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CONTENT

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SCRIPTA MEDICA 2021 | 52 | Q1

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CONTENT

Editorial

Original Articles

History of Medicine

Professional Article

Case Report

CONTENT

Vavić et al. Scr Med 2021 Mar;52(1):1-5. DOI:10.5937/scriptamed52-31453
EDITORIAL

Convalescent COVID-19 Plasma

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Abstract

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a new human disease. December 31, 2019 marked the day the World Health Organization (WHO) first became aware of an infectious outbreak in the Hubei province in China. Until January 2021, more than two million people died from COVID-19. The use of convalescent plasma (CP) has been widely used in different outbreaks as the first therapeutic option given the lack of effective medications or vaccines, and often as a last chance or experimental treatment. CP is a strategy of passive immunisation. Possible mechanisms of CP-COVID-19 action are antiviral and immunomodulatory.

The established protocol for CP-COVID-19 collection defines activities and criteria related to recruiting and informing potential CP donors, clinical and laboratory examination, plasma collection, labelling and storage. Plasma is collected by apheresis/plasmapheresis. Administration of plasma is performed at the request of clinicians, according to the strict indications based on the severity of clinical picture, expressed by precisely determined "scoring" of symptoms. The risks transfusion recipients are likely to be exposed to do not differ from those of standard plasma recipients. At the Blood Transfusion Institute of Serbia, the first plasmapheresis from the recovered patient-donor was performed on 11 April 2020 and so far, collection and distribution of CP-COVID-19 have been performed continuously.

During the observation period, preliminary results of the effect of CP transfusion, along with other applied therapy, indicate its favourable effect, both worldwide and in Serbia. CP-COVID-19 should be used as early as possible in the course of infection in order to achieve the best outcomes.

Keywords: Coronavirus disease 2019 (COVID-19); Acute respiratory syndrome coronavirus 2 (SARS-CoV-2); Convalescent plasma.

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Introduction

Coronavirus disease 2019 (COVID-19) is a new disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The disease is manifested in the human population in a wide range of clinical picture and severity, from almost asymptomatic and mild disease of the upper respiratory tract, to diffuse pneumonia accompanied by acute respiratory failure with a fatal outcome. In addition, it quickly became clear that the disease was characterised by a number of severe and unexpected complications, such as systemic thrombosis, cytokine storm syndrome, multiorgan dysfunction syndrome, as well as long-lasting and severe sequelae after infection, such as pulmonary fibrosis and major or minor damage to many other organs.[1] Patients with COVID-19 infection show similar clinical signs and symptoms as patients with SARS-CoV and

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CONTENT

Middle East respiratory syndrome coronavirus (MERS-CoV) infection in terms of fever, dyspnoea and multilocular invasive changes on lung images, as well as the occurrence of multiorgan failure in severe forms of the diseases.[2]

December 31, 2019 marked the day when the World Health Organization (WHO) first became aware of the COVID-19 infection in the province of Hubei in China and already in January 2021 more than two million people had died from the effects of COVID-19 (https: //coronavirus.jhu.edu/map.html).[3] Given the lack of natural immunity in the population to this virus, as well as the fact that there are currently no established effective therapeutic protocols for this disease and that vaccination of the population is practically just beginning, it can be concluded that, for the time being, the treatment of this infection remains mostly supportive.

Convalescent plasma (CP) has previously been widely used in various epidemics as the first therapeutic option in conditions where there were no effective drugs or vaccines and has often been used as the last resort or experimental treatment.[4] This method, as a strategy of passive immunisation, has been used in the prevention and treatment of infectious diseases since the beginning of the 20th century, although the principle of therapeutic infusion of CP was established in 1890, when it was used in the treatment of diphtheria. It should also be emphasized that the first Nobel Prize in Physiology and Medicine was awarded for the serum therapy of patients with diphtheria in 1901. This therapeutic method has recently been applied to other respiratory viral infections, including SARS-CoV-1, H1N1 influenza, and MERS-CoV, as well as West Nile virus and Ebola virus.[5] In the current pandemic, it can also be said that one of the current therapeutic options for the treatment of COVID-19 infection is CP-anti-COVID-19 (CP-COVID-19) which is collected from people who had recovered from COVID-19 infection and which contains antibodies to SARS-CoV-2.[6, 7]

Possible mechanisms of action of CP-COVID-19

Possible mechanisms of action CP-COVID-19 is antiviral and immunomodulatory. The antiviral efficacy of CP treatment in COVID-19 infection is thought to be related to the activity of plasma neutralising antibodies (NAbs) in the blood of recovered patients. These antibodies are crucial in neutralising and eliminating the virus and play a key role in protecting against viral infections. CP containing these antibodies contributes to the passive immunity and can help the body to fight infection more successfully. It is very important that these neutralising antibodies found in CP in patients infected with SARS-CoV-2 act antivirally by suppressing the replication of the virus even before the infected person begins to establish a humoral immune response to the infection.

Studies have shown that the NAbs titre in patients infected with SARS-CoV-2 was low when measured up to 10 days after the onset of the disease and then increased and that the highest antibody concentration was registered 10 to 15 days after the onset of the disease, after which the NAbs titre remained stable.[8, 9]

The immunomodulatory effect of CP is based on the fact that the plasma of healthy donors contains anti-inflammatory cytokines and antibodies that block complement, pro-inflammatory cytokines and autoantibodies, all of which contribute to the immunomodulatory effect of CP in patients with COVID-19.[4] Immunomodulatory effects of CP-COVID-19, in addition to immunoglobulins and other antibodies that inhibit the complement cascade and limit the formation of immune complexes, are also achieved by specific immunoglobulins G that neutralise cytokines such as IL-1b, IL-6 and TNFα that may play an important role in pathogenesis of uncontrolled degree of the inflammation which is seen in some patients with COVID-19 infection.[10] In addition to the above, other proteins are obtained from donors, such as clotting factors, natural antibodies, defensin, pentraxin and other undefined proteins that may be important in the body's defences. Thus, the passive immunity obtained by infusion of CP-COVID-19 can limit the excessive and unfavourable inflammatory cascade triggered by pathogenic antibodies, as well as cell damage caused by the activation of the complement cascade due to the excessive inflammatory response.[4] It should be emphasized, once again, that the described effect of immunomodulation can be very important, given that excessive activation of the immune system with systemic hyper-inflammation or the "cytokine storm" triggered by IL-1b, IL-2, IL-6, IL-17, IL -8, TNFα in some patients with COVID-19 may be responsible for multiorgan dysfunction and death, as well as chronic damage

CONTENT

to the lung parenchyma with consequent fibrosis and decreased lung function after recovery from COVID-19 infection.[11]

In some severely ill patients, it has been shown that the favourable immunomodulatory effect of CP-COVID-19 could be a consequence of the proven presence of IgA antibodies against cardiolipin and IgA and IgG antibodies to β2-glycoprotein I.[12] By neutralising these proteins, CP-COVID-19 can reduce the risk of thrombo-embolic complications (ie, diseases similar to antiphospholipid syndrome), which are not uncommon in critically ill patients. Moreover, other antibody-mediated immune response mechanisms, such as antibody-induced complement activation and antibody-induced cellular cytotoxicity and phagocytosis, may also be the reason for the beneficial therapeutic effect of CP. Meffre and Ivasaki[13] showed that the defective genome for IFN-1, which is a genetically induced mutation on the X chromosome, is the cause of IFN-1 deficiency resulting in an uncontrolled replication and spread of the virus. INF-1 deficiency results in the suppression of immune-signalling complexes called inflammasomes and the increased production of inflammatory cytokines that are produced without the control of these complexes. These facts may explain the very serious course of COVID-19 infection in people with IFN-1 deficiency, as well as the benefits of using covalent plasma in these patients.[13]

Collection of CP-COVID-19

The CP-COVID-19 collection protocol defines activities and criteria related to the recruitment and information of potential CP donors, clinical and laboratory examination, as well as plasma collection, labelling and storage. These criteria are in line with current versions of the Recommendations of the Council of Europe and the European Committee, the Food and Drug Administration (FDA), the World Health Organization (WHO).[14-16]

CP donors must be eligible for CP donation. They should have undergone COVID-19 infection, whether confirmed by an approved molecular test (eg, nasopharyngeal swab) or by the presence of antibodies to SARS-CoV-2. It is necessary that at least 14 days have passed after the cessation of clinical symptoms of the infection. In addition to this, they must also meet the criteria that apply to regular apheresis procedures. Potential CP donors undergo the same procedure as regular voluntary blood donors. They fill in a standard questionnaire for blood donors and are subject to the usual medical physical examination. They perform laboratory tests as well as for regular plasma donors, including antibodies against SARS-CoV-2. According to FDA guidelines, a sufficient titre of neutralising SARS-CoV-2 antibodies for plasma donation is considered to be greater than or equal to 1:160.[17]

Plasma collection is performed by apheresis procedure - plasmapheresis. This procedure involves effusing the donor's blood, centrifuging the blood, isolating and retaining a certain volume of plasma, usually about 600 mL, and reinfusing the cellular components. Most often, the obtained amount of plasma is divided into 2-3 units that can be transfused separately. The procedure is performed on special devices, separators of blood components. Sets that are sterile, disposable, with the use of anticoagulants and preservatives are used. The procedure lasts about 40-60 min. The usual time period between the two procedures is 15 days, but it can be shorter depending on the concentration of IgG immunoglobulin, but not shorter than 4 days. Plasma units are frozen within 24 h of collection and are issued only after obtaining the results of a standard test of plasma donors. The procedure for storing CP is the same as for fresh frozen plasma - up to 36 months, at a temperature lower than -25 °C.[14] COVID-19 CP must be appropriately labelled. In addition to the usual label for fresh frozen plasma, there must be a label that it is in experimental use or for clinical trials. Plasma recipient blood group and plasma unit must be compatible in the ABO blood group system.

CP-COVID-19 is issued at the request of the doctor treating the patient. Plasma administration is performed according to standard procedure and strict indications. The usual optimal dose is two units CP-COVID-19 or 4-13 mL/kg body weight of the recipient.[18, 19]

Safety and efficiency of application CP-COVID-19

In March 2020, the FDA published a guidance document regarding CP-COVID-19.[16] On 23 August 2020 the FDA issued an Emergency Use Authorization (EUA) for CP-COVID-19 for the treatment of hospitalised patients with COVID-19.[20] The FDA claimed that "overall evidence" suggest-

CONTENT

ed that the benefits of CP would outweigh the risks, and given the lack of effective treatments, the FDA provided guidance on the production and use of CP in hospitalised patients with signs of progressive infection. The American Society of Infectious Diseases and the AABB (American Blood Bank Association) recommend that the use of CP should be limited to clinical trials. They believe that critically ill patients and those in the intensive care unit (ICU) are unlikely to benefit from CP transfusions, but that CP should still be used as early as possible during infection (preferably within 3 days of diagnosis) to achieve the best results. They believe that transfusion may be useful if high-titre anti-spike protein receptor binding domain in CP (RBD) IgG titre ≥ 1:1350 is administered early on admission to the hospital, within 72 h, and that mortality is then reduced within 28 days after transfusion.[21] Libster et al concluded that administration of a high titre of CP-COVID-19 to infected elderly within 72 h after the onset of mild symptoms reduced the progression of COVID-19 to severe disease. This simple and inexpensive intervention can save lives.[22]

The risk of a CP transfusion to recipients is no different from the risk posed by standard plasma transfusion. The risk of transfusion-transmitted infection is very low due to the performed tests. Non-infectious dangers of transfusion, such as allergic reactions, transfusion-related circulatory overload and transfusion-related acute lung injury (TRALI) are possible, but rare. However, routine donor screening includes donor human leukocyte antigen (HLA) antibodies with a history of pregnancy or previous transfusion to reduce the risk of TRALI. There are no reports of transmission of the respiratory virus by blood transfusion. The risk of transmission of SARS-CoV-2 by plasma transfusion is considered to be negligible. At the Mayo Clinic, from 11 July 2020 more than 34,000 patients were transfused with CP-COVID-19 (https://www.uscovidplasma.org) with minimal side effects.[23] The first plasmapheresis of the first recovered patient-donor was performed at the Blood Transfusion Institute of Serbia on 11 April 2020 and, so far, the collection and distribution of CP-COVID-19 has been performed continuously. Finally, from our country there are also published data on antibody investigation and collection of convalescent plasma by plasmapheresis for basic research and/or potential therapeutic use.[24]

Conclusion

CP is a safe and potentially effective strategy for treating new pathogens, especially in those scenarios without proven antiviral agents, vaccines or specific anti-COVID-19 intravenous immunoglobulin (IVIg). Early infusions of CP can provide a bridge for recovery to the risky patients until vaccines become widely available. As IVIg and CP share similar mechanisms of action, the production of anti-COVID-19 IVIg may provide better standardisation in COVID-19 therapy. In this observed period, preliminary results of the effect of CP transfusion, together with other applied therapy, indicate its favourable effect, both in Serbia and worldwide. CP-COVID-19 should be used as early as possible during infection (preferably within 3 days of diagnosis) to achieve the best treatment results.

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None.

Conflict of interest

None.

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CONTENT

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ORIGINAL ARTICLE

Indian COVID-19 Dynamics: Prediction Using Autoregressive Integrated Moving Average Modelling

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Abstract

Background: The forecasting of Coronavirus Disease-19 (COVID-19) dynamics is a centrepiece in evidence-based disease management. Numerous approaches that use mathematical modelling have been used to predict the outcome of the pandemic, including data-driven models, empirical and hybrid models. This study was aimed at prediction of COVID-19 evolution in India using a model based on autoregressive integrated moving average (ARIMA).
Material and Methods: Real-time Indian data of cumulative cases and deaths of COVID-19 was retrieved from the Johns Hopkins dashboard. The dataset from 11 March 2020 to 25 June 2020 (n = 107 time points) was used to fit the autoregressive integrated moving average model. The model with minimum Akaike Information Criteria was used for forecasting. The predicted root mean square error (PreRMSE) and base root mean square error (BaseRMSE) were used to validate the model.
Results: The ARIMA (1,3,2) and ARIMA (3,3,1) model fit best for cumulative cases and deaths, respectively, with minimum Akaike Information Criteria. The prediction of cumulative cases and deaths for next 10 days from 26 June 2020 to 5 July 2020 showed a trend toward continuous increment. The PredRMSE and BaseRMSE of ARIMA (1,3,2) model were 21,137 and 166,330, respectively. Similarly, PredRMSE and BaseRMSE of ARIMA (3,3,1) model were 668.7 and 5,431, respectively.
Conclusion: It is proposed that data on COVID-19 be collected continuously, and that forecasting continue in real time. The COVID-19 forecast assist government in resource optimisation and evidence-based decision making for a subsequent state of affairs.

Keywords: Autoregressive integrated moving average; COVID-19; Epidemic curve; Forecast; Mathematical modelling; Prediction.

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Introduction

Coronavirus Disease-19 (COVID-19), caused by the novel Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), has caused a pandemic with global devastation to human life and health. SARS-CoV-2 is closely related to two bat-derived SARS like coronaviruses, bat-SL-CoVZC45 and bat-SL-CoVZXC21. It is transmitted by human-to-human transmission via droplets or direct contact and the mean incubation period is 6.4 days.[1] According to World Health Organisation, 10,185,374 confirmed cases and 503,862 deaths have been recorded by 1 July 2020.[2] Furthermore, India recorded 568,092 confirmed cases and 17,400 deaths by the same time.[3] The novelty and rapid spread of SARS CoV-2 has challenged medical science across the disciplines of epidemiology, clinical signs and

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symptoms, pathophysiology, disease progression and evolution and management and its preventive protocol. Meanwhile, mathematical models have been used to predict the course of disease and mortality.[4-7] Such models have potential in disease management as well as preventive protocols that are cost-effective that can help optimum allocation of resources to manage the disease.[8-14]

An econometric model has been proposed in the present study to predict and extrapolate the transmission of COVID-19. The model makes use of autoregressive integrated moving average modelling on the epidemiological dataset of COVID-19. The objective of this study was to estimate the forecast of COVID-19 cumulative cases and deaths for India. It is proposed that data on COVID-19 should be collected continuously and that forecasting continues in real time to assist governments in evidence-based decision making.

Methods

A descriptive study was run to forecast COVID-19 evolution in the Indian subcontinent. The time series of COVID-19 cumulative cases and deaths from 11 March 2020 to 25 June 2020 (n = 107 time points) was used in prediction using autoregressive integrated moving average modelling. A useful ARIMA model depends on the number of sample time points, and a good series would have more than 50 sample points.[15]

Data acquisition

The Indian data of COVID-19 cumulative cases and deaths from 11 March 2020 to 25 June 2020 were sourced from the official website of Johns Hopkins University Center for Systems Science and Engineering (https://systems.jhu.edu/) and the repository (https://github.com/CSSEGISandData/COVID-19).[16] Excel 2010 was used to build the database.

To validate the model, dataset was distributed into a training set and a validation set. The training dataset from 11 March 2020 to 4 June 2020 (n = 86) was used to fit autoregressive integrated moving average (ARIMA) model and estimation of parameters. The validation dataset from 5 June 2020 to 25 June 2020 was used to validate the model (Table 1).

Table 1: Distribution of complete datasets of cumulative cases and death cases into training and validation subsets

Sub-datasets	Cumulative cases	Death cases
Training set	11 Mar - 04 Jun 2020 (n = 86) (T×C dataset)	11 Mar - 04 Jun 2020 (n = 86) (T×D dataset)
Validation set	05 Jun - 25 Jun 2020 (n = 21) (V×C dataset)	05 Jun - 25 Jun 2020 (n = 21) (V×D dataset)

Model description

The autoregressive integrated moving average model was proposed to estimate the forecast of COVID-19 evolution.[17-19] Box-Jenkins methodology was followed (Figure 1). The methodology encompasses three phases of identification, estimation and testing and application. The Identification phase involves data preparation and model selection. The data were analysed for trends and seasonal components. The Augmented Dickey-Fuller (ADF) unit root test was performed on time series to examine stationarity. Logarithmic transformation and differencing operations were performed to stabilise the time series. Selection of ARIMA model was required to establish the order of the autoregressive (AR) process 'p', the differencing operator 'd', and the order of moving average (MA) process, 'q'. The succeeding system of mathematical equations delineates the ARIMA(p,d,q) model:

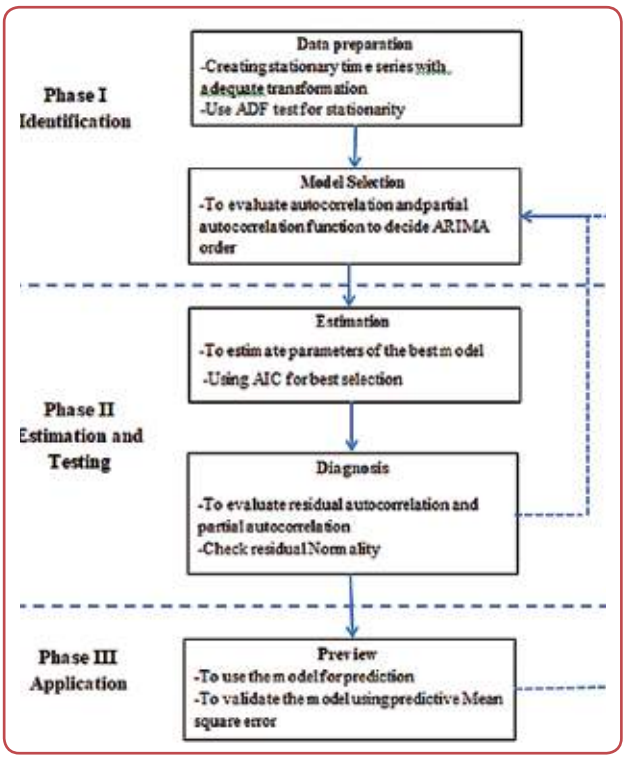


Figure 1: Flowchart outline Box Jenkins Methodology for implementation of autoregressive integrated moving average modeling

$$W_t = \alpha_1 W_{(t-1)} + \alpha_2 W_{(t-2)} + \ldots + \alpha_{(t-p)} W_{(t-p)} + a_t + \beta_1 a_{(t-1)} + \beta_2 a_{(t-2)} + \ldots + \beta_q a_{(t-q)}$$

$$W_t = \nabla^d X_t$$

where $\{X_t\}$ is the original time series, $\{W_t\}$ is the time series acquired after differencing operation on $\{X_t\}$, ∇^d is the difference operator, α_p, β_q are parameters and a_t is the white noise. Since,

$$\nabla^d \equiv (1 - B)^d$$

where B is the backward shift operator,[4] a series $\{X_t\}$ is integrated of order ^{d} if

$$W_t = (1 - B)^d X_t$$

The autocorrelation function (ACF) and partial autocorrelation function (PACF) were used to guide the autoregressive and moving average order, respectively. The Akaike Information Criterion (AIC) was used to select the best model. The AIC considers the maximum log-likelihood estimation (Log L) and number of parameters as the criteria for best model selection. The minimisation of the following equation is required:

$$AIC = -2 \log (\text{maximum likelihood}) + 2k$$

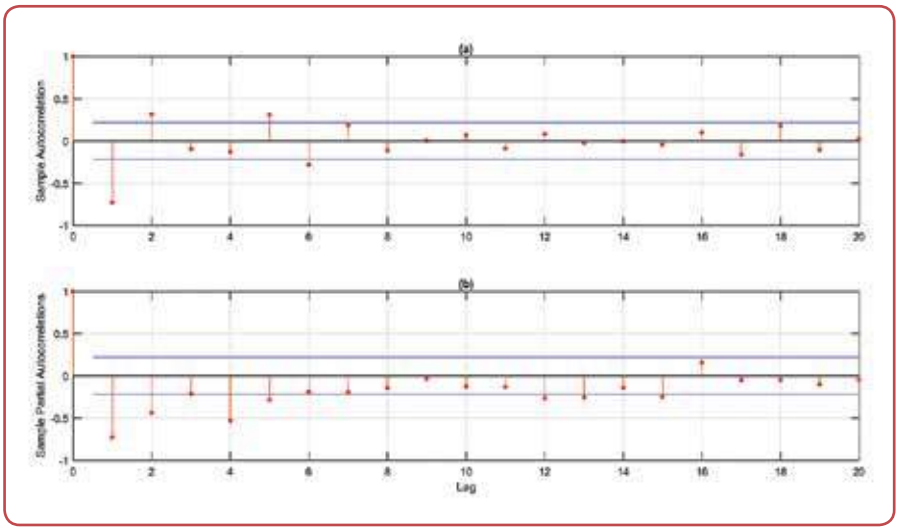
where k = p + q + 1, 2k serves as a penalty function. The model having minimum AIC value was considered the best. Estimation of parameters was accomplished using the method of maximum likelihood estimation. The assumptions of the model were scrutinised with residual diagnostics. The forecasts of cumulative cases and deaths was performed from the best selected ARIMA model. The validation of the model was performed from the validation dataset by enumerating Predicted Root Mean Square Error (PredRMSE) and comparing it with Base Root Mean Square Error (Base RMSE). The forecast of cumulative cases and deaths was performed from 26 Jun to 05 Jul 2020.

