral covering, TAC allows exudate control and approaching the edges of the wound - negetive pressure wound therapy.[13-15]

Aim

Tis work aimed to present 10 years follow up for clinical case diagnosed a 29-year-old patient with Crohn's disease.

Description of the case

A 29-year-old patient with Crohn's disease diagnosed in 2009, treated for many years with azathioprine and mesalazine during periods of exacerbation with glucocorticoids. He did not take medicine for a year to March 2019. From March 2019 to April, 2019. hospitalized in the Gastroenterology Clinic, to which he was admitted due to abdominal pain accompanied by intestinal passage disorders. After performing additional tests, anemia with low iron levels, low protein, albumin, vitamin D levels was found, according to the SGA scale, nutritional status was assessed as severe malnutrition (body weight 52kg, height 175cm, BMI 16.98), increased inflammatory parameters. Gastroscopic examination no pathological changes, colonoscopy found active inflammatory changes in the caecum and obliterated ileocecal valve orifices. Magnetic resonance imaging (MRI) of the abdomen and pelvis were performed revealed the thickening of the walls of the cecum and the ascending colon and the ileocecal valve area with a slight fluid reaction in the environment - inflammatory lesions and the perianal fistula canal (Figure 1). The treatment used empiric antibiotic therapy, systemic glucocorticosteroids, total parenteral nutrition. The patient was then transferred for surgical treatment to the Surgery Clinic. After preparation, a laparotomy was performed. Intraoperatively, inflammatory changes were found with thickening of the terminal wall of the ileum with stenosis, thickening of the caecum wall and fistula features between the jejunum loop and ascending colon. Right hemicoloctomy with ileo-transverse mechanical anastomosis and jejunal segment resection with mechanical anastomosis were performed.

On the 4th day after surgery, the patient's condition deteriorated significantly, which was accompanied by tachycardia, fever and a significant increase in inflammatory parameters, in ultrasound a large amount of liquid content with increased echogenicity. Relaparotomy was performed, diffuse fecal peritonitis was found due

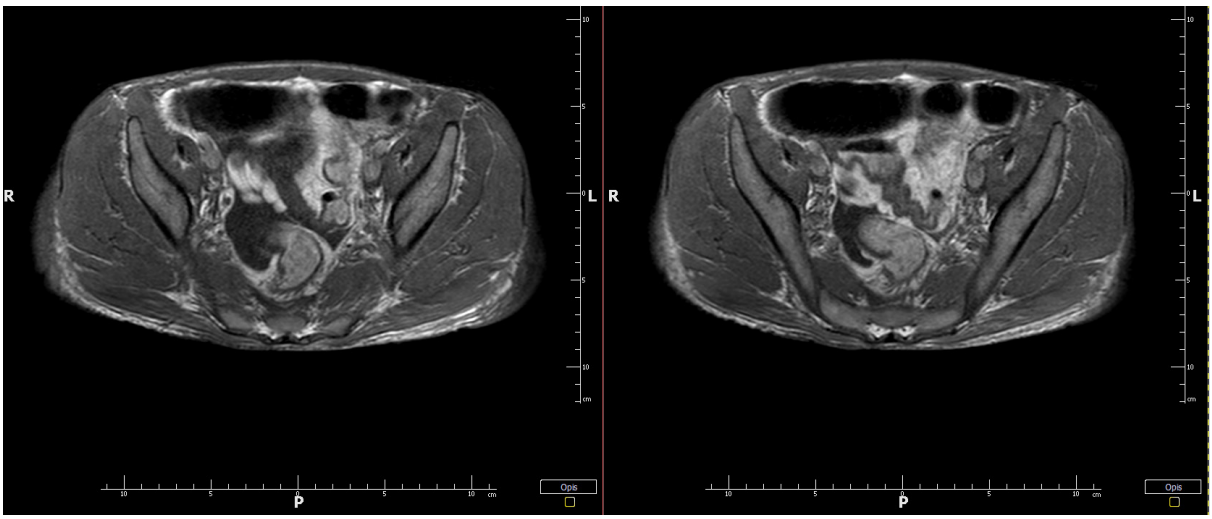

Fig. 1. An MRI Image of the abdomen and pelvis revealed the thickening of the walls of the cecum and the ascending colon and the ileocecal valve area with a slight fluid reaction in the environment - inflammatory lesions and the perianal fistula canal.

to complete separation of ileo-transverse anastomosis, jejunum anastomosis normal without signs of leakage. The abdominal cavity was closed after putting on three drains. In the tenth day after the first surgery, there was an erection, a partial wound separation of the coatings. The patient was qualified for surgery. Intraoperatively, features of fibrinous purulent peritonitis with leakage of the small intestinal anastomosis were found. A fragment of the stapler anastomosis was excised, the abdomen was left open using negative pressure wound therapy.