Statistical analysis

The estimate from the ARIMA model follow a normal distribution, and variation of the forecast was considered within 95 % confidence intervals. The MS Excel 2010 was used to maintain the database and MATLAB 2016a (version 9.0.0.341360) was used for analysis.[20]

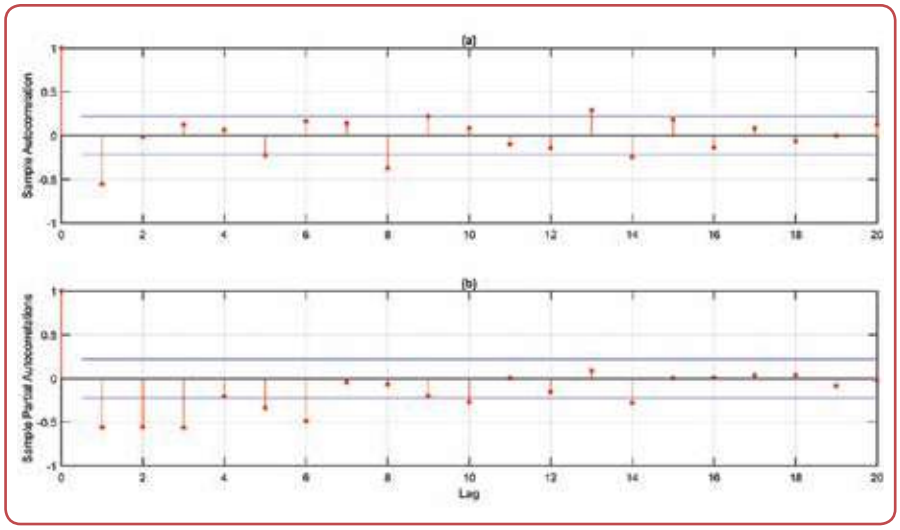
Results

To achieve stationarity, the logarithmic transformation of datasets (cumulative cases and deaths) was performed as they were showing upward trends. Further difference operations were performed on cumulative cases (d = 3) and death (d = 2) datasets. The Augmented Dickey-Fuller (ADF) test showed stationarity (p value < 0.05). The autocorrelation function and partial autocorrelation function were calculated and correlograms were used to guide the autoregressive order and the moving average order of the ARIMA models (Supplemental Figure 1 and 2). The ARIMA

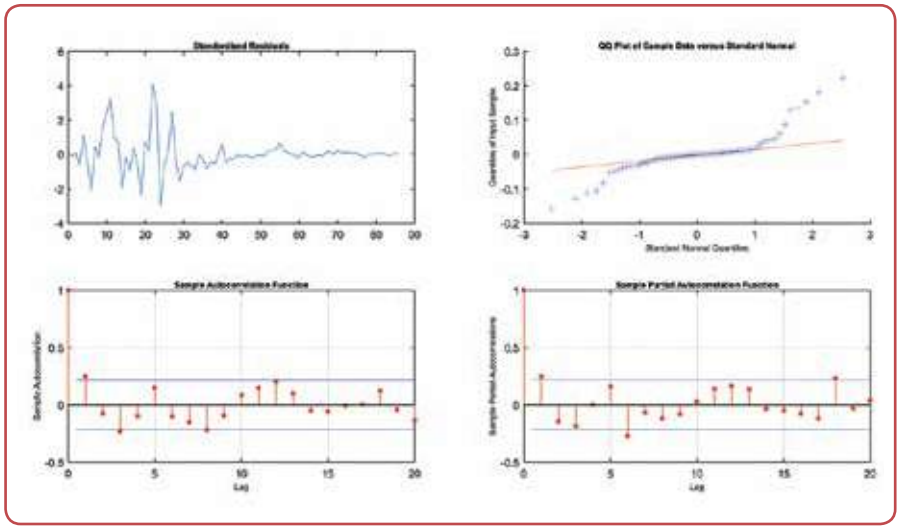


Supplemental Figure S1: Correlograms of time series of cumulative cases of COVID-19 (a) sample autocorrelation function (b) sample partial autocorrelaton function

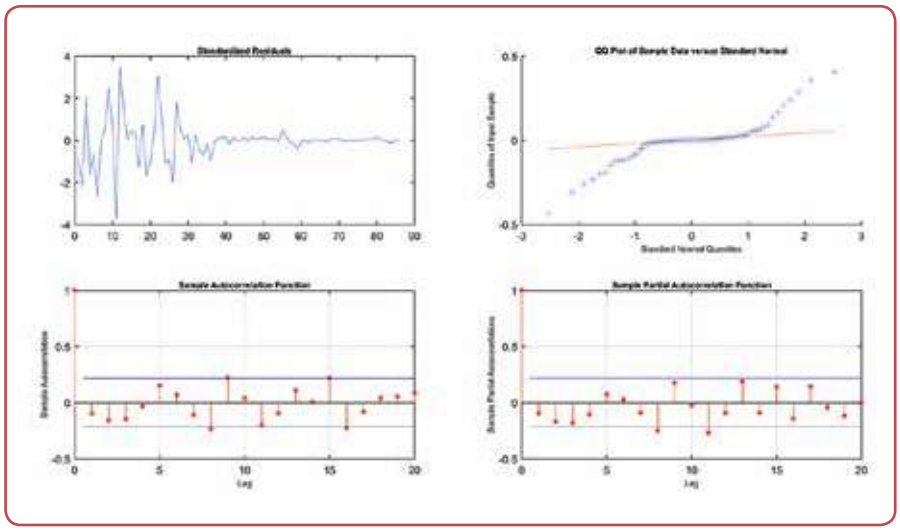
CONTENT



Supplemental Figure S2: Correlograms of time series of death cases of COVID-19 (a) sample autocorrelation function (b) sample partial autocorrelaton function



Supplemental Figure S3: Line plots, QQ plots and correlograms of residual diagnostic of differenced logarithmic transformed time series of cumulative cases of COVID-19. Line plots showing random distribution of standardised residuals and QQ plot showing straight line in the mid portion and the autocorrelation and partial autocorrelation functions are within random limits, represent normality of residuals



Supplemental Figure S4: Line plots, QQ plots and correlograms of residual diagnostic of differenced logarithmic transformed time series of death cases of COVID-19. Line plots showing random distribution of standardised residuals and QQ plot showing straight line in the Mid-portion and the autocorrelation and partial autocorrelation functions are within random limits, represent normality of residuals

CONTENT

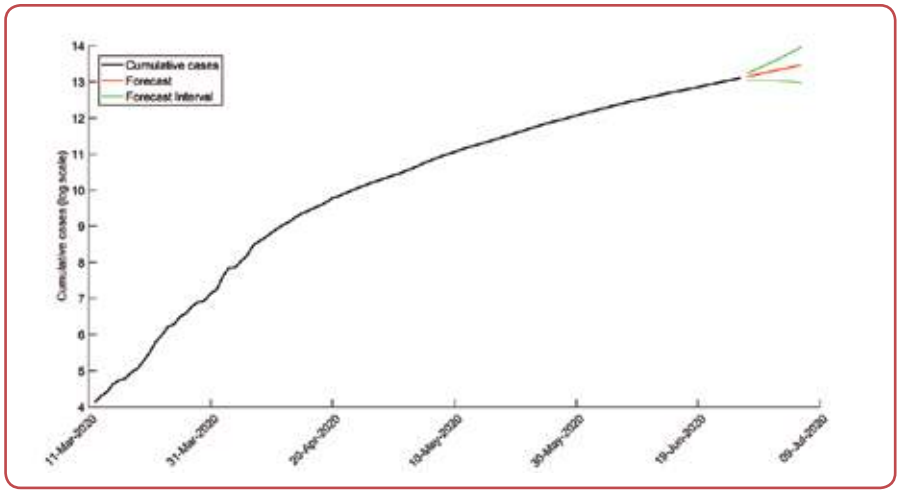


Figure 2: Line plot shows observed (black line plot) and predicted (magenta line plot) cumulative cases of COVID-19 with 95 % confidence interval (green line plot). The forecast is based on ARIMA (1,3,2) model fitted with time series of cumulative cases from 11 March to 25 Jun 2020

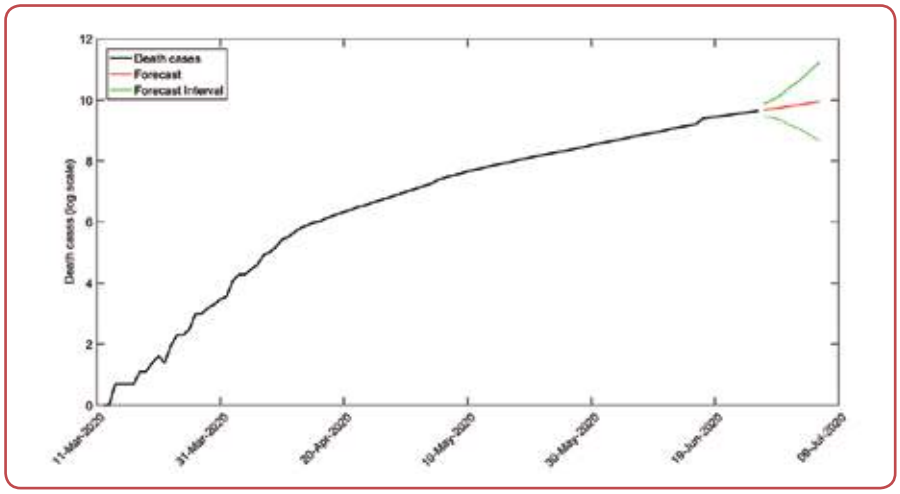


Figure 3: Line plot shows observed (black line plot) and predicted (magenta line plot) death cases of COVID-19 with 95 % confidence interval (green line plot). The forecast is based on ARIMA (3,3,1) model fitted with time series of death cases from 11 March to 25 Jun 2020

Supplemental Table S1: Parameters, errors and statistics of ARIMA (1,3,2) model, fitted from training dataset of cumulative cases of COVID-19

Parameter	Value	Error	Statistics
Constant	0.000	0.0002	0.171
AR {1}	-0.398	0.036	-10.923
MA {1}	-1.756	0.048	-36.406
MA {2}	0.756	0.047	16.092
Variance	0.003	0.0002	10.190

Supplemental Table S2: Parameters, errors and statistics of ARIMA (3,3,1) model, fitted from training dataset of death cases of COVID-19

Parameter	Value	Error	Statistics
Constant	0.0002	0.001	0.158
AR {1}	-1.118	0.052	-21.502
AR {2}	-1.078	0.058	-18.482
AR {3}	-0.606	0.053	-11.430
MA {1}	-1	0.064	-15.525
Variance	0.013	0.001	9.700

Table 2: Goodness of fitted models for cumulative cases and deaths cases using prediction root mean square error and base root mean square error

Dataset	Model	PredRMSE	BaseRMSE
Cumulative cases	ARIMA (1,3,2)	21137	166330
Death cases	ARIMA (3,3,1)	668.70	5431

**ARIMA: autoregressive integrated moving average; PredRMSE: predicted root mean square error; BaseRMSE: base root mean square error*

(1,3,2) and ARIMA (3,3,1) model for cumulative cases (AIC = 2.32) and death cases (AIC = 108.75) was chosen respectively with the lowest AIC values. Goodness of fit and model assumptions were tested with residual analysis. The graph of standardised residuals showed random distribution of residuals. The middle values of the QQ plot were on a straight line. The autocorrelation function and partial autocorrelation functions within random limits showed normal distribution and independence (Supplemental Figure 3 and 4). The

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Table 3: Forecast of cumulative cases and death cases using ARIMA (1,3,2) and ARIMA (3,3,1) models respectively. The dataset used for prediction is from 11 Mar 2020 to 25 Jun 2020 (n = 107 time points)

Date	Cumulative cases			Cumulative deaths		
	Forecast	95 % CI		Forecast	95 % CI	
		LL	UL		LL	UL
26 Jun 20	508210	378270	638150	15732	12399	19065
27 Jun 20	526760	396820	656700	16243	12910	19575
28 Jun 20	546010	416070	675950	16711	13379	20044
29 Jun 20	566050	436100	695990	17198	13865	20530
30 Jun 20	586900	456960	716840	17740	14408	21073
1 Jul 20	608630	478690	738570	18314	14982	21647
2 Jul 20	631290	501350	761230	18873	15540	22205
3 Jul 20	654940	525000	784890	19480	16147	22813
4 Jul 20	679660	549710	809600	20141	16808	23474
5 Jul 20	705500	575560	835440	20818	17485	24150

**CI: confidence interval; LL: lower limit of CI; UL: upper limit of CI*

parameters of both the ARIMA models were estimated (Supplemental Table S1 and S2).

Model validation was conducted by detecting the differences between the observed values and predicted values from the validation dataset. The PredRMSE and BaseRMSE of ARIMA (1,3,2) model for time series of cumulative cases was 21137 and 166330, respectively. Similarly, PredRMSE and BaseRMSE of ARIMA (3,3,1) model for time series of death cases were 668.7 and 5431, respectively. Lower values of PredRMSE than Base RMSE indicated a good fit (Table 2). The estimation of forecast for cumulative cases and death cases were performed at 95 % confidence interval (Figures 2 and 3; Table 3).

Discussion

The dynamics of any infectious disease involve interactions of three elements - host, agent and environment.[21] India lies in both the Northern and Eastern hemispheres, with latitudes to north and longitudes to east results in high environmental variability, and subsequent effects on human behaviour and society.[22] The mathematical models are a centrepiece to forecasting the dynamics of infectious disease pandemics. The fundamental models used in prediction include data-driven,[23-27] empirical,[28-30] hybrid[31-32] models. The parameters of empirical models (incubation period, attack rate, and recovery rate) possess probability distributions such as Ehrlang and the Poisson distribution.[21] The data-driven models include ARIMA, single-input and single-output (SISO) models[33] and AI-based model (machine learning and deep learning techniques).[24] The significance of ARIMA model lies in modelling nonstationary time series. The ARIMA model assumes the trends will continue in the future indefinitely as against the empirical model which assume convergence. Few studies used nonparametric models like Fourier decomposition methods to predict turn-around dates of the epidemic and the results were found to agree with popular SIR models.[34]

SARS-CoV-2 expressed its presence in India with the first case diagnosed on 30 January 2020. On 1 July 2020, the cumulative cases and deaths in India reached 568,092, and 17,400, respectively.[3] India ranked 57th among the 100 countries in Global Health Security Index 2019, a scale to gauge preparedness for the outbreak of serious infectious diseases.[35] The forecast of COVID-19 cases helps government agencies in early preparedness to combat subsequent state of affairs. The patients of COVID-19 can be classified into confirmed cases, recovered cases, admitted, and death cases. All categories have differential importance from the management point of view. The requirement of hospital beds, medical equipment, hospital staff is a function of the number of admitted cases. Similarly, procurement of plasma for antibody therapy is a function of the number of recovered cases.

The present study forecasts the cumulative cases and deaths for India from 26 June 2020 to 5 July 2020. The forecast shows continuously increasing trends for both cumulative cases and deaths and shows no decreasing trends until October 2020.

The ARIMA model was proposed by various authors for forecasting the COVID-19 evolution. The data from 10 January 2020 to 20 February 2020 was used to fit ARIMA (1,0,4) and ARIMA (1,0,3) models for cumulative diagnosis and newer diagnoses to forecast the next two days.[36] The ARIMA and wavelet hybrid model was proposed to forecast ten time points of cumulative cases from 05 Apr 2020 to 14 Apr 2020 for India. The forecast showed oscillations may be due to effects of lockdown.[32] In another data-driven model, using bidirectional LSTM (long short-term memory) model, 15 days prediction of actual cases in India from 30 April 2020 to 14 May 2020 showed an error of less

than 3 %.[31] Susceptible-Exposed-Infectious-Recovered (SEIR) model was used to predict cumulative cases of COVID-19 in India during lockdown and post lockdown. The model predicts the peak of cumulative cases around 43,000 in mid-May. However, 7-21 % increase in peak value of cases was predicted for post lockdown period, reflecting relaxation in control strategies.[30] The evolution of COVID-19 in topmost affected states of India was done using SISO model.[33] The most severely affected states were Maharashtra, Gujarat, Tamil Nadu, Delhi, and Rajasthan.[33,37-39] One time series analysis used genetic programming to predict the COVID-19 evolution in India. On 13 May 2020, the cumulative cases and death cases were 80,000 and 2500, respectively. The prediction for the next ten days was done with 142,000 and 4,200 cumulative cases and deaths, respectively, on 23 May 2020.[40] Sujath used linear regression, multilayer perceptron and vector autoregressive method and 80 time points till 10 April 2020, to predict confirmed cases, deaths, and recovered cases from 11 April 2020 to 18 June 2020. Although prediction varies across the methods and did not seem very accurate.[41] Yadav used six regression analysis-based machine learning models for prediction and found six-degree polynomial models predict very close to observed data.[42]

As the epidemic continues, the effect of different interventional strategies inherited in the time series, and thus data-driven model seem to be more accurate than empirical models. The real-time data modelling has been proposed for monitoring trends of the Indian subcontinent. Evidence-based interventions should be implemented to control the pandemic.

Conclusion

The present study produces encouraging results with the potential to serve as a good adjuvant to existing models for continuous predictive monitoring of the COVID-19 pandemic. The forecast of COVID-19 may assist public health authorities and governmental agencies for early preparedness and evidence-based decision making.

The limitations of the study

Firstly, ARIMA model used in forecasting does not capture the non-linear and chaotic dynamics of the pandemic. Secondly, the parameter selection procedure requires repetition with each time series update.

Contribution of Authors

All authors contributed equally.

Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability of

Data is available on reasonable request from the corresponding author.

Ethics Declarations

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Ethical Committee of the SMS Medical College, Jaipur (No.430/MC/EC/2020 dated 26 Jun 2020) and individual consent for this retrospective analysis was waived.

Acknowledgements

None.

Conflict of interest

None.

Abbreviations

ACF:	Autocorrelation function
ADF test:	Augemented Dickey Fuller unit root test
AIC:	Akaike information criterion
ARIMA:	Autoregressive integrated moving average
BaseRMSE:	Base root mean square error
COVID-19:	Coronavirus disease-2019
PACF:	Partial autocorrelation function
PredRMSE:	Prediction root mean square error
SARS CoV-2:	Severe Acute Respiratory Syndrome Coronavirus 2
T × C dataset:	training dataset of cumulative cases of COVID-19
T × D dataset:	training dataset of death cases of COVID-19

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ORIGINAL ARTICLE

Association of PPARG rs3856806 C>T Polymorphism With Body Mass Index, Glycaemia and Lipid Parameters in Serbian Adolescents

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Abstract

Background/Aim: Peroxisome proliferator-activated receptor gamma (PPARγ) belongs to a family of nuclear hormone receptors and ligand-activated transcription factors. PPARG gene is expressed in many tissues including adipose tissue where it plays a crucial role in differentiation of adipocyte, insulin resistance, blood glucose levels and lipid metabolism. The aim of the study was to examine the association of rs3856806 polymorphism with the body mass index (BMI), fasting glucose levels and lipid parameters in Serbian adolescents.
Methods: This research included 287 adolescents of both genders (143 boys and 144 girls), 14-15 years of age. Genotype detection was done by polymerase chain reaction-restriction fragment length polymorphism (RFLP) assay.
Results: Results showed statistically significant difference in terms of fasting glucose levels among girls (p = 0.013) depending on their genotype. Female carriers of CC genotype had significantly higher level of fasting glucose levels. Also, results showed that in the group of overweight and obese girls, carriers of CT or TT genotype had statistically significant lower values of HDL cholesterol compared to girls - carriers of CC genotype (p = 0.000). However, this result was not confirmed by multiple regression analysis. Statistically significant association of rs3856806 polymorphism was not observed with BMI nor with other lipid parameters.
Conclusion: This polymorphism is associated with fasting glucose level and HDL cholesterol among girls. To draw definite conclusions, further research should be conducted including non-genetic factors and other polymorphisms among this gene.