In the thirteenth day after the first surgery, during replacement of the vacuum dressing, complete separation of the transverse stump and complete separation of the ileostomy from the coatings, jejunum anastomosis was found. Excision of the spleen and descending colon with sigmoid closure by hand suture was performed, fixation sutures for ileostomy were reapplied. The abdomen was left open (open abdomen) using vacuum therapy. The patient was in the Intensive Care Clinic. Due to respiratory failure, pleural puncture was performed, followed by vacuum drainage into the pleural cavity (due to iatrogenic pneumothorax). Mechanically ventilated, circulatory stabilized infusion of levonor at a low dose, antibiotic therapy according to culture, antithrombotic prophylaxis (low molecular weight heparin), intravenous feeding - total parenteral nutricion was continued. In the sixteenth day after the first surgery, the replacement of the vacuum dressing, in the abdomen, exudative-fibrinous in-laws, without intestinal contents (no features of intestinal fistulas). XXIII day postoperative replacement of a vacuum dressing granulation tissue without fibrinous-hypotensive content. Sutures were placed approaching the edges of the wound around both poles. XXVII day replacement of a vacuum dressing granulation tissue with no fibrinous-hypotensive content. Two more stitches were placed approaching the wound poles gradual closing by following sewing seams closing to the poles of the Wound. XXXI day abdominal closure (continuous peritoneo-fascial suture, single sutures on the skin). After discharge, the patient stays under the constant care of the Gastroenterology Clinic (biologically treated) and the Surgery Clinic. After half a year, the patient does not want to try to restore the digestive tract.

Discussion

Leakage and dehiscence of the anastomosis cause about 25% of deaths after bowel surgery. Studies show that patients with Crohn's disease have a higher risk of septic complications. Also important are factors related to the surgical technique, end-to-end anastomosis, handsewn anastomosis, positive resection of the intestine, and penetrating form of Crohn's disease.[16-20] The resection limits are determined by macroscopically assessing inflammatory lesions on the intestine and assessing the thickness and mesenteric infiltration (Fazio symptom) - in the place where the mesentery is soft and thin, there should be no lesions. There are many definitions of the complication of anastomotic leak, which is why the frequency of leakage in literature ranges from 0.5 to 30%. When suspecting anastomotic leaks, it is important to control the patient's basic parameters body temperature, tachycardia, leukocytosis, C reactive protein or procalcitonin. Obvious evidence of leakage of the anastomosis is the presence of intestinal contents in the drainage or visible in imaging tests (e.g. ultrasound) fluid content in the peritoneal cavity with increased echogenicity, it is obvious that physical examination - peritoneal symptoms cannot be omitted. Treatment of septic condition resulting from anastomotic leak requires fluid and electrolyte equalization, antibiotic therapy, and surgical intervention. The surgical procedure for finding leaks involves: adding several sutures (77.5%), sealing the anastomosis with "sealants" (17.5%), selecting an ileo- or colostomy (10%) and performing a new anastomosis (9.4%) or combining these procedures. If the leakage is not controlled and the patient manifests the symptoms of peritonitis and septic state, the safest and most common procedure is the emergence of a stoma above the leak, and the defect in the anastomosis is provided with additional sutures and/or local sealing agents (e.g. Tacho-Sil, tissue adhesives). The anastomosis can be resealed by closing the distal intestine and the proximal segment emerging in the form of a final stoma (Hartmann method). In the patient presented above on the 4th day after resection of the ileum fragment with right hemicolectomy, ileo-transverse anastomosis and resection of the jejunum fragment) and small intestinal anastomosis with mechanical suture (two linear staplers), the ileo-transverse anastomosis anastomosis almost completely. Due to diffuse fecal peritonitis, the distal part of the intestine was closed and the proximal one emerged in the form of a final ileostomy. In the tenth day after the first surgery, the treatment occurred. Intraoperatively, anastomosis of the jejunum anastomosis was found proximal to the ileostomy, a portion of the anastomosis was resected with a linear stapler.[17-23] The abdomen was left open (laparostomy) using a commercial VAC kit for pressure therapy. For a patient in a severe condition with symptoms of abdominal sepsis after a second or subsequent laparotomy, open abdominal therapy is the best solution. Vacuum therapy is currently the most commonly used method of temporary abdominal closure. It is often the case that primary fascia closure (after the end of vacuum therapy) is impossible. Planning for laparostomy closure should start from day one when the surgeon leaves the abdomen open. The edges of the fascia should be approached as soon as possible. Vacuum therapy alone brings the edges of the wound closer together. Various methods are described in the literature to increase the percent-