Keywords: PPARG; rs3856806; Lipid profile; BMI; Fasting glucose level.

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Introduction

Childhood and adolescent obesity is becoming a major problem worldwide, reaching epidemic proportions. It is estimated that one out of five youth is overweight and out of them 9.5 % are severely obese.[1, 2] Adolescent obesity is associated with various disorders of which the most common are metabolic disorders and diabetes mellitus type II.[3] Recently, many studies are focused on the molecular-genetic causes of obesity and their comorbidities. To the already known factors, brown adipose tissue (BAT) is gaining an increased interest since the discovery that this type of tissue is present not only in newborns but also in adolescents and adults. Due to this fact many studies were conducted in order to give answers on its activation, physiology, impact on

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CONTENT

human health and possible treatment options.[4, 5] The main role of BAT is to dissipate chemical energy to produce heat.[6] Its activation depends on gender, age, temperature and obesity. However, activation and volume of BAT increases during puberty, probably due to effects of sex steroids and growth hormone.[7, 8] It has been shown that adults with detectable BAT had significantly lower levels of total cholesterol, low-density lipoprotein cholesterol and glycaemia in comparison to the individuals without BAT.[9–11]

Differentiation and activation of BAT is very complex mechanism which involves multiple transcriptional factors, among which peroxisome proliferator-activated receptor gamma (PPARγ) is a crucial regulatory factor. PPARγ is essential not only for differentiation of BAT, but also for differentiation and activation of white adipose tissue.[12] PPARγ belongs to family of nuclear hormone receptors and ligand-activated transcription factors and is encoded by PPARG gene. PPARG gene is expressed in many tissues including adipose tissue where it has a key role in lipid metabolism, adipocyte differentiation, regulation of insulin resistance and blood glucose levels.[13]

Preadipocyte differentiation into either white or brown adipose tissue depends on PPARγ and its coactivators. PPARγ in interaction with Positive regulatory domain containing 16 (PRDM16) transcriptional factor leads to the expression of genes specific for BAT and beige adipose tissue and to the expression of uncoupling protein 1 (UCP1).[14, 15] On the other side, binding to the Transducin-like enhancer protein 3, PPARγ is able to suppress expression of brown and beige adipose tissue and to stimulate expression of selective genes for white adipose tissue.[14, 16] In humans, obesity leads to the decreased expression of PPARG, thus causing an increased degree of inflammation, angiogenesis and fibrosis in white adipose tissue. In line with these facts, individuals with mutations within the PPARG gene are prone to insulin resistance and lipodystrophy. However, increased PPARG expression results in improved insulin sensitivity.[17]

Within the PPARG gene, several mutations have been described which are linked to the metabolic disorders and lipodystrophy.[18] One of the common polymorphic variants in this gene is C1431T silent mutation (rs3856806) found in the exon 6. This polymorphic variant is related with lower body mass index and favourable lipid profile.[19, 20] Even though this polymorphic variant is associated with better metabolic state, results obtained from different research are still contradictory.

Methods

Study design

Participants enrolled in this study (n = 287) were healthy adolescents, 15 years of age. Participants were selected randomly from a total of 6000 adolescents who were included in Yugoslav Study of the Precursors of Atherosclerosis in School Children (YUSAD). YUSAD study lasted for 21 years and it ended in 2008. Data of anthropometric measurements (height and weight), gender, fasting blood glucose (FBG) levels and values of lipid parameters such as total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG) levels were recorded during 2003 at 11 paediatric departments of primary health care centres (Čukarica, Palilula, Požarevac, Užice, Kraljevo, Knjaževac, Bor, Niš, Subotica, Arilje and Despotovac) in Serbia. According to the previously published BMI charts for Serbian adolescents, participants were classified in 3 groups: normal weight (< 85th percentile), overweight (≥ 85th percentile) and obese (≥ 95th percentile).[21] Criteria for exclusion from the study were presence of neurodegenerative malformations, malignant and cardiovascular disease, congenital genetics malformations, cerebral palsy, chronic immobility and diabetes mellitus type 1 or 2.

Ethical principles

This research was permitted by the Ethics Committee of Faculty of Medicine, University of Belgrade, Serbia. Signed authorisation form was obtained from each participant's parent or guardian.

Genotyping

Genotype analysis were conducted at the Institute of Human Genetics, Faculty of Medicine, University of Belgrade, Serbia. DNA extraction was performed from 5 mL of peripheral blood by salting out method.[22] Genotypes of rs3856806 polymorphism were investigated by classical PCR followed

CONTENT

by restriction fragment length polymorphism (RFLP) analysis. The cycling conditions were performed by the standard genotyping protocol which included 37 cycles (5 min at 95 °C, 30 s at 94 °C, 30 s 63 °C, 30 s at 72 °C and 7 min at 72 °C). Two set of primers were used (Forward: 5'-CAG GTT TGC TGA ATG TGA AGC-3', and Reverse: 5' -TGG CTC AGG ACT CTC TGC TAG -3'). After PCR, fragments of 191 bp were digested with NlaIII restriction enzyme at 37 °C for 2 hours. The 8.0 % polyacrylamide gel electrophoresis was used to detect fragments of 197 bp and 62 bp which corresponds to CC genotype, and fragments of 156, 62 and 41 bp were noticed in TT genotype.

Blood analysis

Before blood sampling, each participant fasted for 12 h. Levels of TG, TC, HDL-C were measured as described previously.[21] Friedewald's equation was used to calculate levels of LDL-C concentrations.[23]

Statistical methods

Numerical variables were presented as mean ± standard deviation. Student`s t-test or Mann-Whitney U-test were used to examine the association of selected parameters with rs3856806 genotypes. Multivariate linear regression analysis was also performed to assess the association of lipid levels and PPARG genotypes with gender, using BMI and FBG as covariates.

Results

This research included 287 randomly selected school children 15 years of age. There were 143 boys (49.8 %) and 144 girls (50.2 %). In the group of boys 24 (16.8 %) had BMI ≥ 85th percentile, and 18 (12.6 %) had BMI ≥ 95th percentile. Among girls 29 (20.1 %) had BMI ≥ 85th percentile and 18 were with BMI ≥ 95th percentile (12.6 %). Average values of selected parameters between group of boys and girls are summarised in Table 1.

Table 1: Analysis of selected parameters according to gender

Parameters	Total	Boys	Girls	p
BMI (kg/m^2)	21.99 ± 4.30	21.64 ± 4.26	22.34 ± 4.32	0.088
Fasting blood glucose (mmol/L)	4.69 ± 0.65	4.78 ± 0.61	4.61 ± 0.67	0.031
Triglycerides (mmol/L)	0.99 ± 0.56	1.00 ± 0.64	0.98 ± 0.47	0.213
Total cholesterol (mmol/L)	4.34 ± 0.86	4.17 ± 0.75	4.50 ± 0.94	0.001
HDL-Cholesterol (mmol/L)	1.41 ± 0.41	1.38 ± 0.42	1.45 ± 0.39	0.171
LDL-Cholesterol (mmol/L)	2.44 ± 0.84	2.33 ± 0.74	2.54 ± 0.91	0.033

BMI - body mass index; HDL - high-density lipoprotein; LDL - low–density lipoprotein; red colour - statistically significant

Statistically significant differences were observed in mean values of glycaemia, TC and LDL-C between males and females. Observed differences in values of total and LDL-C (p = 0.01; p = 0.033, respectively) means that girls had higher values of these parameters in comparison to boys. On the other side, statistically significant difference was noticed in the mean values of FBG in the group of boys. Boys had statistically higher mean values of FBG in comparison to girls (p = 0.031). Allele and genotype frequencies of rs3856806 are summarised in Table 2.

Table 2: Genotypes and alleles frequencies of PPARG polymorphism

PPARG rs3856806 Genotypes	Frequency n (%)	Alleles	Frequencies %
CC	233 (81.2)	C	90.1
CT	52 (18.1)	–	–
TT	2 (0.7)	T	9.9

The distributions of genotypes were in Hardy-Weinberg equilibrium. No statistically significant difference was observed between male and female adolescents in frequencies of PPARG genotypes (p = 0.527).

Average values of BMI, FBG, TG, TC, HLD-C, and LDL-C according to PPARG genotype on whole sample as well as in the group of overweight and obese adolescents are summarised in Tables 3 and 4. Since only two adolescents were carriers of TT genotype, grouped genotypes as CC/CT+TT were grouped.

Lower mean values of FBG are noticed in carriers of CT or TT genotype compared to carriers of CC genotype (p = 0.054). Multiple linear regression analysis was performed to evaluate this result. The obtained p value on the total sample was p = 0.07. Since it has been previously observed that FBG levels are influenced by gender the association between the PPARG genotype and FBG separately in the groups of boys and girls was analysed. However, statistical significance was noticed only in the group of girls. No statistical association was found in mean values of other analysed parameters.

Furthermore, the association of selected parameters with PPARG polymorphism was analysed in

CONTENT

Table 3: Mean values of BMI and biochemical parameters according to PPRAG rs3856806 genotype

Parameters	Genotype	Total	p	Boys	p	Girls	p
BMI (kg/m^2)	CC	21.98 ± 4.20	0.716	21.50 ± 4.00	0.810	22.46 ± 4.36	0.426
	CT+TT	22.01 ± 4.73		22.19 ± 5.20		21.80 ± 4.18	
Fasting blood glucose (mmol/L)	CC	4.73 ± 0.65	0.054	4.79 ± 0.64	0.689	4.67 ± 0.66	0.013
	CT+TT	4.54 ± 0.62		4.74 ± 0.50		4.30 ± 0.67	
Triglycerides (mmol/L)	CC	1.00 ± 0.59	0.968	1.01 ± 0.68	0.583	0.98 ± 0.49	0.617
	CT+TT	0.94 ± 0.41		0.99 ± 0.44		0.97 ± 0.37	
Total cholesterol (mmol/L)	CC	4.33 ± 0.86	0.796	4.15 ± 0.77	0.535	4.50 ± 0.92	0.987
	CT+TT	4.36 ± 0.87		4.25 ± 0.68		4.50 ± 1.06	
HDL-Cholesterol (mmol/L)	CC	1.43 ± 0.41	0.111	1.40 ± 0.44	0.214	1.46 ± 0.39	0.364
	CT+TT	1.33 ± 0.38		1.29 ± 0.37		1.38 ± 0.39	
LDL-Cholesterol (mmol/L)	CC	2.41 ± 0.85	0.198	2.29 ± 0.76	0.179	2.52 ± 0.92	0.498
	CT+TT	2.57 ± 0.76		2.50 ± 0.66		2.66 ± 0.87	

BMI - body mass index; HDL - high-density lipoprotein; LDL - low–density lipoprotein; red colour - statistically significant

Table 4: Mean values of biochemical parameters according to PPRAG rs3856806 genotype in adolescents with BMI ≥ 85th percentile

Parameters	Genotype	BMI ≥ 85th pc	p	Boys	p	Girls	p
Fasting blood glucose (mmol/L)	CC	4.80 ± 0.51	0.104	4.90 ± 0.44	0.446	4.73 ± 0.55	0.030
	CT+TT	4.55 ± 0.60		4.77 ± 0.54		4.18 ± 0.49	
Triglycerides (mmol/L)	CC	1.11 ± 0.67	0.164	1.12 ± 0.74	0.538	1.12 ± 0.65	0.561
	CT+TT	1.15 ± 0.45		1.15 ± 0.51		1.04 ± 0.40	
Total cholesterol (mmol/L)	CC	4.41 ± 0.86	0.796	4.11 ± 0.66	0.492	4.61 ± 0.95	0.419
	CT+TT	4.24 ± 1.01		4.27 ± 0.63		4.24 ± 1.50	
HDL-Cholesterol (mmol/L)	CC	1.33 ± 0.35	0.052	1.22 ± 0.28	0.620	1.41 ± 0.38	0.000
	CT+TT	1.14 ± 0.25		1.29 ± 0.46		1.08 ± 0.11	
LDL-Cholesterol (mmol/L)	CC	2.54 ± 0.84	0.847	2.31 ± 0.70	0.512	2.65 ± 0.94	0.964
	CT+TT	2.58 ± 0.93		2.47 ± 0.65		2.67 ± 1.34	

BMI - body mass index; HDL - high-density lipoprotein; LDL - low–density lipoprotein; red colour - statistically significant

the group of overweight and obese adolescents. Statistically significant lower mean values of HDL-C were noticed in adolescents with CC+CT genotype in comparison to adolescents with CC genotype (p = 0.052). The result is on the border of statistical significance and multiple linear regression analysis did not confirm these results (β = -0.125, p = 0.315).

However, overweight and obese girls who were carriers of CT+TT genotype revealed statistically significant lower mean values of fasting blood glucose and HDL-C in comparison to the girls who were carriers of CT+TT genotype (p = 0.030; p = 0.000, respectively). Multiple linear regression analysis confirmed only the results for FBG (β = -0.302, p = 0.050). Statistically significant difference in mean values of other analysed parameters was not observed.

CONTENT

Discussion

BAT is very important organ which role is reflected on the lipid and glucose metabolism, energy homeostasis and weight regulation. Its differentiation and activation is a complex mechanism in which PPARγ has very important role.[5] Researches have shown that lean adolescents have more functional and non-functional BAT compared to children and adolescents with BMI ≥ 85th percentile.[24] Overweight and obese adolescents with no metabolically active BAT had three times more subcutaneous fat, and six times more visceral fat compared to children and adolescents with metabolically active BAT.[5, 25, 26] Bearing in mind that volume and activation of BAT increases during puberty, the aim of this research was to investigate whether single nucleotide polymorphism rs3856806 within PPARG gene could affect the mean values of TC, HLD-C, LDL-C, TG, FBG levels and BMI in adolescents. Even though results from previous researches showed an association of rs3856806 polymorphism with favourable metabolic traits and lower BMI, studies are still rare especially in children and adolescent population.[9, 20]

Results from this study revealed statistically significant difference in mean values of FBG, TC and LDL-C between the group of boys and girls. Higher mean values of fasting blood glucose were noticed in boys, while girls had higher mean values of TC and LDL-C. These differences could be explained by the increase in production of growth hormone, growth factors, gonadotropins and sex steroid hormones. Thus, the differences in obtained results between the group of boys and girls might come from difference in growth and sexual maturity between these two groups.[4]

Statistically significant association of rs3856806 with BMI was not observed in this study, which corresponds to the data obtained in research of Leon-Mimila et al conducted in population of 1218 healthy children 6-18 years of age.[27] Besides, no association of this polymorphism with BMI was found in the study of Luo et al,[28] Parra et al[29] and in the research of Kim et al.[19] Analysing the obtained results regarding lipid parameters, the authors came to the conclusion that in the group of overweight and obese adolescents, carriers of CT or TT genotype had lower mean values of HDL-C compared to the carriers of CC genotype, although significance was borderline. The reasonable explanation for the statically significance found only among girls could be derived from the fact that secretion of oestrogen and other sex hormones is increasing during puberty. Due to rise of oestrogen, girls tend to have higher BMI compared to boys as well as increase of subcutaneous adiposity. On the other hand, testosterone has impact on lipase activity of liver, thus causing lower levels of lipid parameters in boys. Also, there were no available data on eating habits or level of physical activity of adolescents, which are also very important parameters when assessing lipid parameters.[30, 31]

To the authors' knowledge, there is no available studies which examined the effects of this polymorphism on metabolic parameters in the population of children and adolescents. However, the study of Wei-min Wei and colleagues[32] investigated the association of rs3856086 with ischaemic stroke and lipid status in patients with this disease as well as with normal controls. Results revealed that in both groups carriers of CC genotype had significantly higher values of total and LDL-C compared to carriers of CT or TT genotype, while the association between this polymorphism and mean values of TG and HDL-C was not observed.[32] Research of Butt et al showed that higher mean values of LDL-C were recorded in carriers of CC genotype in comparison with carriers of CT or TT genotype.[33] In the same research the association of rs3856806 with levels of TG and HDL-C was not found. On the other side, some studies showed opposite results. Also, results from study of Fan et al showed that carriers of CT or TT genotype had higher values of LDL-C compared to carriers of CC genotype.[34] However, study of Dujic et al conducted in the population of Bosnia and Herzegovina in patients with metabolic syndrome and healthy controls did not find association of rs3856806 with lipid profile parameters in neither group.[35]

Regarding association of this polymorphism with FBG, these results showed that girls who were carriers of T allele had lower values of FBG in comparison to the girls who revealed CC genotype.

Relieving the literature results obtained in previous studies, data are contradictory. However, Zhou et al revealed association of this polymorfism with lower values of blood glucose in patients with coronary artery diesease, but not in healthy controls.[36] The study of Butt et al and Grygiel-Górniak et al results have shown that carriers of CC genotype had significanlty lower values of FBG

compared to carriers of T allele.[33, 37] On the other hand, Parra et al investigated the association of 11 different polymorphisms including rs3856806 with diabetes mellitus type 2 and BMI in the Latinoamerican population. Their results have shown that presence of T allele is significantly associated with diabetes mellitus type 2, but haplotype analysis with another polymorphisms within this gene did not show any significant association.[29]

However, inconsistencies in results obtained from different studies in terms of metabolic traits might be explained by the fact that this polymorphism is silent and is not causing change in amino acid sequence, thus is unlikely to have any direct link to certain phenotype, or it could be in linkage disequilibrium with another functional polymorphism such as Pro12Ala polymorphism, or with still unidentified functional variation. Also, other reasons for contradictory results could be different study designs, the selection criteria of patients as well as differences in ethics origins.

Conclusion

Taking into account all available data of this polymorphism and its correlation with the levels of lipid parameters and FBG it could be concluded that rs3856806 plays significant role in lipid and glucose metabolism. However, the present study is limited with data about non-genetic factors that could influence the BMI, glucose level and lipid profile of adolescents. It is known that lifestyle, primarily eating habits, physical activity and usage of certain drugs can modify lipid status. Thus, to draw definite conclusions it would be of great interest to further investigate the impact of this polymorphism on lipid parameters of adolescents and adults on a larger population with taking in consideration the non-genetic factors as well.

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Conflict of interest

None.

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CONTENT

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ORIGINAL ARTICLE

Assessment of the Haematological Profile After Appendectomy Using Linear Titanium Stapler Clips: an Experimental Study in Rats

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Abstract

Introduction: The technique of closing the appendiceal stump using linear titanium stapler clips is being used more and more frequently in laparoscopic surgery, despite the good practice in the use of resorbable single endoloop vicryl ligatures and/or the non-resorbable plastic hem-o-lok clips. No light has been shed on potential undesirable effects on the haemathological profile of the use of titanium stapler clips. This experimental study aimed at establishing any possible undesirable effect of linear titanium stapler clips and single resorbable vicryl endoloop ligatures on the blood cells in rats following appendectomy.
Methods: A total of 70 adult rats were used, divided into a control group (n = 10), and two experimental groups (n = 30 + 30). The appendices were removed from the rats in the first experimental group using titanium stapler clips and in the second experimental group the appendiceal stump using resorbable endoloop vicryl ligatures was closed. In both experimental groups three sub-groups with ten animals each were formed, from which peripheral blood from a tail vein on days 7, 28 and 60 was removed postoperatively, in order to assess the usual haematological parameters. Stained blood smears were also analysed in order to establish any poikilocytotic erythrocytes present.
Results: In the first experimental group, with the titanium stapler clips, more than 25 % neutrophils were found on day 7, which is a significantly different result ($p < 0.05$) to the control group. In the second experimental group, there were more neutrophils than in the titanium stapler clips group, especially on days 7 and 60 and the results of these two sub-groups differ statistically significantly, $p < 0.05$. Hypochromia was found in the endoloop vicryl ligatures group, as well as in the titanium stapler clips sub-group on day 28, due to lower haemoglobin values which were significantly different to the control group, $p < 0.05$. Moderate levels of annulocytes, spherocytes and stomatocytes were found in most experimental groups.
Conclusion: The results of this study favour the use of linear titanium stapler clips over resorbable single endoloop vicryl ligatures, because a less unfavourable effect was established on the blood cells of the experimental rats with their use.

Keywords: Appendectomy; Titanium stapler clips; Annulocytes; Neutrophils; Hypochromia.