age of primary fascia closures: Vacuum assisted wound closure and mesh mediated fascial traction , Wittmann Path, dynamic retention suture-DRS. In the present patient, the fascial edges were gradually brought closer by suturing the poles of the wound during subsequent abdominal revisions and replacing the vacuum dressing. Among other things, it was possible to close the fascia originally.[24-27] After six months of follow-up, no hernia was found in the postoperative scar, which is a common complication following open abdomen treatment. A very important element of the patient's comprehensive treatment was the therapy in the Intensive Care Clinic (mechanical ventilation, circulatory stabilization, antibiotic therapy, parenteral nutrition).

Conclusion

After the original surgery, a number of complications occurred: dissolution of the anastomoses, recurrence of inflammation in fragments of the left intestine, which was accompanied by diffuse fecal peritonitis. The patient was re-operated many times with excision, secondary anastomosis suturing, ileostomy, open abdomen using vacuum therapy. Repeated surgical procedures, intensive therapy in the Intensive Care Clinic led to septic state control, healing of anastomotic leaks and healing of abdominal wall wounds without postoperative hernia.

Leakage, dehiscence of intestinal anastomoses, especially in patients with chronic inflammatory process. Such treatment requires a multi-specialist and comprehensive approach to subsequent complications. Such behavior is a very big challenge, but only such behavior can lead to therapeutic success.

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CASUISTIC PAPER

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Fibromatosis-like spindle-cell metaplastic carcinoma of the breast – a case report

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ABSTRACT
Introduction. Metaplastic breast carcinoma is expressing epithelial and/or mesenchymal tissue within the same tumor.
Aim. The aim of this study is to evaluate metaplastic breast carcinoma in a case report and literature review.
Description of the case. The presented case desribes metaplastic carcinoma of the breast in 65 years old female patient.
Conclusion. Fibromatosis-cell metaplastic carcinoma of the breast presents a particularly large diagnostic challenge. Malignant variants of this disease have been described in the literature.
Keywords. breast cancer, fibromatosis, metaplastic breast carcinoma

Introduction

Metaplastic carcinoma of the breast is a heterogeneous group of malignant tumors. It is composed of neoplastic cells that exhibit epithelial and/or mesenchymal differentiation. This form of breast cancer may be aggressive and accounts for less than 1% of all breast cancer diagnoses.[1] Fibromatosis-like spindle-cell metaplastic carcinoma (FLSpCC) is a variant of metaplastic carcinoma that needs to be distinguished from other cancers due to its favorable outcome.[2] It rarely metastases to axillary lymph nodes and has very low potential for distant metastases.[3] On the other hand it presents a high risk of local recurrence.[4] FLSpCC cannot be reliably diagnosed by Ultrasound or Magnetic Resonance Imaging alone and therefore requires histological examination.[5] It also requires a different therapeutic approach than other metaplastic carcinomas of the breast.[6]

Aim

This study cover histopatological examination in order to diagnose metaplastic carcinoma. We studied histopatology in order to assess the degree of cytologic and histologic correlation in the cytology based diagnosis.

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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Description of the case

65 years old female patient has been admitted to an Oncological Surgery Clinic because of a palpable right breast tumor. In the performed ultrasound examination, the lesion was identified and defined as grade 4a on the BIRADS scale. No enlarged axillary lymph nodes were visualized. The patient was qualified for surgical treatment.

Postoperative material was sent to our Pathology Department for final diagnosis. On gross examination tumor was white, solid, with well-defined borders. It measured 55 x 42 x 30 mm. On microscopic examination the lesion appeared deceptively benign. It consisted of bland spindle cells in a prominent collagenous stroma with infiltrative borders (Figure 1).