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Introduction

Laparoscopic appendectomy as a surgical technique for dealing with the appendix, has widespread use in abdominal surgery, and its roots go back to the 1980's.[1]

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CONTENT

The most frequently used technique for closing the appendiceal stump is the single endoloop ligature,[2, 3] whilst non-resorbable polymer plastic clips are an alternative technique used to close the appendiceal stump more quickly and cheaply than standard methods.[1] In contrast to these, titanium stapler clips may also be used successfully to treat all forms of acute appendicitis.[4]

The effects on the tissue and organism in general caused by this laparoscopic method have not yet been fully discussed. Differences between the degree of inflammation and the foreign body reaction to different materials used in laparoscopic appendectomy favour the use of titanium stapler clips.[5-7]

The fact is that the development of various diagnostic scores,[8] and the mass use of diagnostic aids, for example C-reactive protein,[9] have often been the cause of confused diagnoses made by a large number of clinicians.[10] Practically no laboratory or radiological test is 100 % accurate.[11] As a result it is important to compare these tests and monitor the haematological parameters. Kamaran et al (2008)[12] mention the importance of analysing the total leukocyte count in patients in whom appendectomy is indicated.

There are reports whose results indicate monitoring local inflammatory responses.[2, 7, 13, 14] Reports describing changes in haematological parameters in the peripheral blood in the post-operative period in treated patients are few. As a result, little is known about how non-resorbable linear titanium stapler clips affect blood cells after appendectomy.

Monitoring haematological parameters, not only in cells of the leukocyte order, but also all other closely related parameters, for example in relation to erythrocytes in the post-operative period, is also extremely important for the successful post-operative care of patients.

The Aim of the Study

The aim of this experimental study was to establish any possible, undesirable effect of linear titanium stapler clips and single resorbable endoloop vicryl ligatures on the blood cells of experimental rats.

Methods

Approval by the Ethics Committee

This research was approved by the Ethics Committee for the Protection and Welfare of Experimental Animals in Biomedical Research of the Faculty of Medicine, University of Banja Luka No 18/2/20.

Study Design and Animals

The experimental study was conducted on an animal model using albino Wistar rats. A total of 70 adult rats were used, weighing 250-300 g. The animals were kept in polypropilene cages under optimal conditions: temperature 22 ± 2 °C, relative humidity 43 % to 67 %, and a light:dark cycle of 12:12 hours. The rats consumed briquette food and drinking water *ad libitum*. Twenty-four hours before the surgery, the animals were denied food.

The rats were divided into three groups: the control (n = 10), which did not undergo appendectomy, and two experimental groups. The animals in the first experimental group (1, n = 30) had their appendix removed, with the use of non-resorbable titanium stapler clips. In the second experimental group (2, n = 30), the appendiceal stump was closed off with resorbable endoloop vicryl ligatures. In both experimental groups, three sub-groups were formed, with ten animals each, from which the peripheral blood was removed on days 7, 28 and 60.

Surgical Procedures

Ketamine hydrochloride (50 mg/kg) (International B.V. Boxmeer, The Netherlands) was used, as the general anaesthetic, intramuscularly. The rats earmarked for surgery were previously prepared by shaving the fur in the abdominal region. The skin was then disinfected with a povidon-iodine solution. The animals were fixed to the operating table in a supine position. The laparotomy was performed using a medial incision. After the caecum was located, a large sac in the lower third of the abdominal cavity which mimics the appendix in humans, it was resected.

The rats in the control group (n = 10) did not undergo surgery. The rats in the first experimental group, 1 (n = 30) had their appendix removed using non-resorbable linear 45 mm titanium stapler clips, (thick filling) (Ethicon, Endosurgery, Cincinnati, OH). In group 2 (n = 30) the blind-end

CONTENT

of the appendix (about 0.7 cm long) was closed with a Vicryl 2-0 ligature (Vicryl Ethicon, polyglactin 910). The laparotomy and closure were performed with a continuous suture of 3-0. During the surgical procedure and in the post-operative period no antibiotic therapy was used.

Haematological Procedure

Ten animals from experimental groups had peripheral blood drawn from the caudal vein three times: on the 7th, 28th and 60th postoperative days. Rats from the control group (n = 10) had blood drawn only once, and it was used for comparison. Using an "Idexx Laser Cyte" flow haemocytometer, results were obtained for: haemoglobin (Hb) (g/dL), haematocrits (HCT) (%), Mean Corpuscular Volume (MCV) (fL), Mean Corpuscular Haemoglobin (MCH) (pg), Mean Corpuscular Haemoglobin Concentration (MCHC) (g/dL), total erythrocyte count (RBC) (10^{12}/L) and total leukocyte count (WBC) (10^{9}/L).

Microscopic Examination of Peripheral Blood

Samples of peripheral blood were collected from the *vena caudalis* with the intention of creating at least two sample blood smears, using the standard Giemsa laboratory staining procedure. Analysis of the stained blood smears was based on the standard morphology and performed by two independent researchers. Differentiation and percentage expression of the blood cells observed were conducted on a sample, with a single layer field of vision, where only the edges of the the blood cells touched one another, and they did not overlap.[15]

The poikilocytotic erythrocytes were classified semi-quantitatively according to similar research,[16] following the criteria: non-existent (0 %), scarce (0.05 - 0.5 %), mild (> 0.5 - 3 %), moderate (> 3 % - 10 %), or strongly expressed (> 10 %). The number and type of poikilocytotics were noted as a percentage of the RBC.

For each original stained smear, 1000 erythrocytes were counted and analysed, using a binocular light microscope, Motic Type 102 M, with 900 x magnification. The average values from two independent researchers, that is, two sets of measurements were taken.

Lymphocytes (L) (%), neutrophils (N) (%), monocytes (M) (%), basophils (B) (%) and eosinophils (E) (%), were differentiated according to similar studies[3, 17] and are shown as percentages after analysis of 1000 cells of the leukocyte order, for each blood smear sample in the experimental and control groups, also using a Motic Type 102 M binocular light microscope, with 900 x magnification.

Statistical data analysis

For statistical data processing the IBM program SPSS Statistics for Windows, Version 24 was used. Data processing was performed using the ANOVA / Kruskal Wallis test (depending on whether the data were normally distributed - the Shapiro-Wilk test was used to examine normality), and $p < 0.05$ was considered statistically significant.[18]

Results

After statistical processing of the data, it was established that a statistically significant difference existed in almost all the tested parameters in the experimental groups in comparison with the control (Graph 1 and Table 1). The RBC values did not change significantly in all three endoloop vicryl ligatures subgroups, but there were differences in the titanium stapler clips groups on days 7 and 60 in comparison with the control. It was similar with the HCT values, where Table 1 shows that there were no differences between the endoloop vicryl ligatures sub-groups, but there was a difference in the stapler group on day 60 in comparison with the control. The values of the parameters HB, MCV and MCHC changed statistically significantly in comparison with the control in all three experimental vicryl sub-groups, whilst in the first experimental titanium stapler clips group, HB was < 0.05 on day 28, MCV in the stapler groups on days 28 and 60.

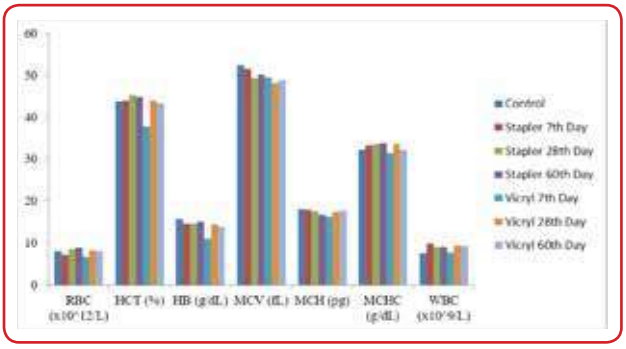

Figure 1. Haematological parameters in the experimental stapler and vicryl groups, and the control group

CONTENT

Table 1: Statistical analysis of haematological parameters

Haematological parameters	p value					
	Group I			Group II		
	Stapler 7th Day	Stapler 28th Day	Stapler 60th Day	Vicryl 7th Day	Vicryl 28th Day	Vicryl 60th Day
RBC (x 10^{12}/L)	0.011	0.113	0.002	0.161	0.243	0.604
HCT (%)	0.531	0.153	0.028	0.085	0.870	0.870
HB (g/dL)	0.06	0.05	0.306	0.000	0.022	0.004
MCV (fL)	0.700	0.001	0.024	0.004	0.000	0.003
MCH (pg)	-			-	-	-
MCHC (g/dL)	0.015	0.004	0.000	0.001	0.002	0.01
WBC (x 10^9/L)				-	-	-

p < 0.05 is statistically significant, indicated in red

A significant difference was found in the titanium stapler clips sub-group on day 7 in relation to the control group for the parameters N, L, M. For the stapler sub-group, on day 60 significant differences were noticed for the L and M results. In the stapler sub-group, no statistically significant difference was noticed on day 28 in comparison with the control group for any parameters (Figure 2 and Table 2).

In the endoloop vicryl ligatures sub-group, none of the parameters showed any statistically significant difference in comparison with the control on day 28, whilst in the vicryl groups on days 7 and 60 the differences in the values of N and L were p < 0.05 (Figure 2 and Table 2).

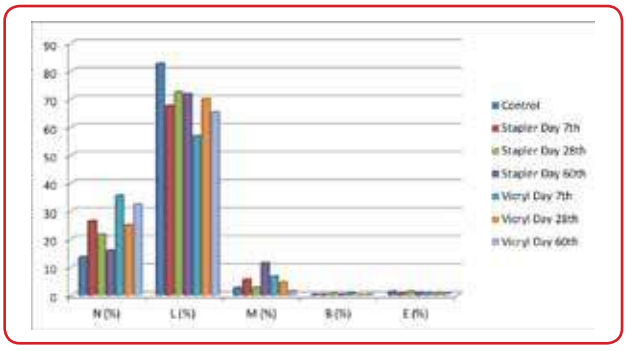

Figure 2. Leukogram results (%) of the experimental and control groups

Table 2: Statistical analysis of the control group in relation to the experimental groups in relation to the leukogram

Leukocyte cells	p value					
	Group I			Group II		
	Stapler Day 7	Stapler Day 28	Stapler Day 60	Vicryl Day 7	Vicryl Day 28	Vicryl Day 60
Neutrophils	0.01	0.22	0.412	0.001	0.172	0.006
Lymphocytes	0.001	0.164	0.01	0.000	0.104	0.013
Monocytes	0.021	0.901	0.000	0.063	0.662	0.946
Basophils	-	-	-	-	-	-
Eosinophils	0.055	0.965	0.233	0.065	0.075	0.259

p < 0.05 is statistically significant, indicated in red

Table 3: The average values of the poikilocytotic forms per 1000 erythrocytes

Poikilocytotic forms of erythrocytes	Follow-up	Stapler Day 7	Stapler Day 28	Stapler Day 60	Vicryl Day 7	Vicryl Day 28	Vicryl Day 60
OVALOCYTES	0.195	1.4	0.52	0.485	0.49	0.485	0.795
DACRYOCYTES	0.16	0.63	0.445	1.985	1.11	0.535	1.055
ANNULOCYTES	1.025	6.59	5.16	4.23	3.445	4.56	5.14
ECHINOCYTES	0.495	0.075	0.575	0.02	1.085	1.46	0.67
STOMATOCYTES	0.27	1.005	1.735	7.785	0.5	1.765	0.635
DREPANOCYTES	0	0.04	0.035	0.545	0.02	0	0.115
SCHISTOCYTES	0.035	0.11	0.095	0.035	0.135	0.08	0.085
TARGET CELLS	0	2.56	0.885	0.025	3.735	0.815	7.725
ACANTHOCYTES	0	0.02	0.235	0.03	3.03	0	10.135
SPHEROCYTES	0.01	4.92	1.63	1.805	2.56	1.565	12.48
RETICULOCYTES	0.24	0.75	0.05	0.39	0.635	0.115	0.01

Red colour indicates results with moderate (> 3 % –10%), and strong (> 10 %) values

If the appearance of poikilocytotic forms (Table 3) is considered, it may be seen that in all the experimental groups there is a moderate number of annulocytes, in the stapler sub-group on day 60 there is moderate stomatocytosis, whilst moderate spherocytosis appears in the titanium stapler clips sub-group on day 7. In the experimental endoloop vicryl ligatures group, there was strong acanthocytosis and spherocytosis in the vicryl subgroup on day 60, and moderate values for target cells in the endoloop vicryl ligatures sub-groups on days 7 and 60. All the other poikilocytotic forms were mildly expressed (0.05 - 3 %) within all the experimental groups.

Discussion

The tissue of the small and large intestines reacts to sutures and clips, as to any other foreign body. It has been established that non-resorbable linear titanium stapler clips cause less reaction in the surrounding tissue than resorbable endoloop vicryl ligatures.[2, 14, 19] The results of the leukogram in this study completely correspond with the results of these researchers. In the first experimental group, titanium stapler clips, more than 25 % neutrophils were found, on day 7, which is a significantly different result in comparison with the control group. The appearance of neutrophils is the expected reaction in the peripheral blood to the titanium stapler clips used to close the appendiceal stump. In the sub-groups of rats that underwent appendectomy where titanium stapler clips were used, mild neutrophilia was noticed on day 28, whilst the neutrophils retur-

CONTENT

ned to normal values on day 60, and their percentage values were equal to the values in the control group. In the second experimental group, there were more neutrophils than in the titanium stapler clips group, especially on days 7 and 60, and the results of these two sub-groups differ statistically significantly to the results in the control group. If these results are compared with similar research, appendectomy using plastic hem-o-lok clips and the accompanying effects in the peripheral blood, by Bajrić et al (2020),[3] it can be seen that the presented results partially correspond with theirs. In this study, neutrophilia, mild and expected monocytosis, was established in the vicryl sub-group on day 28, and also in the titanium stapler clips sub-group on day 60, which is a statistically significant result in comparison with the control group.

The results obtained regarding the total number of WBC are within the physiological reference intervals but tend towards the lower physiological level. The trend of lower values in the present study continues for lymphocytes, which were the most numerous cells in terms of percentage in the leukogram of the rats. The lowest physiological limit of lymphocytes in adult rates is 75 %,[20] and the results obtained from both experimental groups in this study were slightly below this low physiological limit. The percentage of lymphocytes in the leukogram of the experimental animals differs significantly from the control group at all time periods in both experimental groups, except within the sub-groups on day 28.

The obvious mild lymphopenia in this case completely corresponds with the similar research by Bajrić et al (2020)[3] for appendectomy using hem-o-lok plastic clips. The reasons for the appearance of lymphopenia have not been completely explained and could possibly be linked to the stressful circumstances for the experimental rats during the appendectomy, as well as the possible effect of the anaesthetic, as iatrogenic factors.

Lower percentage values of basophils and eosinophils were found, appropriate to their physiological reference ranges, and they were in line with relatively similar research.[3, 21]

The total number of RBC varied within the physiological intervals in relation to both experimental groups. There were no significant differences between the results obtained for vicryl on days 7, 28 and 60 in comparison with the results from the control group. In relation to the total number of RBC in the experimental titanium stapler clips group, significant differences were found on days 7 and 60 in comparison with the control group. The HCT results in both experimental groups are compatible with the results obtained for RBC.

From the analysis of the results obtained for HB, it was noticed the obvious undesirable effect of the surgical closure of the appendiceal stump in both experimental groups, in the sense of mildly lower values in relation to the control. The results were found to be significant at $p < 0.05$ in all the animals in the second experimental endoloop vicryl ligatures group, as well as in the titanium stapler clips sub-group on day 28. The values obtained are at the lower limits of the physiological range, or lower in some sub-groups, which clearly indicates the beginning of hypochromia, in line with similar research by Lelovas et al (2017),[22] and Bajrić et al (2020).[3]

The MCHC and MCV count parameters varied within the physiological range, but they were also significantly different to the control in all three experimental vicryl groups. The parameter MCV was significantly different in titanium stapler clips sub-groups on days 28 and 60 in comparison with the control results, whilst the MCHC parameter was significantly different in all three titanium stapler clips groups.

This study also monitored the poikilocytotic forms of RBC, and the results support the possible finding of anaemia, or mild hypochromia. That is to say, moderate findings were recorded of hypochromic erythrocytes (annulocytes) in the first experimental group in all three titanium stapler clips sub-groups on days 7, 28 and 60. Also, in the titanium stapler clips sub-group, moderate stomatocytosis was found on day 60, whilst moderate spherocytosis appeared in the stapler group on day 7. The experimental endoloop vicryl ligatures group had strong spherocytosis on day 60, as well as a moderate target cell result on day 60. The presence of all these poikilocytotic forms of RBC suggests the possible presence of anaemia.

All other poikilocytotic forms were mildly expressed (0.05 - 3 %) within both experimental groups, but this finding can be ignored since it is irrelevant due to its small percentage value. Moreover, a small number of these poikilocytotic forms probably occurred as artefacts, by mechanical trauma when creating the blood smear.

CONTENT

Conclusion

The results of this study favour the use of linear titanium stapler clips over resorbable single endoloop vicryl ligatures, because a less unfavourable effect was established on the blood cells of the experimental rats with their use. The results obtained indicate the possible occurrence of mild inflammation, post-operatively, but to a significantly lower extent than when using the other techniques mentioned. Also, titanium stapler clips cause the beginnings of hypochromic anaemia post-operatively, but in a significantly lower scope than when using the other conventional methods mentioned.

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None.

Conflict of interest

None.

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CONTENT

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ORIGINAL ARTICLE

Professional Stress and Burnout Syndrome in Teachers: Are There Differences Among the Republic of Srpska Regions?

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Abstract

Introduction: Burnout syndrome occurs as a result of prolonged exposure to emotional and interpersonal stressors in the workplace and is characterised by three dimensions: emotional exhaustion, depersonalization and personal achievements. Research and prevention of this phenomenon are of public health importance due to numerous consequences it leaves on the health of the working population and work organisation, and among teachers, on children as direct users of their services. The research aims to examine the presence of the stress and burnout syndrome at work in teachers in the regions of the Republic of Srpska, as well as to determine the existence of differences between regions.

Methods: The research was conducted as a cross-sectional study in the period August-September 2018, in the territory of the Republic of Srpska. The target population was represented by teachers of primary and secondary schools. The response rate in all regions was 75 % or above it. For this research, a special questionnaire was constructed, consisting of sociodemographic data, data on economic characteristics of the respondents, characteristics of the work environment, as well as data on satisfaction with equipment and resources at work, support from family and friends and presence of work-life conflict. In addition to the general questionnaire, the Maslach Burnout Inventory-Human Services Study (MBI-HSS) questionnaire for the assessment of the burnout syndrome at work, and Karasek's questionnaire (Job Content Questionnaire) for the assessment of stress at work were used.

Results: A statistically significant difference in the prevalence of occupational stress among teachers between RS regions was found. Also, a statistically significant difference was found between the regions of the Republic of Srpska related to the dimensions of burnout syndrome at work. A high level of emotional exhaustion was more often reported by teachers from the territory of the region of East Sarajevo and Prijedor (8.0 % and 7.7 %) comparing to teachers from other regions. Teachers from the territory of Prijedor more often showed moderate and high levels of depersonalization, as well as a low level of personal achievements comparing to teachers from other regions.

Conclusion: This research shows the presence of burnout syndrome in teachers of all the Republic of Srpska regions at the beginning of the school year, as well as statistically significant differences between the regions. This indicates the need for additional research on risk factors by regions to form targeted and thus more effective prevention measures.

Key words: Burnout syndrome; Occupational stress; Professional stress; Teachers; Educators; Workplace.

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CONTENT

Introduction

Burnout syndrome has intrigued the scientific public since the 1970s.[1, 2] According to Christina Maslach, it is traditionally defined as a three-dimensional concept and is characterised through emotional exhaustion, depersonalisation and personal achievements.[1] Since 2019, it has been included in the international classification of diseases and is defined as a mental state characterised by lack of energy or exhaustion, increased mental distance from work or a feeling of negativity or cynicism related to work, as well as a reduction of professional success.[3]

Burnout syndrome occurs as a response to prolonged exposure to emotional and interpersonal stressors in the workplace and is mostly related to helping/support professions whereas employees are exposed to constant emotional demands (health workers, social workers, psychologists, and teachers).[1]

Research and prevention of this phenomenon are of public health significance due to numerous consequences it has on the mental and physical health of the working population (insomnia, depression, psychosomatic diseases, cardiovascular disease, musculoskeletal disorders, etc.), but also on work organisations (job dissatisfaction, absenteeism, presentism, disability pensions).[4] At the same time, various authors point out that it is inevitable that burnout syndrome at work also represents a significant economic burden for the state.[5, 6] However, more recent specific data on economic consequences have not been found in the literature. The significance of this phenomenon could bear an even higher burden in the population of teachers. Researchers have shown that it is associated with long-term consequences on the academic achievements of students and a negative impact on their emotional development and mental health.[7, 8]

An analysis shows that the prevalence of burnout syndrome in the teacher population has a wide range (eg, emotional exhaustion 20-40 %, depersonalization 9-20 %).[9, 10] Data show that the system of values and beliefs, influencing behaviour and attitudes, have a significant impact on the variance of the burnout syndrome.[11] Thus, the authors on the study conducted in Germany and Syria noticed the existence of the difference between countries and the question of the possible influence of culture is raised.[12] Bearing in mind that the Republic of Srpska, as part of Bosnia and Herzegovina (BiH), is multinational and multi-ethnic, the goal of this research was to determine the frequency of occupational stress and burnout syndrome at work in the Republic of Srpska regions, as well as the possible existence of differences among the Republic of Srpska regions.