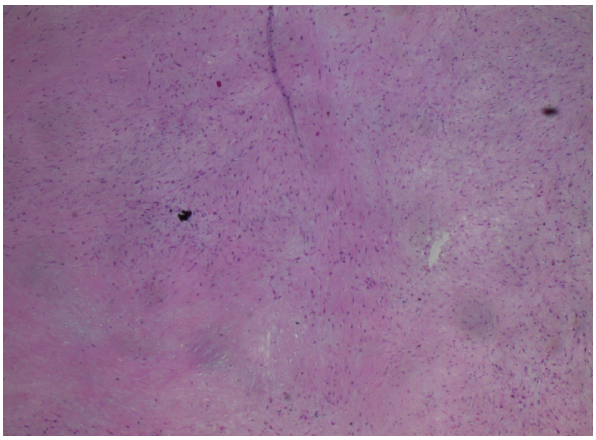

Fig. 1. Bland spindle cells in prominent collagenous stroma with infiltrative borders (H&E, 40X)

On high magnification low-grade atypical cytologic features including hyperchromasia, polymorphism and irregular contours of cell nuclei could be identified (Figure 2).

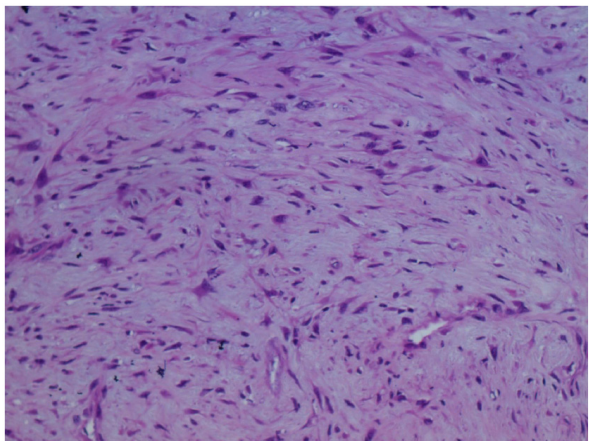

Fig. 2. Low-grade atypical cytologic features (H&E, 200X)

After further examination the tumor proved to be biphasic. Positive immunohistochemical stains for Smooth Muscle Actin (SMA) (Figure 3) and p63 (Figure 4), suggested myoepithelial differentiation, while stains such as CK7 (Figure 5) pointed to epithelial differentiation.

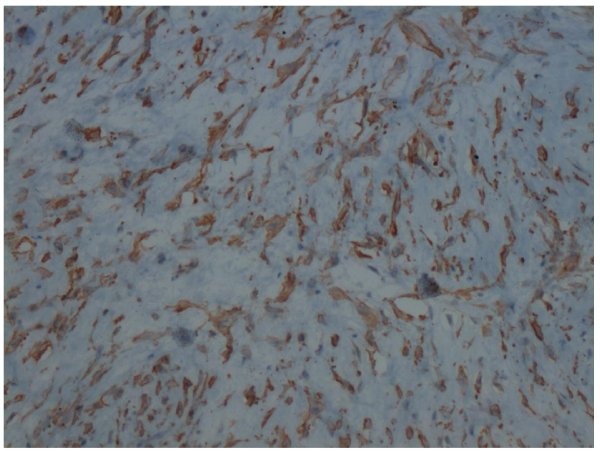

Fig. 3. Postivie SMA immunohistochemical stain (200X)

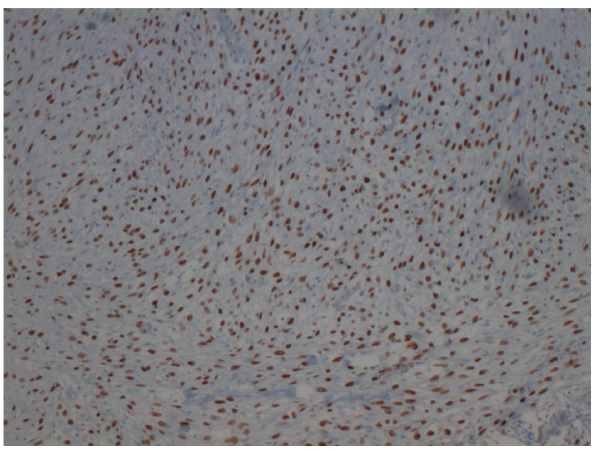

Fig. 4. Positive p63 immunohistochemical stain (100X)

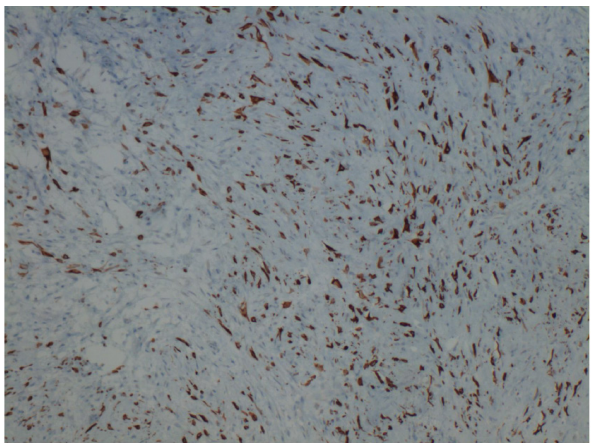

Fig. 5. Positive CK7 immunohistochemical stain (100X)

At least one cytokeratin stain must be positive in order to diagnose metaplastic carcinoma. In the absence of any keratin a different diagnosis should be made.[7,8] Stains for estrogen receptor (Figure 6), progesterone re-

ceptor (Figure 7) and HER-2 receptor (Figure 8) where all negative. The lesion turned out to be a "triple-negative" carcinoma. Ki67 staining was about 10%.

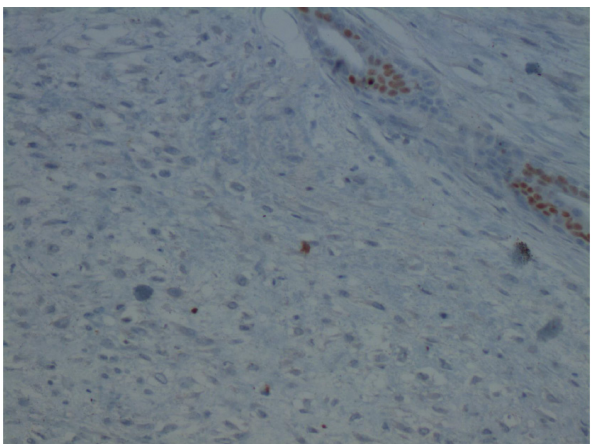

Fig. 6. Negative ER receptor immunohistochemical stain (200X)

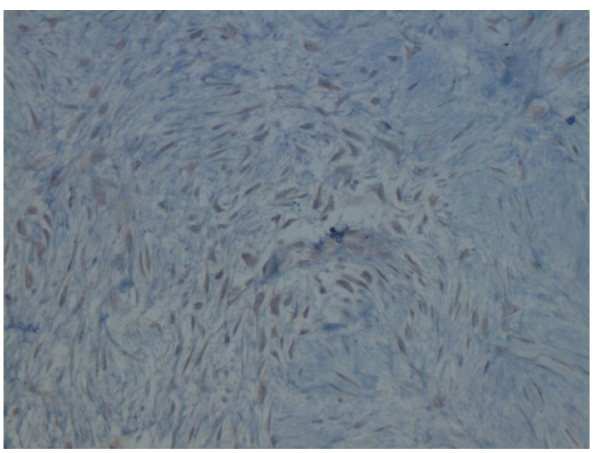

Fig. 7. Negative PR immunohistochemical stain (200X)

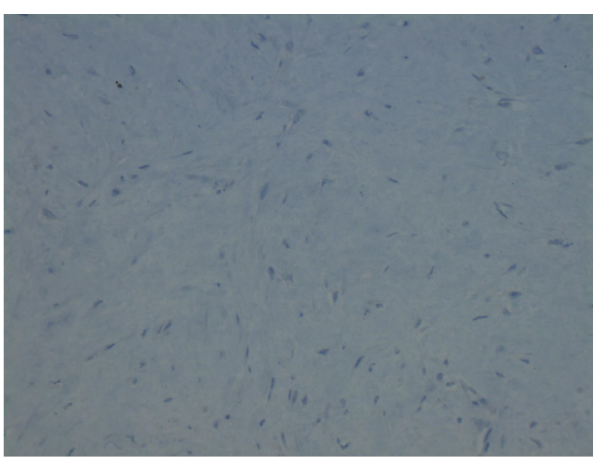

Fig. 8. HER-2 receptor status (200X)

Angiosarcoma was ruled out with negative CD31 and CD34 immunohistochemical staining (Figure 9).