Methods

The research was conducted as a cross-sectional study before the start of the 2018/2019 school year in the period August-September on the territory of the Republic of Srpska regions at the Institute of Occupational Health and Sports Medicine of the Republic of Srpska in Banja Luka and regional centres (Prijedor, Doboj, Bijeljina, East Sarajevo and Trebinje). It was approved by the Ethics Committee of the Institute of Occupational Medicine and Sports of the Republic of Srpska.

According to the Law on Primary Education and Upbringing of the Republic of Srpska and the Law on Secondary Education and Upbringing of the Republic of Srpska, before the beginning of the school year all school workers are obliged to undergo a medical examination. The research was conducted during the medical examination, and the target population consisted of primary and secondary school teachers. Participation was offered to one out of four teacher who applied for the examination randomly and was conducted anonymously and voluntarily.

The response rate in all regions was 75 % or above it (Banja Luka 84 %, Prijedor 75 %, Doboj 78 %, Bijeljina 91 %, East Sarajevo 79 %, Trebinje 81 %).

A special questionnaire was constructed for this research. It consisted of a general questionnaire that included standard sociodemographic data (gender, age, marital status, parenthood), data on the economic characteristic of the respondents (material condition of the household assessed by five-points Likert scale). Also, variables on the characteristics of the work environment (length of the service, overtime work) and variables on personal satisfaction with equipment and resources for work, family and friends support (estimated

CONTENT

by five-points Likert scale) were included and the presence of work-life conflict was determined. For the determination of work-life conflict, the standard question which is already used in the literature was used "*In the last three weeks, how often were you annoyed or upset because of the inability to reconcile work with family and/or partner commitments?*".[13]

In addition to the general questionnaire, linguistically adapted and validated questionnaires have been used, Maslach Burnout Inventory-Human Services Study (MBI-HSS) for the assessment of the burnout syndrome at work and Karasek's (Job Content Questionnaire) for the assessment of the stress at work. MBI-HSS has a total of 22 items and consists of three scales: the emotional exhaustion scale (9 items), the depersonalisation/cynicism scale (5 items) and the personal achievement scale (8 items). Each scale consists of a series of statements that express the range of agreement with the statements and the categories of answers are given through a six-point Likert scale. The overall attitude of every examinee was obtained by summing, specific key, the matrix for each of the three previously mentioned scales. High exhaustion and cynicism frequency contribute to burnout syndrome, while the high frequency of personal achievements reduces it.[14] Karasek's questionnaire is frequently used in the world for assessing the stress level in the work environment and has shown satisfactory psychometric characteristics.[15] The Serbian version has 22 questions and measures two basic constructs: psychological demands of the workplace (job demand) (5 questions) and autonomy in the workplace ie, freedom of decision-making (job control) (9 questions). The job control scale has two subscales: skill-discretion (6 items) and decision latitude (3 items). The questionnaire also measures two additional constructs that are considered to be moderators of the demand-control relationship, which are: co-worker support (4 items) and superiors support (4 items). Also, the job strain calculated by a specific formula as the ratio job demands-job control, where a score above 1 indicates the existence of the workload.[16]

Methods of descriptive and inferential statistics were used in the work. From the methods of descriptive statistics, measures of central tendency (arithmetic mean and median), variability measures (standard deviation), and relative numbers depending on the type of data were used.

Chi-square test was used to compare the frequency between the groups, and the Fisher test when the expected frequency was less than 5. The normality of the distribution was assessed using the Kolmogorov-Smirnov test, and the Shapiro-Wilk test. Depending on the type of data, adequate Student t test or Mann-Whitney tests were used to assess the difference in the distribution of independent variables between different categories of source variables and ANOVA or Kruskal Wallis test were used for variables with more modalities (eg, differences between the regions). In the case of more than two groups, pairwise comparison adjusting for multiple testing was also performed (Tukey in case the row-variable was normally distributed or Benjamini-Hochberg method if this condition was not met). IBM SPSS Statistics 25 software was used for statistical data analysis. The results are shown in tables and graphs for statistical significance of $p < 0.05$ and high statistical significance $p < 0.01$.

Results

Sociodemographic characteristics of respondents

The gender distribution of teachers of the Republic of Srpska regions was similar. Almost half of the teachers (48.6 %) from the Prijedor region were 46 and older, while in other regions the number of teachers aged over 46 ranged from 26.2 % to 36.6 % and this difference between the regions was statistically significant ($p = 0.007$). Besides, the largest percentage of teachers in the Prijedor region showed divorced status or their spouses had died (13.6 %). In other regions, the same data gave 2.5-9.7 % and this difference between the regions was statistically significant ($p = 0.009$). Sociodemographic characteristics of teachers in the regions of the Republic of Srpska are presented in Table 1.

Life and work characteristics of the respondents

A statistically significant difference between regions was found in all examined life and work characteristics of teachers ($p < 0.01$). All of the teachers who participated in the research in the Doboj region worked in primary schools. In other regions, the number of teachers working in primary schools ranged from 44.8 % in Bijeljina to 72.9 % in Banja Luka. A small proportion of teachers (5

CONTENT

Table 1: Sociodemographic characteristics of the participating teachers in various regions in the Republic of Srpska

Sociodemographic characteristics	Banja Luka N (%)	Prijedor N (%)	Doboj N (%)	Bijeljina N (%)	East Sarajevo N (%)	Trebinje N (%)	p
Gender							
• Male	76 (26.1)	55 (25.1)	16 (20.0)	27 (28.1)	30 (26.5)	46 (30.3)	0.669
• Female	215 (73.9)	164 (74.9)	64 (80.0)	69 (71.9)	83 (73.5)	106 (69.7)	
Age (years)							
• 25-35	98 (33.7)	49 (22.3)	30 (37.5)	35 (36.5)	34 (30.1)	40 (26.3)	0.007*
• 36-45	101 (34.7)	64 (29.1)	29 (36.2)	35 (36.5)	42 (37.2)	56 (36.8)	
• 46-55	53 (18.2)	**63 (28.6)**	12 (15.0)	15 (15.5)	16 (14.2)	27 (17.8)	
• 56 and high	39 (13.4)	**44 (20.0)**	9 (11.2)	11 (11.5)	21 (18.6)	29 (19.1)	
Marital status							
• Single	58 (19.9)	35 (15.9)	18 (22.5)	25 (26.0)	29 (25.7)	42 (27.6)	0.009*
• Married	215 (73.9)	155 (70.5)	60 (75.0)	65 (67.7)	73 (64.6)	99 (65.1)	
• Divorced/Widower	18 (6.2)	**30 (13.6)**	2 (2.5)	6 (6.2)	11 (9.7)	22 (7.2)	
Children							
• Yes	205 (70.4)	166 (75.5)	56 (70.0)	62 (64.6)	75 (66.4)	94 (61.8)	0.091
• No	86 (29.6)	54 (24.5)	24 (30.0)	34 (35.4)	38 (33.6)	58 (38.2)	

Chi-squared test and Fisher exact test were used for statistical analysis of the data. Bolded numbers denote values significantly different from the ones for other regions

Table 2: Work-life characteristics of the participating teachers in various regions in the Republic of Srpska

Work-life characteristics	Banja Luka N (%)	Prijedor N (%)	Doboj N (%)	Bijeljina N (%)	East Sarajevo N (%)	Trebinje N (%)	p
Type of school							
• Primary	212 (72.9)	128 (58.2)	**80 (100.0)**	43 (44.8)	63 (55.8)	89 (58.6)	0.000*
• Secondary	76 (26.1)	91 (41.4)	0	51 (53.1)	50 (44.2)	62 (40.8)	
Length of work (years)							
• ≤ 10	119 (40.9)	74 (33.6)	38 (47.5)	47 (49.0)	46 (40.7)	52 (34.2)	0.000*
• 11 - 20	106 (36.4)	51 (23.2)	26 (32.5)	34 (35.4)	36 (31.9)	52 (34.2)	
• > 20	66 (22.7)	**95 (43.2)**	16 (20.0)	15 (15.6)	31 (27.4)	48 (31.6)	
Overtime work (per week)							
• Never	234 (80.4)	165 (75.0)	72 (90.0)	60 (62.5)	85 (75.2)	106 (69.7)	0.000*
• Up to 10 h	56 (19.2)	53 (24.1)	8 (10.0)	**32 (33.3)**	28 (24.8)	**44 (28.9)**	
• > 10 h	1 (0.4)	2 (0.9)	0	**4 (4.2)**	0	**2 (1.4)**	
Work-life conflict							
• Yes	140 (48.1)	**184 (83.6)**	27 (33.8)	57 (59.4)	56 (49.6)	77 (50.7)	0.000*
• No	151 (51.9)	36 (16.4)	53 (66.2)	39 (40.6)	57 (50.4)	75 (49.3)	
Satisfaction with equipment							
• Very unsatisfied	**18 (6.2)**	8 (3.6)	**2 (2.5)**	2 (2.1)	1 (1.8)	1 (0.7)	0.000*
• Unsatisfied	**99 (64.0)**	46 (20.9)	**6 (7.5)**	25 (26.0)	35 (31.0)	44 (28.9)	
• Neutral	59 (20.3)	57 (25.9)	19 (23.8)	19 (19.8)	24 (21.2)	34 (22.4)	
• Satisfied	108 (37.1)	105 (47.7)	47 (58.8)	49 (51.0)	51 (45.1)	65 (42.8)	
• Very satisfied	7 (2.4)	4 (1.8)	6 (7.5)	1 (1.0)	1 (0.9)	8 (5.3)	
Satisfaction with monthly income							
• Very unsatisfied	12 (4.1)	**41 (18.6)**	**1 (1.2)**	2 (2.1)	9 (8.0)	11 (7.2)	0.000*
• Unsatisfied	101 (34.7)	**107 (48.6)**	**11 (13.8)**	23 (24.0)	47 (41.6)	46 (30.3)	
• Neutral	64 (22.0)	45 (20.5)	14 (17.5)	19 (19.8)	17 (15.0)	32 (21.1)	
• Satisfied	112 (38.5)	26 (11.8)	50 (62.5)	51 (53.1)	39 (34.5)	61 (40.1)	
• Very satisfied	2 (0.7))	1 (0.5)	4 (5.0)	1 (1.0)	1 (0.9)	2 (1.3)	
Satisfaction with Supports of friends/ family							
• Very unsatisfied	8 (2.7)	6 (2.7)	3 (3.8)	2 (2.1)	2 (1.8)	3 (2.0)	0.000*
• Unsatisfied	7 (2.4)	15 (6.8)	0	0	0	2 (1.3)	
• Neutral	21 (7.3)	33 (15.0)	3 (3.8)	5 (5.2)	7 (6.2)	16 (10.5)	
• Satisfied	142 (48.8)	119 (54.1)	32 (40.0)	46 (47.9)	59 (52.2)	78 (51.3)	
• Very satisfied	113 (38.8)	**47 (1.4)**	**42 (52.4)**	43 (44.8)	45 (39.8)	53 (34.9)	

Chi-squared test and Fisher exact test were used for statistical analysis of the data. Bolded numbers denote values significantly different from the ones for other regions

CONTENT

Table 3: Karasek's model of stress at work of the participating teachers in various regions in the Republic of Srpska

Karasek's model of stress	Banja Luka X (SD)	Prijedor X (SD)	Doboj X (SD)	Bijeljina X (SD)	East Sarajevo X (SD)	Trebinje X (SD)	p
Job demand	28.31	29.31	26.78	27.55	28.70	28.08	0.001*
	(4.22)	(3.90)	(5.89)	(4.03)	(5.17)	(5.71)	
Skill-discretion	37.13	35.70	39.62	38.19	38.92	37.74	0.000*
	(5.09)	(5.33)	(4.14)	(3.92)	(4.74)	(4.17)	
Decision latitude	3.98	32.04	32.45	32.89	33.49	31.71	0.008*
	(4.08)	(4.85)	(3.43)	(3.90)	(4.21)	(4.38)	
Supervisor support	11.91	10.94	12.21	12.07	12.62	11.81	0.000*
	(2.14)	(2.56)	(2.58)	(2.21)	(2.18)	(2.40)	
Co-workers support	11.79	11.00	12.20	11.57	11.71	11.36	0.000*
	(1.69)	(1.79)	(1.66)	(1.60)	(1.99)	(1.94)	

teachers) worked in both an elementary as well as a high school and they were excluded from analyses that consider the workplace. In the territory of the Prijedor region, teachers more often had a longer work experience, ie, work experience > 20 years (43 %), while in other regions the frequency of teachers with the same work experience was 15-31 %. Overtime work was mostly reported by teachers from the Bijeljina region, 37 % (up to 10 h 33 % and > 10 h 4 %), while in other regions the frequency ranged from 10 % in the Doboj region (only up to 10 hours) to 29 % in the region of Trebinje (up to 10 h 28 % and > 10 h 1 %). The conflict between the roles of work and family life were mostly reported by teachers belonging to the Prijedor region (83.6 %), and least often in Doboj (33.8 %). Dissatisfaction with the equipment at work was mostly expressed by teachers from the territory of the Banja Luka region (70 %) and least often in Doboj (10 %). With the personal monthly income, teachers from the territory of Prijedor showed great dissatisfaction (67 %), while in Doboj was the least (15 %). In the Prijedor region, teachers reported that they were the least satisfied with the support they had from family/friends (56.5 %), while in other regions the same parameter ranged 86 %-92 %. Work-life characteristics are presented in Table 2.

Professional stress

A statistically significant difference among the Republic of Srpska regions in the frequency of job strain among teachers was noticed ($p < 0.05$) and results are presented in Figure 1.

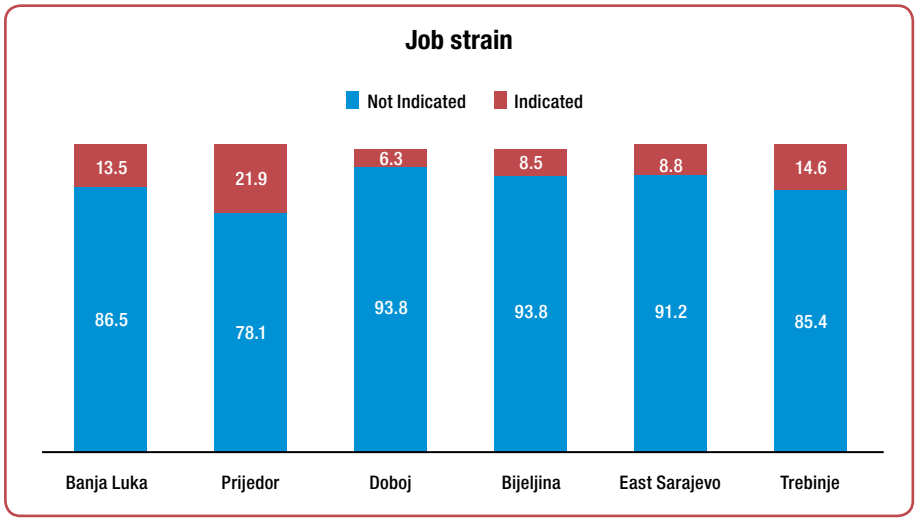

Figure 1: Job strain of the participating teachers in various regions in the Republic of Srpska

Results of Karasek's model of stress at work by regions of the Republic of Srpska are presented in Table 3. A statistically significant difference was found between regions on all scales of Karasek's model of stress at work ($p < 0.05$).

Burnout syndrome at work

A statistically significant difference among the regions of the Republic of Srpska in the dimensions of the burnout syndrome was found ($p < 0.01$). Compared to other regions, a high level of emotional exhaustion was more frequently reported by teachers from the territory of the East Sarajevo and the Prijedor region (8.0 % and 7.7 %). Emotional exhaustion of teachers in the Republic of Srpska regions is presented in Figure 2.

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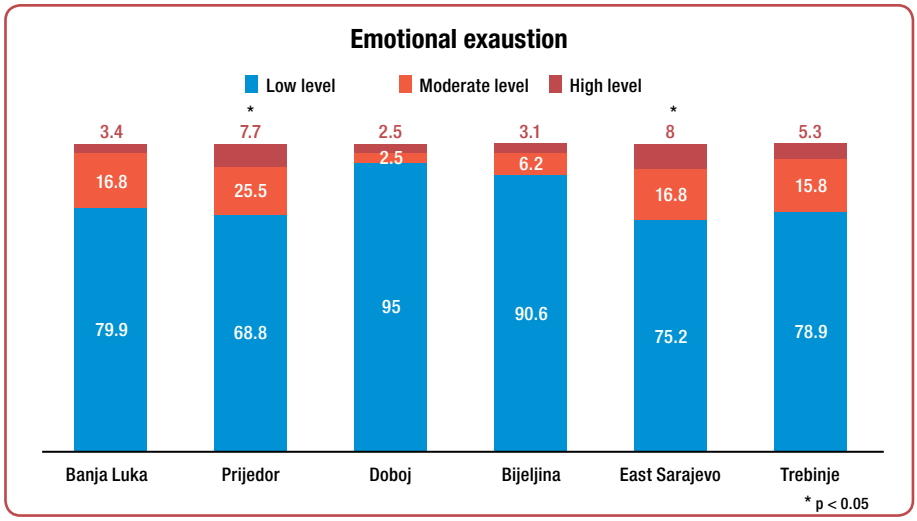

Figure 2: Levels of emotional exhaustion of the participating teachers in various regions in the Republic of Srpska

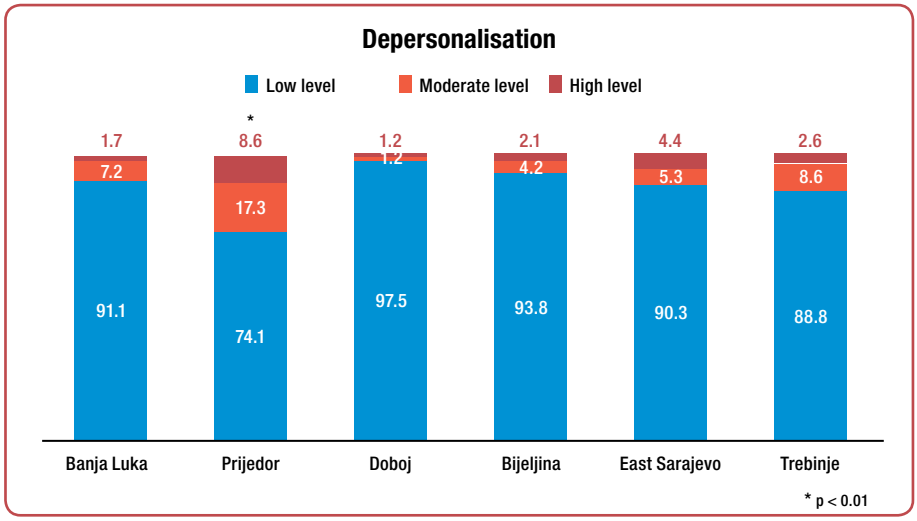

Figure 3: Levels of depersonalisation of the participating teachers in various regions in the Republic of Srpska

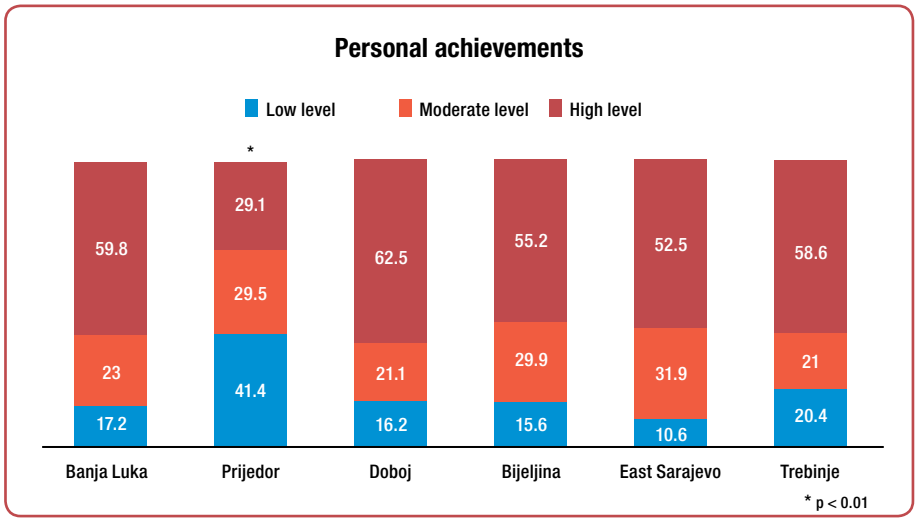

Figure 4: Levels of personal achievements of the participating teachers in various regions in the Republic of Srpska

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Table 4: Burnout syndrome at work of the participating teachers in various regions in the Republic of Srpska

Regions	Emotional exhaustion			Depersonalisation			Personal achievements		
	X (SD)	Med (Min-Max)	p	X (SD)	Med (Min-Max)	p	X (SD)	Med (Min-Max)	p
Banja Luka	9.10 (8.27)	6.00 (0.00-39.00)	0.000*	1.89 (3.11)	0.00 (0.00-21.00)	0.000*	38.05 (8.27)	40.00 (0.00-48.00)	0.000*
Prijedor	13.07 (9.14)	11.00 (0.00-50.00)		4.46 (5.17)	3.00 (0.00-25.00)		32.35 (8.78)	32.00 (3.00-48.00)	
Doboj	5.90 (6.40)	5.00 (0.00-35.00)		0.96 (2.12)	4.52 (0.00-15.00)		38.99 (8.71)	41.00 (12.00-48.00)	
Bijeljina	7.98 (6.93)	6.00 (0.00-34.00)		1.64 (2.72)	0.50 (0.00-14.00)		38.68 (7.11)	39.50 (12.00-48.00)	
East Sarajevo	11.55 (8.83)	9.00 (0.00-37.00)		2.35 (4.06)	1.00 (0.00-21.00)		38.53 (6.46)	39.00 (12.00-48.00)	
Trebinje	10.38 (8.96)	9.00 (0.00-42.00)		2.35 (3.65)	1.00 (0.00-19.00)		37.67 (8.94)	40.00 (4.00-48.00)	

A statistically significant difference was also found in the prevalence of depersonalisation among the Republic of Srpska regions ($p < 0.01$). Depersonalisation of teachers by the Republic of Srpska regions are presented in Figure 3.

A statistically significant difference was found in the scale of personal achievements among teachers from different regions ($p < 0.01$). Personal achievements of teachers by regions of the Republic of Srpska are presented in Figure 4.

A statistically significant difference was also found concerning the scores of the dimensions of the burnout syndrome. The scores of the dimensions of the burnout syndrome at work are presented in Table 4.

Discussion

This is the first study exploring the difference in burnout syndrome at work and occupational stress between teachers in different regions of the Republic of Srpska.

The gender distribution of teachers in the regions was similar. In all regions, the larger fraction consisted of female teachers (Table 1). These results correspond to the data of the Republic of Srpska Institute of Statistics, according to which mostly female teachers work in the Republic of Srpska. [17,18] In research on the teacher population, other authors also noticed this population is mostly made up of female teachers[19-21] and pointed out the reason for this is that teaching is considered to be a female profession.[22, 23]

A statistically significant difference in the age of the teachers among regions has been found. Almost half of the teachers in the region of Prijedor were 46 and older (Table 1). The results of age distribution by regions are similar (except for the Prijedor region) to the data of the Republic of Srpska Institute of Statistics, according to which the majority of teachers are under the age of 44. It is not known why older teachers participated in the research more often in the Prijedor region. A statistically significant difference in the marital status of teachers was found among regions. In the Prijedor region, teachers were more commonly divorced or widowed compared to other regions (Table 1). Also, in the Prijedor region, teachers reported they were least satisfied with the support they have from family/friends. A conflict between the roles of work and family life was most often reported by teachers belonging to the Prijedor region (Table 2), which could explain the significantly more frequent status of divorced people in this region compared to others. These results indicate the need for further research on the quality of life, especially in the Prijedor region, and possible factors that affect the quality of life of this region population.

The analysis by the regions showed a statistically significant difference in the school type among

regions. In the Doboj region, all teachers worked in primary education, while in the region of Bijeljina half of teachers worked in secondary education (Table 2). It is not known why in the Doboj region in the research participated only teachers working in primary schools.

Statistically significant differences between the Republic of Srpska regions were also determined by other examined work and life characteristics (Table 2). Teachers from the region of Prijedor were most dissatisfied with their monthly income (67.2 %), and the least in Doboj (15 %). These results also raise questions for further research on teacher dissatisfaction and factors influencing differences in satisfaction among the regions.

A statistically significant difference regarding the presence of professional stress was found among teachers from different regions. Teachers from the Prijedor region most often reported the presence of job strain (21.9 %), while in other regions the frequency ranged from 6.3 % in Doboj to 14.6 % in Trebinje (Figure 1). By analysing Karasek's model of stress at work, the results showed that teachers from the Prijedor region have the highest score on the scale of demands at work, and the least on the scales of freedom to choose skills and support in the workplace (Table 3). Having in mind the above results, it is necessary to conduct additional researches within the working environment of teachers on the territory of the Prijedor region.

The results of the research show the differences in the frequency of burnout syndrome at work between the regions of the Republic of Srpska. High level of emotional exhaustion was mainly reported by teachers belonging to the region of East Sarajevo and Prijedor (Figure 2). In the research conducted in the Republic of Srpska, concerning the factors that influence the development of burnout syndrome at work among teachers,[24] conflicts of roles and satisfaction with the support of family and friends proved to be one of the important factors. Bearing in mind teachers from the Prijedor region had conflict roles much more often and were more dissatisfied with the support, that can explain the reason for the much more frequent emotional exhaustion in this region. Also, teachers from this region who participated in the research were mostly teachers older than 45 years (Table 1) and had more years of work experience (Table 2) which can also be an explanation for the more frequent manifestation of emotional exhaustion. Other authors[21, 24, 25] also pointed out that older teachers more often report emotional exhaustion, explaining that with age, it is more and more difficult to face student's problems on daily basis. Teachers from the territory of the Prijedor region more often showed a moderate and high level of depersonalization (Figure 3), so as a low level of personal achievement (Figure 4) comparing to teachers who belong to other regions. Having in mind the results of Karasek's model of stress at work for the Prijedor region, where the teachers had a high score of demands at work and low scores on the subscale of autonomy in the workplace, the frequent occurrence of burnout syndrome at work is expected. According to Karasek's model of stress at work, the most vulnerable occupations for the development of burnout syndrome at work are the one where the workers are exposed to high demands on the workplace, and a little possibility of control. As moderators of this demand-control connection are the support employees have from their colleagues and superiors.[15, 26] Earlier, the authors emphasized that various aspects of the work environment at school affect the perception of the stress at work and the development of the burnout syndrome at work, such as administrative (hierarchy, management), and physical (cleanliness, space).[27] Given the above results on the difference between regions, additional research within the regions on living and working characteristics is inevitably needed, as well as the inclusion of other factors (eg, individual).

However, in addition to the region of Prijedor, teachers from the territory of East Sarajevo often had higher levels of emotional exhaustion compared to teachers from other regions, and their sociodemographic and life-work characteristics were similar to those of teachers from other regions, which opens the need for further research in this region to find reasons for these results. It is possible that, in the development of the burnout syndrome in teachers in this region, a significant role is played by individual characteristics, for which previous research has also shown to have a significant contribution.[28]

This is the first research regarding the differences between regions in the Republic of Srpska in the burnout syndrome in teachers. One of the advantages is the use of linguistically adapted, validated, and standardised questionnaires. The disadvantage of the research is primarily the design of the study, which makes it impossible to determine the causal relationship. Another disadvantage is that some factors (role conflict,

CONTENT

self-assessment of satisfaction) were measured based on only one question. One part of the teachers did not fill in the questionnaires, which could have had an impact on the results. In the region of Doboj, the teachers from secondary schools did not participate.

Conclusion

This research has pointed out the presence of burnout syndrome at work in all the Republic of Srpska regions at the beginning of the school year, to the existence of differences between them. An earlier proposal to introduce a screening programme as part of mandatory medical examinations is significant in all regions. Also, due to presented differences, this research indicates the need for additional research of risk factors by regions to form targeted and thus more effective prevention measures.

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Conflict of interest

None.

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CONTENT

Saadat. Scr Med 2021 Mar;52(1):38-41. DOI:10.5937/scriptamed52-30739
ORIGINAL ARTICLE

Evaluation of Associations of *GSTM1/GSTT1* Null Genotypes with the Susceptibility to Age-Related Macular Degeneration, a Meta-Analysis

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Abstract

Background: The relationship between glutathione S-transferase M1 (*GSTM1*) and T1 (*GSTT1*) null genotypes (homozygotes for the null alleles) and the susceptibility to age-related macular degeneration (ARMD) have been reported and revealed inconsistent results. Therefore, the current meta-analysis was carried out.
Methods: Eligible published articles (before December 2020) were found by searching 8 databases. The data was extracted from articles. The heterogeneity across studies was estimated using Q and I^2 statistics and the odds ratios (ORs) and its 95 % confidence intervals (95 % CI) were estimated.
Results: In total, 6 independent studies including 1089 participants (634 controls and 455 patients) were used in the current study. There was no heterogeneity between studies for both polymorphisms. Statistical analysis showed that the null genotypes of the *GSTM1* (OR = 1.18, 95 % CI: 0.91 - 1.53, p = 0.191) and *GSTT1* (OR = 0.84, 95 % CI: 0.60 - 1.18, p = 0.328) loci were not correlated with the susceptibility to ARMD.
Conclusion: The *GSTT1* and *GSTM1* genetic polymorphisms did not associated with the risk of ARMD in Caucasian populations.

Keywords: *GSTM1*; *GSTT1*; Age-related macular degeneration; Meta-analysis.

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Introduction

Age-related macular degeneration (ARMD) is an extensively studied disease as a leading cause of visual disability in the aging population. It has been reported that both genetic and environmental elements have roles in the development of the disease.[1, 2] The heritability of ARMD seems to be about 15-65 %.[1] Retina has the highest oxygen-consuming among human tissues.[3] Based on the epidemiologic, genetic, and molecular pathologic studies it has been hypothesised that oxidative stress may play a key role in the aetiology of ARMD.[2, 4]

The members of glutathione S-transferases (GSTs, EC 2.5.1.18) superfamily belongs functionally to cellular detoxification system and they are classified to some classes, including theta (GSTT) and mu (GSTM) classes. The *GSTT1* (MIM: 600436) and *GSTM1* (MIM: 138350) genes are polymorphic. The null alleles of these loci have been reported in human populations. Several meta-analyses revealed that these polymorphisms were associated with numerous human complex diseases, such as cancer.[5-9] It should be noted that the risks of cataract and glaucoma are associated with *GSTM1/GSTT1* polymorphisms.[10-12]

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CONTENT

The relationship between the *GSTM1/GSTT1* null genotypes (homozygotes for the null alleles) and the risk of ARMD have been reported and revealed inconsistent results.[13-17] To evaluate the relationship between these genetic variations and the susceptibility to ARMD, the current meta-analysis was carried out.

Methods

Relevant published articles (before December 2020) were found by searching several databases, including PubMed, Scopus, Index Copernicus, DOAJ, Academic Journals Databases, SID, KoreaMed, and Google scholar. The following search terms were used: *GSTT1, GSTM1,* null genotype, age-related macular degeneration. Only articles published in English were included in the study. In addition, the bibliographies of the retrieved studies were screened to identify relevant articles.

The eligible studies had raw data on genotype distributions in both patient and control groups. The exclusion criteria were related to reviews, editorials, abstracts, comments and studies with same or overlapping data. The application of the above-mentioned criteria yielded five reports.[13-17] Study of Hunter et al[17] had been reported two case-control groups, therefore, considered as two studies. The following data were extracted: author's name, publication year, country, ethnicity of the participants and the frequencies of the genotypes for each polymorphism in ARMD patients and control subjects.

The heterogeneity across studies was estimated using Cochran's Q and I^2 statistics. If there was no heterogeneity between the studies (I^2 < 50 % and p > 0.10 for Q statistics), the fixed effects model[18] was used for estimation of the odds ratios (ORs) and its 95 % confidence intervals (95 % CI).

Results

Tables 1 and 2 summarised the extracted data from 6 studies which were including in the current study. The studies were published between 2006 and 2016. In total, 6 eligible independent studies with 1,089 participants (634 controls and 455 patients) were used. All of the studies were conducted in Caucasian populations.

Table 1: Characteristics of the studies included in the meta-analysis

Frist author	Total	Country	Ethnicity	Control group			Patient group		
				n	Age*	MP**	n	Age	MP
Oz	2006	Turkey	Caucasian	159	62.0 ± 8.6	0.566	35	63.0 ± 8.1	0.457
Guven	2011	Turkey	Caucasian	198	73.0 ± 10.0	0.450	120	75.0 ± 8.0	0.440
Liu	2011	USA	Caucasian	103	69.6 ± 9.8	0.408	131	79.4 ± 8.3	0.519
Othman	2012	Iran	Caucasian	112	63.2 ± 9.4	-***	112	69.5 ± 8.9	0.607
Hunter, 1	2016	USA	Caucasian	50	77.8 ± 8.4	0.460	37	80.9 ± 6.5	0.420
Hunter, 2	2016	USA	Caucasian	50	77.3 ± 10.5	0.438	48	79.4 ± 8.0	0.520

**mean ± SD of age (Years); **Male proportion, ***Sex-matched controls*

Table 2: Genotypes of the studied polymorphisms in age-related macular degeneration patients and controls

Frist author/ Polymorphisms	Control group		Patient group	
GSTM1 Genotypes	Positive genotype	Null genotype	Positive genotype	Null genotype
Oz	18	17	96	63
Guven	102	96	45	75
Liu	33	47	40	48
Othman	71	41	72	40
Hunter, 1	28	22	22	15
Hunter, 2	36	14	32	16
GSTT1 Genotypes	Positive genotype	Null genotype	Positive genotype	Null genotype
Oz	25	10	118	41
Guven	157	41	100	20
Liu	63	17	71	15
Othman	81	31	85	27

CONTENT

The associations of the null genotypes of the *GSTM1* (Figure 1A) and *GSTT1* (Figure 1B) with the risk of ARMD were investigated. There was no heterogeneity across studies for any of the polymorphisms (For *GSTM1*: Q statistics = 5.84, df = 5, p = 0.322, I^2 = 14.3 %; For *GSTT1*: Q statistics = 0.70, df = 3, p = 0.873, I^2 = 0.00). Statistical analysis showed that *GSTM1* (OR = 1.18, 95 % CI: 0.91 - 1.53, p = 0.191) and *GSTT1* (OR = 0.84, 95 % CI: 0.60 - 1.18, p = 0.328) polymorphisms were not associated with the susceptibility to ARMD.

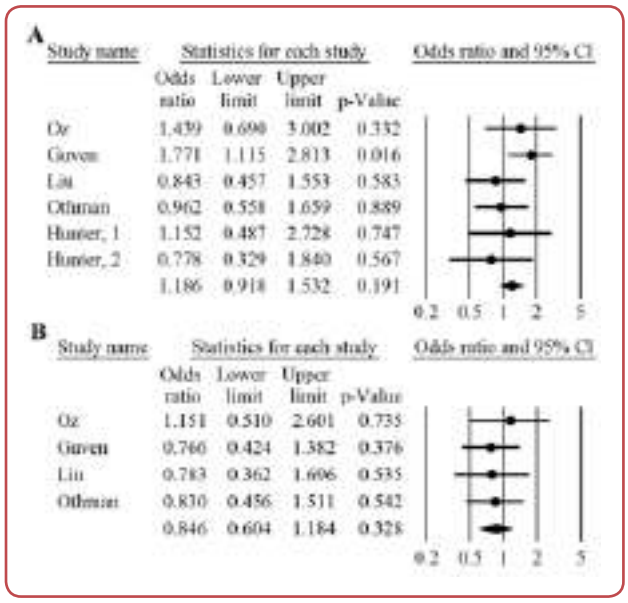

Figure 1: Forest plots of the relationship between the null genotypes versus active genotypes of the GSTM1 (A) and GSTT1 (B) and the susceptibility to age-related macular degeneration

For evaluation of the publication bias, the funnel plot and Egger's test were used. There was no evidence for publication bias (p > 0.320, data not shown). The stability of the findings was evaluated by removing of individual studies sequentially. Sensitivity tests indicated that the present findings were stable.

Discussion

Numerous studies indicated that ARMD has many risk factors. High oxidative stress level is one of the most important risk factors for development of ARMD.[23] Many reactive oxygen species (ROS) such as superoxide, hydrogen peroxide etc, are generated in the retina during transformation of light into vision.[23] The GST superfamily plays a key roles in defence against ROS and oxidative stress. Considering that the null genotypes of *GSTT1* and *GSTM1* were associated with several multifactorial human complex diseases.[5-12]

There were studies investigated the relationship between these polymorphisms and the risk of ARMD,[13-17] the results were inconsistent. Considering that association studies usually were done on a relatively small samples size, in order to increase the sample size and aid the generalisation of the results to larger populations, the present meta-analysis was carried out. To the best of author's knowledge, the current study is the first meta-analysis undertaken to investigate the relationship between the susceptibility to ARMD and null-genotypes of *GSTT1/GSTM1* loci. Overall, the present findings indicated that there was no association between the study null genotypes and the risk of ARMD. Due to some limitations of this study, which are described below, the present results should be interpreted with caution.

Study Limitations

A number of limitations of the current study should be acknowledged. All of the studies used for the present meta-analysis were conducted in Caucasian participants. Therefore, there was no data from Asian and African populations. Considering that the risk of other multifactorial complex traits (such as diabetes mellitus and gastric cancer) with polymorphisms (such as the *GSTT1* and *GSTM1*) were not similar between the ethnic groups[10, 11, 19-22] further association studies from African and Asian populations are required. Considering that environmental factors in the pathogenesis of the ARMD is involved[1, 2] and reports which were included in the analysis did not report the environmental factors, further studies are necessary to examine the interaction between genes and environments, as well as the combinations of polymorphisms.

Conclusion

The current study suggests that the *GSTM1/GSTT1* null genotypes are not significantly associated with the susceptibility to age-related macular degeneration.

CONTENT

Acknowledgements

None.

Conflict of interest

None.

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CONTENT

Svolacchia & Svolacchia. Scr Med 2021 Mar;52(1):42-8. DOI:10.5937/scriptamed52-30316
ORIGINAL ARTICLE

Adipose Tissue Micrograft in a Scaffold of Plasma-Gel Combined With Platelet-Derived Growth Factors in Dermal Wrinkle Regeneration

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Abstract

Background: The dermal aging process and the formation of deep wrinkles are a biological involution that also involves the regeneration system of cells immersed in the extracellular matrix and the papillary dermis. The progressive loss of niches of adult stem cells (MSCs) is more evident after the first third of life; it increases the phenotypic expression and the characteristics of the tissue senescence process. The purpose of this study was to clinically demonstrate that in viable micrograft there may be an improvement of deep wrinkles and surrounding tissues.
Methods: This study involved 11 female patients who underwent the correction of deep dermal wrinkles through a suspension containing 0.8 mL of viable micrografts in a 5 mL plasma gel scaffold, obtained from the centrifugation of a 20 cc venous sample peripheral blood, gelled by heat in a dry steriliser and the buffy coat coming from the same venous sample, in order verify overtime the improvement of the interested anatomical area. Individual signs of wrinkles and the degree of correction obtained for each treatment and each area were objectively evaluated by using a 10-0 visual analog scale (VAS), Modified Vancouver scale and Berardesca's scale.
Results: With this technique excellent results were obtained. In fact, wrinkles were improved, as well as surrounding tissues, even after 60 days, as shown by the Berardesca's, VAS and Modified Vancouver scales.
Conclusion: This retrospective clinical evaluation allowed us to consider the excellent clinical results obtained with this method for the treatment of deep wrinkles and surrounding tissues, through a suspension of progenitors with MSCs derived from adipose tissue (ADSCa) in a not inflammatory plasma gel scaffold combined with buffy coat.

Keywords: Adult Tissue Progenitor; Cluster of Differentiation (CD); Side Population; Buffy coat; PRP, STBA.

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Introduction

Unlike embryonic stem cells (ESCs), adult stem cells (MSCs) have a lower self-maintaining capacity due to lower levels of telomerase activity.[1] MSCs in adult tissues can be able to avoid the differentiation process which is typical for other cells and to colonise appropriate niches with the function of maintaining their potential in adult life and to limit their differentiation processes.[1] The loss of dermis tone, with the formation of wrinkles during the aging process, are favored by the worn out of niches of MSCs; ROS and UV of exogenous and endogenous origins are the primary causes of these biological events.[2] Over time there will be a reduction of natural fibroblasts, with an inver-

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CONTENT

sion of the balance between type III-collagen and type I-collagen, with a decrease in the production of elastin and extracellular matrix (ECM).[3] Over time, the dermis will be colonised by senescent fibroblasts with poor secretory capacity, which will be directed towards type I or fibrotic collagen.[4]

These pathophysiological events will be implicated in the loss of elasticity of the tissue and in the formation of wrinkles and deep furrows on the face. The positive effects on the use of MSCs in pathologies that involve a dermal regenerative stimulus have already been demonstrated.[5] It has also been shown that, according to Tonnard 2013, a disaggregated adipose tissue contains viable cells with characteristics of adult stemness; it has been demonstrated that cellular fragments and fibrous shoots contained in a disaggregated adipose according to Tonnard 2013 are potentially inflammatory and that the filtration of disaggregate adipose tissue at 50 microns allows to maintain a vital and numerically high the Side Population (SP).[6-8] SP in flow cytometry is a sub-population of cells that is distinct from the main population based on the markers employed, cluster of differentiation (CD).