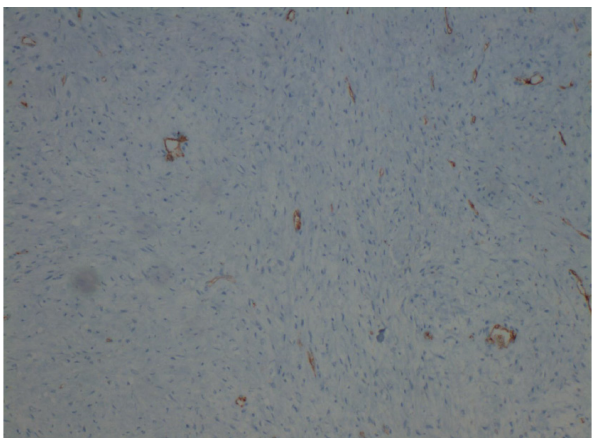

Fig. 9. Tumor cells negative for CD31 immunohistochemical stain (100X)

Discussion

Different spindle cell changes were considered in the differential diagnosis.[9] Mild fibromatosis, nodular fasciitis, squamous metaplasia or granulation tissue reaction show less nuclear and cellular atypical features and lower mitotic activity. Low-grade sarcomas ie. fibrosarcoma should not express keratins.[10-15]

The adenomyoepithelioma presents a particularly large diagnostic challenge. This change morphologically may be very similar to FLSpCC and malignant variants of this disease have also been described.[16-20] In most cases of this lesion, the myoepithelial component is the dominant one. Epithelial cells usually form glandular spaces and can show apocrine, sebaceous or squamous metaplasia or can have papillary epithelial proliferation.[20-23] Such changes were not observed in the described case.[23-29]

Conclusion

FLSpCC is a rare malignancy, however, this diagnosis carries significant clinical consequences. It has a better prognosis compared to other metaplastic breast cancers, as well as a significantly lower percentage of axillary lymph node metastases compared to „classic" breast cancers, ie invasive ductal carcinoma of no special type or lobular carcinoma. Some authors even propose to distinguish a group of lesions called „breast lesions of limited metastatic potential" to emphasize a good prognosis in the case of changes such as FLSpCC, low-grade adenosquamous carcinoma or encapsulated papillary carcinoma. On the other hand, due to the risk of underdiagnosis of FLSpCC as a benign lesion, it is recommended that the use of immunohistochemical studies, especially for cytokeratins and SMA, is necessary to properly assess spindle cell changes in the breasts.

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Article from a journal, number of authors more than 6	Gonzalez ME, Martin EE, Anwar T, et al. Mesenchymal stem cell-induced DDR2 mediates stromal-breast cancer interactions and metastasis growth. Cell Rep. 2017;18:1215-28. Jordan J, Toplak H, Grassi G, et al.Joint statement of the European Association for the Study of Obesity and the European Society of Hypertension: obesity and heart failure. J Hypertens. 2016;34:1678-88.
Article from an online journal	Coppinger T, Jeanes YM, Hardwick J, Reeves S. Body mass, frequency of eating and breakfast consumption in 9-13-year-olds. J Hum Nutr Diet. 2012;25:43-9. doi: 10.1111/j.1365-277X.2011.01184.x. Cogulu O, Schoumans J, Toruner G, Demkow U, Karaca E, Durmaz AA. Laboratory Genetic Testing in Clinical Practice 2016. Biomed Res Int. 2017;2017:5798714. doi: 10.1155/2017/5798714.
Websites	Cholera in Haiti. Centers for Disease Control and Prevention Web site. http://www.cdc.gov/haiti-cholera/. Published October 22, 2010. Updated January 9, 2012. Accessed February 1, 2012. Address double burden of malnutrition: WHO. World Health Organization site. http://www.searo.who.int/mediacentre/releases/2016/1636/en/. Accessed February 2, 2017.
Book	Naish J, Syndercombe Court D. Medical Sciences. 2nd ed. London, Elsevier;2015. Modlin J, Jenkins P. Decision Analysis in Planning for a Polio Outbreak in the United States. San Francisco, CA:Pediatric Academic Societies;2004.
Chapter in a book	Pignone M, Salazar R. Disease Prevention & Health Promotion. In: Papadakis MA, McPhee S, ed. Current Medical Diagnosis & Treatment. 54th ed. New York, NY: McGraw-Hill Education; 2015:1-19. Solensky R. Drugallergy: desensitization and Treatment of reactions to antibiotics and aspirin. In: Lockey P, ed. Allergens and Allergen Immunotherapy. 3rd ed. New York, NY: Marcel Dekker; 2004:585-606.

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