Many recent studies also show that the platelet-rich plasma (PRP) that comes from a centrifuged venous sampling, produces positive effects when it is injected into the dermis, despite the persistence of these effects over time,[9] in addition, the portion of tissue used for the treatments appears to be in the buffy coat.[10] Therefore, a portion of autologous plasma for PRP treatments is not used.[11] This study involved the use of a viable micrograft of adipose tissue containing progenitors in suspension, with markers of adult stem cell on the surface (CD) from 2 mL of subcutaneous fat, which was taken and disrupted as indicated by Tonnard 2013, then microfiltered through a 50-micron filter combined with a plasma gel scaffold (STBA), suitable for use as an autologous biological tissue support since it is not inflammatory.[5-7, 13-15]

It is also envisaged the union of the buffy coat with the suspension obtained immediately before, order allow the physiological activation of progenitors through the surface CDs.[7] Given the ability of MSCs derived from adipose tissue (ADSCa) to induce physiological neocollagenogenesis, our intention was to clinically verify whether the viable micrograft, mixed with a non-inflammatory STBA scaffold to which it was added immediately prior to injection into the deep dermis the buffy coat of PRP, could allow a clinical improvement of wrinkles and surrounding tissues, by considering the mechanical protection capacity possessed by a support of autologous gel against the effects of ROS and UV and the properties of the buffy coat.[10, 16, 17]

An improvement of the physiological neo-collagenogenesis would be activated through the reaction capacity of the CD 44 present on progenitors, triggered by the PDGF contained in alpha granules of the platelets suspended in the buffy coat, by subsequently improving tissues through the increase of production of elastin, collagen and other components of the extra cytoplasmic matrix.[9, 10, 18, 19] It is also hypothesized that the expression on the progenitors of other markers such as CD 73, 90 and 105 would affect neo-vasculogenesis and consequently improve the papillary dermis.[7, 20] Hypothesis of improving vitality and longer functionality of progenitors, once injected into dermis through this new technique, was linked to the re-establishment of the physiological niches of viable cells, as protected from ROS by an autologous anti-inflammatory biological tissue support (STBA), and physiologically activated by the buffy coat of venous centrifugate.[17] Therefore, an increase in the number of active and protected progenitors present in dermis would have allowed an increase in biological parameters with a consequent increase in cell turnover with a reduction in deep wrinkles observable in a follow-up.

The study of this method began in 2017, and it allowed us to clinically evaluate the effects of an increase in the production of elastin, collagen and other components of the extra cytoplasmic matrix, evaluated with the Berardesca's and VAS scales. The Modified Vancouver Scale was also used to evaluate the quality of the tissues adjacent to the imperfections.

Methods

This study involved 11 female patients who underwent the correction of deep dermal wrinkles through a suspension containing 0.8 mL of viable micrografts in a 5 mL plasma gel scaffold, obtained from the centrifugation of a 20 mL venous sample

peripheral blood, gelled by heat in a dry steriliser and the buffy coat coming from the same venous sample, in order verify overtime the improvement of the interested anatomical area. All 11 volunteer patients (between 57 and 70 years of age) attended the clinic between 2017 and 2020. To participate in the study, the only obligatory criteria was the presence of deep wrinkles and severe signs of skin aging. The study was performed by following standards of the local ethics committee and in accordance with the Declaration of Helsinki (2000). All patients were female and did not have any specific dermal pathologies or other systemic pathologies not pharmacologically controlled.

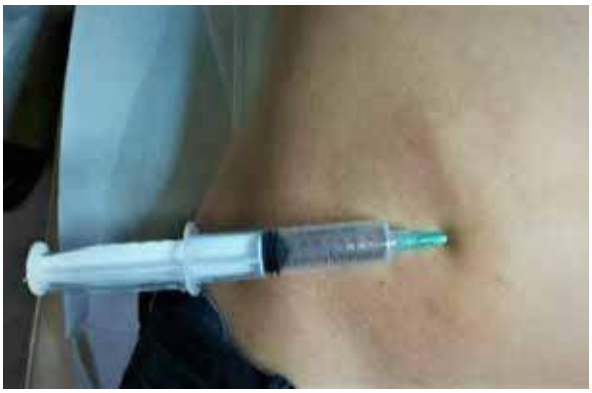

Figure 1: Extraction of adipose tissue with a syringe and a 16 G needle

Figure 2: Microfiltration at 50 microns of adipose tissue after the Tonnard's 2013 disruption

For the extraction of the adipose tissue, a 10 mL syringe with luer lock connection combined with a 16 G needle (Figure 1) was used, while for filtration a 50-micron filter was used in order obtain vital progenitors in the suspension, by excluding fibrous shoots and cellular fragments.[8] After performing a local anesthesia with Klein solution on the tissue-removal area, procedure involved the extraction of 3 mL of adipose tissue. The extraction with a needle allowed a survival of the progenitors,[7] as well as an overlap in the quantity and quality of viable cells extracted versus the extraction with a multi-hole cannula, but with less trauma for the cells.[21, 22] After the extraction of the adipose tissue, the suspension was settled for 10 minutes to eliminate anesthesia fluids and, finally, 2 mL of fat was obtained.

Figure 3: Fibrous shoots and cellular fragments by filter retained

At this point, the tissue was disrupted as indicated by Tonnard 2013, and it was filtered at 50 microns (Figure 2) to better preserve the SP, from which 0.8 mL of final suspension (Figure 2) was obtained.[8] During the disintegration and filtration phase there is a loss of vital elements, but their therapeutic potential is higher because the fibrous shoots and cellular fragments are eliminated (Figure 3).[2, 21, 22] In fact, fibrous shoots and cellular fragments are responsible for the activation of inflammatory process in dermis through the Toll-like receptors expressed on dendridic cells and macrophages which increase in number during aging in dermis and it is incompatible with our regenerative procedure.[7] We obtained the 5 mL STBA from a centrifuged peripheral venous blood sample (Figure 4) from which the buffy coat was excluded and gelled by denaturation through heat in a dry steriliser at 70 ^{0}C for 10 minutes (Figure 5), and it was left to cool. The scaffold was joined to the 0.8 mL vital suspension through a three-way tap with gentle movement 5-7 times (Figure 6).

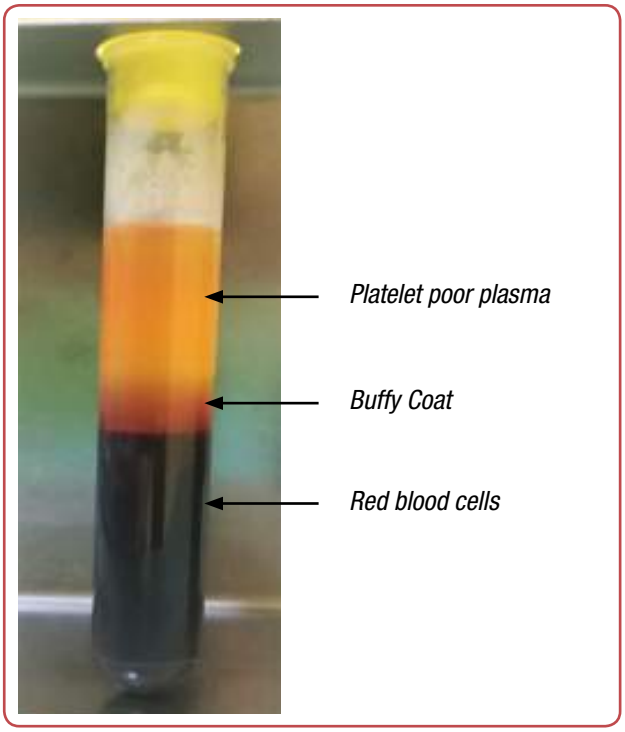

Figure 4: Peripheral blood centrifugation. The platelet poor plasma was gelled and the buffy coat was combined immediately before injecting the wrinkles and surrounding tissues

Figure 5: STBA, Autologous Biological Tissue Support. Plasma gel obtained in a dry oven at 70 °C for 10 minutes and left to cool

Figure 6: Mixing of autologous tissues through a three-way tap

The buffy coat was joined with the same three-way tap and with the same movements order to obtain a homogeneous gel immediately before injecting it into dermis (Figure 7). The suspension was injected into the deep dermis through a 25 G needle and a microcannula. The whole procedure took 30 minutes for each patient.

Figure 7: Correction of deep wrinkles and surrounding tissues

Subjects evaluated their satisfaction or dissatisfaction by giving scores on the appearance of firmness and wrinkles, by using a scale of 0 - 4 for each criteria (0 = unsatisfactory; 4 = satisfactory), as suggested by Berardesca et al, after the first and second month and a six-month follow-up.[12]

Table 1: Vancouver Modified Scale

Baseline				Follow-up 60 Days
Vascularity:	Red	Purple	Grey	Normal
Pigmentation:	Hyperpigmentation	Hypopigmentation		Normal
Pliability:	Firm	Ropes		Normal/Supple

Individual signs of wrinkles and the degree of correction obtained for each treatment and each area were objectively evaluated by using a 10-0 visual analog scale (VAS) with separate scores for each site. Surrounding tissues were also evaluated with the Modified Vancouver scale (Table 1).

Results

Subjects evaluated their satisfaction or dissatisfaction by giving scores on the appearance of firmness and wrinkles, by using a Berardesca's scale. Results are reported in Figure 10.

With the VAS scale the results are shown in a Figure 11 (10 = no correction; 5 = satisfactory correction; 0 = complete correction). Figure 8 and figure 9, represents photographic examples of results.

With this technique we were able to obtain excellent results. In fact, wrinkles were improved even

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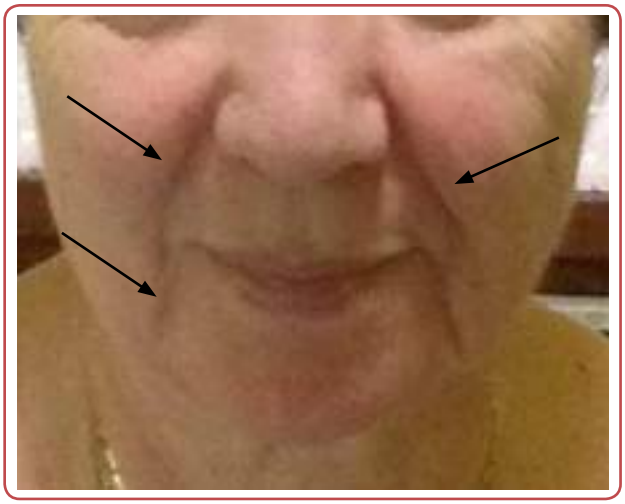

Figure 8: *Before treatment*

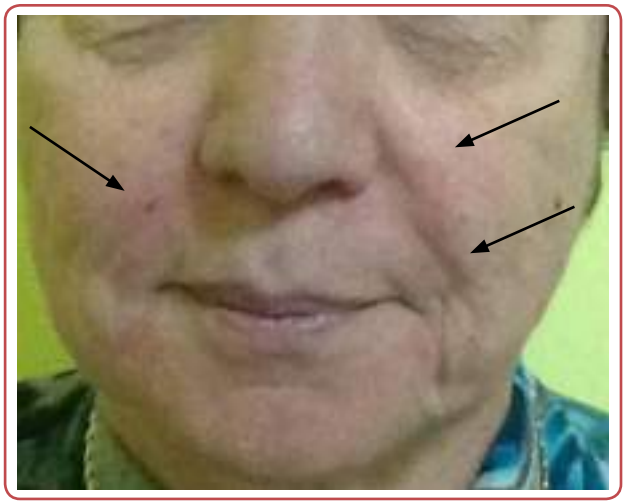

Figure 9: *After treatment*

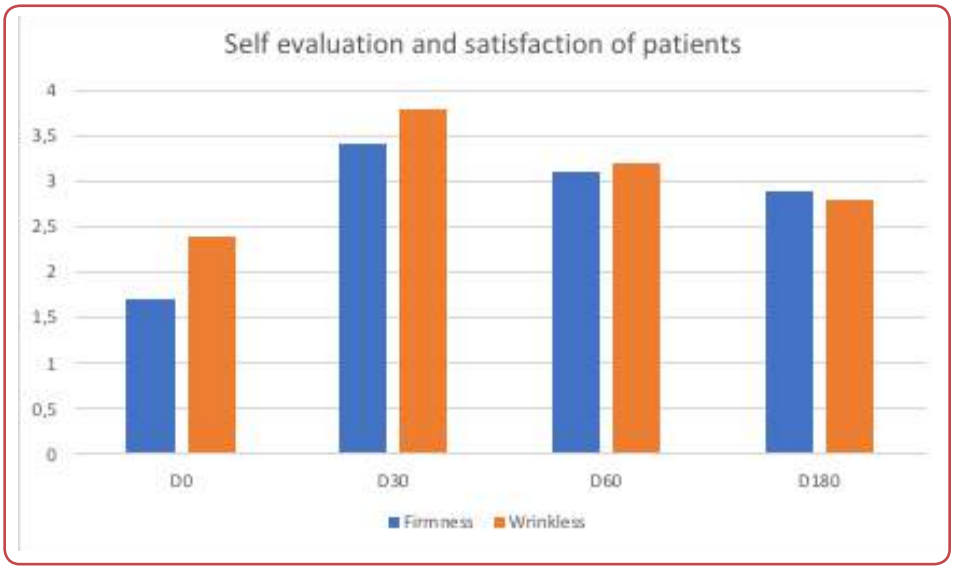

Figure 10: *Self-evaluation and patient satisfaction based on Berardesca's Scale*
D0: after treatments, D30: after 30 days, D60: after 60 days, D180: after 180 days

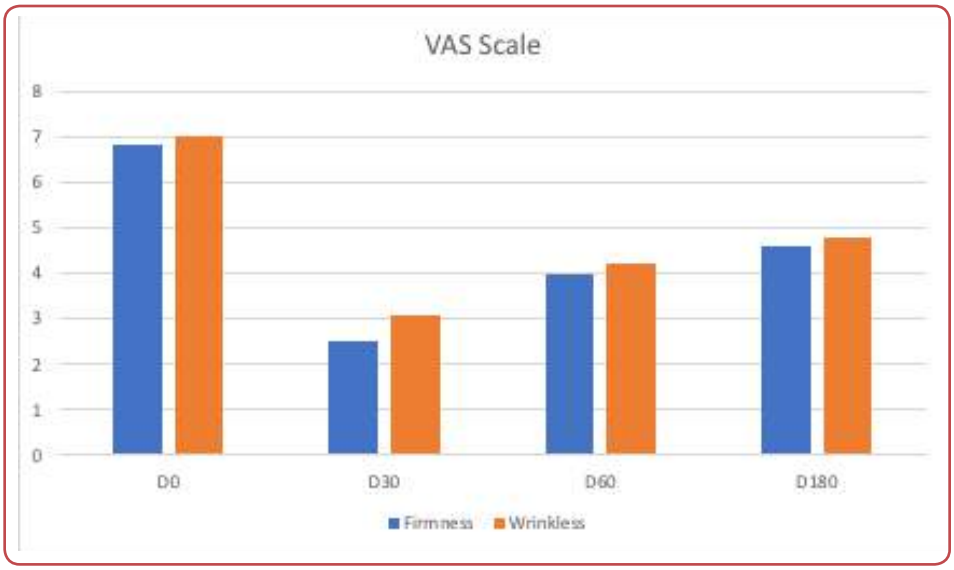

Figure 11: *Evaluation based on visual analog scale (VAS)*
D0: after treatments, D30: after 30 days, D60: after 60 days, D180: after 180 days

CONTENT

after 60 days, as shown by VAS scale after the first and second month and Berardesca's scale after a six-month follow-up.

Discussion

Aim of this study was to clinically demonstrate that a viable tissue micrograft joined to a non-inflammatory STBA, to which the buffy coat was added immediately prior to dermal injection, could be used in regenerative medicine and for the correction of deep wrinkles. We had the idea that the union of the autologous materials, prepared with this technique, could allow the activation the Progenitors with MSCs characteristics through growth factors and buffy coat, by allowing a more regulated and lasting action due to the ability of the protective plasma gel, and an induction to a more regular plasticity through the activation of CDs 44, 73, 90 and 105.

The possibility of exploiting the ability to bind growth factors to CD 44 receptors order to normalize the extra cytoplasmic matrix through a physiological neocollagenogenesis and the CD 73, CD 90 and CD 105 for the improvement of the vascularisation of the papillary dermis induced to enroll a sufficient number of patients and to study them for a suitable time, to be able to express a retrospective clinical evaluation in vivo.[8, 23]

This procedure lasted for 30 minutes for each patient. All patients were satisfied with the treatment. The physical examination that the patients underwent during the follow-up was in line with their self-assessment.

There are no similar therapeutic approaches in the literature that combine the heat-gelled plasma used as a medium with a protective agent for the viable micrografts identified in the progenitors with the characteristics of MSCs and activated with growth factors joined immediately before infiltration to activate their tissue plasticity. With this technique we were able to obtain excellent results.

Conclusion

This prospective clinical evaluation allowed us to ascertain excellent results, that have been obtained with our method for the treatment of deep wrinkles and dermal surrounding tissues through a suspension of Progenitors with ADSCa in a not inflammatory STBA combined with buffy coat. This phenomenon can be biologically explained with a better regulation of the Progenitor Plasticity phenomenon through the signaling pathways of CD 44 of CD 73, CD 90 and CD 105. Furthermore, the STBA could physiologically mimic the formation of natural niches in which the adult mesenchymal stem cells are included, by providing them with a protective action and a longer stay at the injection site with beneficial effects on the regulation of ECM, neocollagenogenesis and neovasculogenesis. The illustrated method opens new therapeutic solutions in the regenerative correction of deep wrinkles and surrounding tissues with autologous materials. Moreover, this method can be performed by the physician very quickly and by providing extreme satisfaction to patients, by avoiding the inflammatory component of a nanofat consisting of fibrous shoots and cells fragments.

Acknowledgements

None.

Conflict of interest

None.

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CONTENT

Maksimović et al. Scr Med 2021 Mar;52(1):49-58. DOI:10.5937/scriptamed52-31191
ORIGINAL ARTICLE

Onset Rate and Intensity of Signs of Organophosphate Poisoning Related to Paraoxon Dose and Survival in Rats

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Abstract

Introduction: Oganophosphorus compounds (OP) bind to acetylcholinesterase (AChE) and inactivate it. In the synaptic cleft, undestroyed and accumulated acetylcholine produce the acute cholinergic effects. The aim of this study was to determine the frequency, speed of onset and intensity of certain signs of paraoxon poisoning depending on dose and outcome of poisoning.
Methods: The study was conducted in adult Wistar rats. The median lethal dose (LD_{50}) of paraoxon as well as protective ratio (PR) of atropine (10 mg/kg intramuscularly) was determined. Clinical signs of poisoning were observed: fasciculations, tremor, seizures, ataxia, piloerection, lacrimation, exophthalmos, bizzare/stereotypic behaviour and dyspnoea. The time from paraoxon injection to the first appearance of the sign of poisoning was recorded as well as the intensity of poisoning with evaluation at 10 time intervals throughout the 4 h observational period.
Results: The LD_{50} of paraoxon was 0.33 mg/kg (subcutaneously) and PR of atropine was 2.73. Dose-dependent, piloerection occurred more often ($p = 0.009$) and at higher intensity ($p = 0.016$) at higher doses. Fasciculations, tremor, seizures and ataxia occurred significantly earlier at higher doses of paraoxon ($p = 0.015$, 0.002, 0.021 and 0.016, respectively), as well as the intensity of seizure, tremor and fasciculation. Piloerection ($p = 0.002$) and seizures occurred more frequently ($p = 0.009$) in non-survivors. Fasciculations, tremor, seizures and ataxia occurred significantly earlier and at higher intensity in non-survivors ($p < 0.001$, for all parameters), as well as dyspnoea ($p = 0.009$ and $p = 0.048$). In atropine-protected rats, nicotinic effects persevered, so they were the prognostic parameter of the severity of the poisoning.
Conclusion: Seizures and fasciculations followed by tremor were strong prognostic parameters of the probability of lethal outcome of paraoxon poisoning. Also, the mentioned poisoning signs were with their intensity and speed of occurrence in a clear positive correlation with the administered dose of paraoxon. Even at high doses of paraoxon, atropine blocked the muscarinic (but not nicotinic) effects and somewhat mitigated the CNS toxic effects.

Keywords: Organophosphate; Acetylcholinesterase inhibitor; Paraoxon; Insecticide; Poisoning; Atropine.

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Introduction

In the body, organophosphosphorus compound (OPC) binds to the serine group of the enzyme acetylcholinesterase (AChE, EC 3.1.1.7), phosphorylates it and creates a stable covalent bond

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CONTENT

with it.[1] By binding to it, AChE becomes inactive and cannot perform its function, which is the breakdown of acetylcholine in the synaptic cleft.[2] Accumulated acetylcholine (ACh) stimulates various postsynaptic cholinergic receptors and exhibits its acute cholinergic effects: muscarinic (bronchorrhoea, bronchoconstriction, bradycardia, hypotension, miosis, hypersalivation, lacrimation, nausea, vomiting, increased motility of the bowels and of the bladder), nicotinic (mydriasis, tachycardia, hypertension, fibrillation, fasciculation and necrosis of skeletal muscles) and central (tremor, convulsions, motor incoordination, respiratory depression and coma).[3] Besides acute cholinergic crisis, OP poisoning can manifest itself as intermediate syndrome, organophosphate-induced delayed neuropathy (OPIDN) and chronic organophosphate-induced neuropsychiatric disorder due to long-term low-level exposure.[4]

The OP-AChE bond does not spontaneously dissociate (hydrolyses) in the body, which is why OPs are called irreversible AChE inhibitors. The bond can be separated using AChE reactivators, so-called oximes, which are causal antidotes in OP poisonings.[5] Unfortunately, the diversity of OPs makes oximes insufficiently universal and effective antidotes.[6] An additional limitation of its efficacy is the process of dealkylation (so-called "ageing") through which the OP-AChE bond passes, after which it is not possible to separate OP and AChE.[7] Ageing in some OPCs like the nerve agent soman is measured in minutes, while in organophosphorus insecticide (OPI) it is mostly measured in days.[8]

Although oximes are the only causal antidotes, their insufficient efficacy, especially in clinical practice, makes anticholinergic drugs still the main antidotes in OP poisoning. The most commonly used and most available anticholinergic drug is atropine, which blocks the muscarinic effects of OP poisoning, but not the nicotinic ones.[9] The contribution to the protective effect of atropine is its lipophilicity, ie the ability to penetrate the CNS. There is an assumption whether more lipophilic anticholinergics would have better protection against OP poisoning than atropine. However, there are no clinical studies to corroborate this notion.

In addition to atropine and oximes (pralidoxime and obidoxime), anticonvulsants, primarily diazepam and midazolam, are also used in the treatment of OP poisoning. Anticonvulsants are important, especially when the fact that the fatal outcome in OP poisoning most often occurs due to seizures and respiratory failure is taken into account.[10, 11]

Although most developed countries have banned the use of the most toxic pesticides, OPI poisonings, especially the intentional ones, are still common, especially in undeveloped and developing countries.[12, 13] Unfortunately, due to the high toxicity of OPCs, they continue to be abused for terrorist purposes and as nerve agents (tabun, sarin, soman and VX).[14] Paraoxon (diethyl (4-nitrophenyl) phosphate) is the active metabolite of the OPI parathion.[15] Due to its toxicity and chemical characteristics, it serves as a good experimental tool in the field of research of toxicity of nerve agents.

The aim of the study was to determine whether increasing doses of paraoxon affect the rate and intensity of signs of poisoning with an AChE inhibitor. The aim was also to determine whether the speed of onset and intensity of the appearance of certain signs of poisoning can be a prognostic sign of the fatal outcome of poisoning. In addition, the goal was to determine how clinical signs are manifested at high (> LD_{100}) doses of paraoxon in the presence of an antidote (atropine).

Methods

Animals

The study was conducted in adult male and female Wistar rats, weighing 200-240 g, purchased from Faculty of Natural Sciences and Mathematics, University of Banja Luka, the Republic of Srpska. The animals had access to food and water *ad libitum*. The room temperature was maintained at 20-22 ^{0}C, with a 12 h light and dark cycle. The study was approved by Ethics Committee for the Protection and Welfare of Experimental Animals in Biomedical Research, Faculty of Medicine, University of Banja Luka (Decision No 18/1/20). During the entire experiment, the "Guiding principles in the care of and use of laboratory animals" have been observed.

Chemicals

Paraoxon was purchased from Sigma Aldrich, St Louis, MO, USA. Application volume of the chemical was 1 mL/kg. Paraoxon was injected subcutaneously (sc) into the abdominal region, while atro-

pine was administered intramuscularly (im) into the right thigh. Stock solution of paraoxon (100 mg/mL) was dissolved in isopropyl alcohol. Final dilution for sc administration was made from saline (0.9 % NaCl) before injection. Atropine sulphate monohydrate was dissolved in saline up to the concentration of 10 mg/mL

Study design

In these experiments, lethality of paraoxon and an antidote potential of atropine was based on the 24 h survival.

a) Paraoxon and saline

Increasing doses of paraoxon were administered sc. Each group consisted of 6 rats and doses were determined by the "up and down" method (doses were: 0.2, 0.3, 0.35, 0.4 mg/kg). One minute after paraoxon injection, rats were injected with 1 mL/kg saline im. Based on the 24 h lethality, median lethal dose (LD_{50}) was calculated.

b) Paraoxon and atropine

Increasing doses of paraoxon were administered sc (doses were: 0.6, 0.9, 1.2 mg/kg). Each group consisted of 6 rats and doses were determined by the "up and down" method. One minute after the injection of paraoxon, the rats were injected with 10 mg/kg of atropine im. The outcome was protective ratio (PR) ie, ratio of LD_{50} in protected and in unprotected rats.

c) Clinical signs of poisoning

The following clinical signs of poisoning were observed: fasciculations, tremor, seizures, ataxia, piloerection, lacrimation, exophthalmos, bizzare/stereotypic behaviour, dyspnoea. The time from paraoxon injection to the appearance of the sign of poisoning was recorded. The intensity of poisoning was recorded as: 0 - absent, 1 - mild / moderate, 2 - severe. Evaluation was performed in the 5th, 10th, 15th, 30th, 60th, 90th, 120th, 150th, 180th, 210th, and 240th minute. To analyse the intensity of symptoms throughout the observed period (4 h), ToxScore was introduced, as a parameter that summed the intensity of each sign in all time intervals. TotToxScore was a measure that represented the sum of all ToxScore for one animal.

Statistics

The LD_{50} values were computed by means of the Pharm/PCS statistical software, according to the Litchfield and Wilcoxon (1949).[16] IBM SPSS 21.0 software was used for other statistical procedures. After the normality of data distribution was analysed (Kolmogorov–Smirnov test), the appropriate parametric / nonparametric test was used (Student t-test / Man-Whitney U test, One Way ANOVA / Kruskal-Wallis test) as well as Chi-Squared test for categorical data.

Results

Paraoxon

a) Dose

The LD_{50} of paraoxon was 0.33 mg/kg sc (95 % CI: 0.31 - 0.36). In all rats, death occurred in the first hour of poisoning (mean ± SD: 19.19 ± 6.19, 95 % CI: 16.37 - 22.01).

Fasciculations occurred in 85.71 % of all rats, piloerection in 50.00 %, exophthalmos in 95.23 %, lacrimation in 54.76 %, tremor in 97.62 %, seizures in 83.33 %, ataxia in 66.67 %, stereotypic behaviour in 69.05 % and dyspnoea in 26.19 % rats.

The frequency of symptoms at different doses of paraoxon was not significant, except in the case of piloerection, where it occurred after doses of paraoxon 0.2, 0.3, 0.35 and 0.4 mg/kg in 0 %, 33.33 %, 66.67 % and 75.00 %, respectively (χ^2 = 11,667, p = 0.009). The onset rate of poisoning clinical signs at different doses of paraoxon is shown in Table 1. Bizzare-stereotypic behaviour, although frequent (69.05 % of all animals), was not significantly different in either the intensity or the rate of sign on any parameter (data not shown).

Fasciculation, tremor, seizures, and ataxia occurred significantly earlier at higher doses of paraoxon. The intensity of signs measured 15 min and 4 h after paraoxon application relative to the dose applied is shown in Table 2. The time-point of 15th minute was taken as the time period when most of the signs of poisoning were manifested, yet most of the rats were still alive, and the time period of 4th hour was taken as the end of the observation period.

In the presented time intervals (15 min and 4 h after paraoxon), the intensity of seizures, tremor and fasciculations increased significantly with increasing doses. Piloerection was significantly

CONTENT

Table 1: Onset rate of clinical signs of poisoning at different doses of paraoxon (minutes after application of paraoxon)

Sign (ToO)	Paraoxon dose (mg/kg sc) 0.2	0.3	0.35	0.4	p value
Fasciculations	35.67 ± 17.95	34.25 ± 22.17	20.83 ± 1.84	12.70 ± 5.08	0.015*
Tremor	24.60 ± 7.92	19.33 ± 14.18	19.67 ± 31.29	7.67 ± 1.83	0.002*
Seizures	35.25 ± 20.81	24.78 ± 25.67	18.10 ± 18.73	10.42 ± 3.23	0.021*
Ataxia	47.60 ± 28.02	41.43 ± 51.82	33.89 ± 63.30	9.43 ± 3.10	0.016*
Piloerection	-	6.50 ± 3.32	7.75 ± 6.23	7.00 ± 2.83	0.972*
Exophthalmos	7.00 ± 3.46	5.82 ± 3.63	6.67 ± 3.75	3.92 ± 2.07	0.120*
Lacrimation	28.60 ± 24.34	39.50 ± 27.5	19.67 ± 9.58	17.00 ± 9.74	0.412*
Dyspnoea	29.50 ± 4.95	22.75 ± 12.53	27.33 ± 24.85	15.00 ± 7.07	0.795**

ToO – time of occurrence (minutes after application of paraoxon)
** Kruskal-Wallis test, **One-Way ANOVA, Red colour – statistically significant*
Values: mean ± standard deviation

Table 2: Intensity of clinical signs of poisoning 15 min and 4 h after administration of increasing doses of paraoxon

Sign (Intensity)	Paraoxon dose (mg/kg sc) 0.2	0.3	0.35	0.4	p value*
Fasciculations					
15th min	0	0.45 ± 0.82	0.58 ± 0.67	1.11 ± 0.93	0.052
240th min	0.33 ± 0.52	0.56 ± 0.52	0.56 ± 0.73	2.00 ± 0.00	0.061
Tremor					
15th min	0.33 ± 0.52	0.82 ± 0.87	1.50 ± 0.67	1.78 ± 0.44	0.002
240th min	0.17 ± 0.41	0.56 ± 0.53	1.00 ± 0.00	1.00 ± 0.00	0.044
Seizures					
15th min	0	0.91 ± 0.94	0.92 ± 0.79	1.67 ± 0.50	0.004
240th min	0	0.11 ± 0.33	0.40 ± 0.55	1.00 ± 0.00	0.069
Ataxia					
15th min	0	0.45 ± 0.69	0.33 ± 0.49	0.44 ± 0.52	0.329
240th min	0.33 ± 0.52	0.44 ± 0.88	1.00 ± 0.71	1.00 ± 0.00	0.271
Piloerection					
15th min	0	0.27 ± 0.47	0.83 ± 0.72	0.67 ± 0.50	0.016
240th min	0	0	0	0	-
Exophthalmos					
15th min	1.00 ± 0.63	1.09 ± 0.53	1.50 ± 0.52	1.67 ± 0.50	0.055
240th min	1.17 ± 0.98	1.22 ± 0.67	1.40 ± 0.55	2.00 ± 0.00	0.721

** Kruskal-Wallis test, Red colour – statistically significant. Values: mean ± standard deviation (Intensity: 0 - absent, 1 - mild/moderate, 2 - severe)*

higher at higher doses at the 15th min, however, it disappeared in all rats 45 min after poisoning.

To analyse the intensity of symptoms throughout the observed period (4 h), ToxScore was introduced, as a sum of the intensity of each sign in all time intervals. A significant difference was found in ToxScore values relative to dose regarding fasciculations and seizures (Figure 1). Other parameters were not significant.

TotToxScore is a measure that represents the sum of all ToxScores for one animal. TotToxScore values are shown in Figure 2. Although the difference was not statistically significant, an increase in the value of TotToxScore is noticeable, as a joint parameter of the intensity and duration of signs of

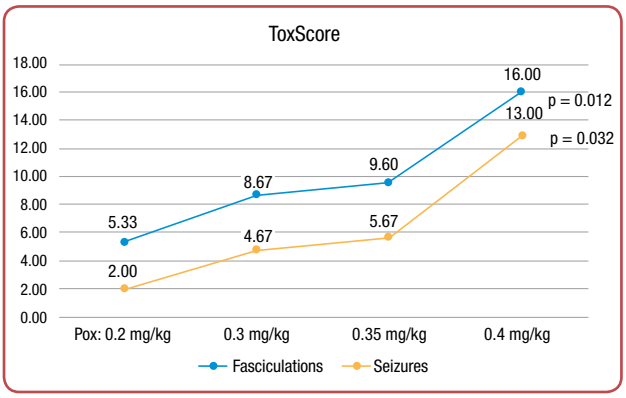

Figure 1: ToxScore for fasciculations and seizures relative to dose of paraoxon (Pox)
** Kruskal-Wallis test; ToxScore: the sum of the values of each sign in each point of time*

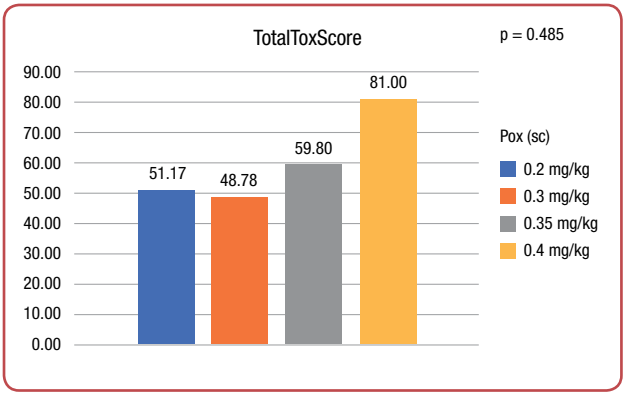

Figure 2: Mean TotToxScore relative to dose of paraoxon (Pox)
**TotToxScore: the sum of the values of all sign in each point of time; Kruskal-Wallis test*

poisoning, especially at high doses.

b) Survival

Analysing the frequency of clinical signs in relation to whether the rat survived or not, a significant difference was found regarding the piloerection: 23.81 % of survivors and 76.19 % of non-survivors had piloerection (χ^2 = 11,524, p = 0.002). The same goes for seizure frequency (66.67 % in survivors vs 100.00 % in non-survivors have had seizures, χ^2 = 8,400, p = 0.009). The speed of onset of clinical signs of poisoning relative to whether or not the animals survived is shown in Table 3. All signs of poisoning occurred earlier in non-survivors compared to survivors.

The intensity of the signs at 15 min after paraoxon application in relation to whether the rat survived

Table 3: Onset rate of clinical signs of paraoxon poisoning in relation to whether rats survived or not

Sign (ToO)	Survived		p-value
	Yes	No	
Fasciculations	35.44 ± 16.94	12.61 ± 4.25	<0.001*
Tremor	26.40 ± 24.02	7.38 ± 1.91	<0.001*
Seizures	33.14 ± 23.74	9.81 ± 3.12	<0.001*
Ataxia	52.33 ± 55.77	8.77 ± 2.05	<0.001*
Piloerection	8.40 ± 5.64	6.81 ± 3.97	0.646*
Exophthalmos	6.60 ± 3.35	4.70 ± 3.16	0.025*
Lacrimation	32.33 ± 22.28	14.37 ± 6.59	0.086*
Dyspnoea	33.67 ± 11.18	12.00 ± 5.83	0.004**

ToO – time of occurrence (minutes after application of paraoxon)
**Man-Whitney U-test, ** Student t-test, Red colour – statistically significant*
Values: mean ± standard deviation

Table 4: Signs intensity at 15 min after paraoxon administration in relation to whether the rat survived or not

Sign (ToO)	Survived		p-value*
	Yes	No	
Fasciculations	0.09 ± 0.44	1.17 ± 0.73	<0.001
Tremor	0.71 ± 0.78	1.76 ± 0.44	<0.001
Seizures	0.43 ± 0.75	1.59 ± 0.51	<0.001
Ataxia	0.19 ± 0.40	0.53 ± 0.62	0.059
Piloerection	0.19 ± 0.40	0.88 ± 0.60	0.646
Exophthalmos	1.14 ± 0.48	1.59 ± 0.62	0.011
Lacrimation	0.33 ± 0.48	0.18 ± 0.39	0.416
Dyspnoea	0	0.24 ± 0.56	0.048

** Man-Whitney U-test, Red colour – statistically significant. Values: mean ± standard deviation (Intensity: 0- absent, 1 - mild/moderate, 2-severe)*

or not is shown in Table 4.
Apart from lacrimation and stereotypy, all other signs were of higher intensity in non-survivors. The most significant difference in intensity was recorded in fasciculations, tremor and seizures, where high significance was found ($p < 0.001$).

Paraoxon and atropine

a) Dose

The PR of atropine was 2.73 (LD_{50} of paraoxon when atropine was administered 1 min after was 0.91 mg/kg sc (95 % CI: 0.67 - 1.25)). In all rats, death occurred during the first hour of poisoning (mean ± SD: 14.00 ± 3.74, 95% CI: 10.87 - 17.13). Fasciculations occurred in 77.78 % of all rats, piloerection in 33.33 %, exophthalmos in 94.44 %, lacrimation in 22.22 %, tremor in 100.00 %, seizures in 94.44 %, ataxia in 44.44 %, stereotypic behaviour in 38.89 % and dyspnoea in 55.56 % of rats.

In atropine-protected rats, the dose had a significantly smaller effect on the speed and frequency of symptoms. The frequency of any poisoning signs was not affected by the dose. As for the onset of clinical signs manifestation, a significant difference was found only in fasciculations ($p = 0.038$) and tremor ($p = 0.007$) (Table 5).

The intensity of the signs persisted throughout the observation period. The intensities of certain symptoms at 15th min and after 4 h relative to the dose are shown in Table 6. The cross-section at the 15th minute was taken as the time period when most of the signs of poisoning were present, yet most of the rats were still alive, while the 4th h was taken as the end of the observation period.

Table 5: Onset rate of clinical signs of poisoning at different doses of paraoxon with atropine protection (10 mg/kg im) (one minute after application of paraoxon)

Sign (ToO)	Paraoxon dose (mg/kg sc)			p-value
	0.6	0.9	1.2	
Fasciculations	28.83 ± 30.75	7.60 ± 1.95	10.33 ± 4.73	0.038*
Tremor	12.83 ± 5.85	8.33 ± 1.03	5.00 ± 2.19	0.007*
Seizures	18.20 ± 15.21	9.50 ± 1.64	8.50 ± 4.64	0.170**
Ataxia	51.50 ± 79.20	8.00 ± 2.83	70.50 ± 85.56	0.444*
Piloerection	4.33 ± 1.15	6.00 ± 3.60	5.17 ± 2.56	0.507*
Exophthalmos	3.20 ± 1.64	4.00 ± 2.89	6.00 ± 0.89	0.134*
Lacrimation	111.00 ± 0.00	105.00 ± 140.00	6.00 ± 0.00	0.632*
Dyspnoea	63.40 ± 46.47	25.33 ± 31.77	52.50 ± 44.55	0.504**

ToO – time of occurrence (minutes after application of paraoxon)
** Kruskal-Wallis test, **One-Way ANOVA. Red colour – statistically significant*
Values: mean ± standard deviation

Table 6: Intensity of clinical signs of poisoning 15 min and 4 h after the application of paraoxon and atropine (10 mg/kg, im, one minute after) depending on the dose of paraoxon

Sign (Intensity)	Paraoxon dose (mg/kg sc)			p-value*
	0.6	0.9	1.2	
Fasciculations				
15th min	0.89 ± 0.37	0.71 ± 0.32	0.55 ± 0.24	0.613
240th min	1.00 ± 0.00	1.00 ± 0.00	2.00 ± 0.00	0.011
Tremor				
15th min	1.17 ± 0.41	2.00 ± 0.00	1.8 ± 0.45	0.015
240th min	0.67 ± 0.52	1.00 ± 0.00	1.00 ± 0.00	0.472
Seizures				
15th min	0.83 ± 0.98	2.00 ± 0.00	2.00 ± 0.00	0.017
240th min	0	0.50 ± 0.71	2.00 ± 0.00	0.022
Ataxia				
15th min	0.33 ± 0.52	0.40 ± 0.55	0.20 ± 0.45	0.797
240th min	0.33 ± 0.52	0.50 ± 0.71	2.00 ± 0.00	0.073
Dyspnoea				
15th min	0.17 ± 0.41	0.20 ± 0.45	0.40 ± 0.89	0.961
240th min	0.33 ± 0.52	0.50 ± 0.71	1.00 ± 0.00	0.301

** Kruskal-Wallis test, Red colour – statistically significant. Values: mean ± standard deviation (Intensity: 0 - absent, 1 - mild/moderate, 2 - severe)*

CONTENT

ToxScore, although without statistical significance, showed a clear increase in fasciculations, tremor, seizures, ataxia, and dyspnoea with increasing doses of paraoxon (Figure 3).

TotToxScore values for paraoxon poisoning followed by atropine administration are shown in Figure 4. Total Tox Score, although without statistical significance, showed a clear increase with higher dose.

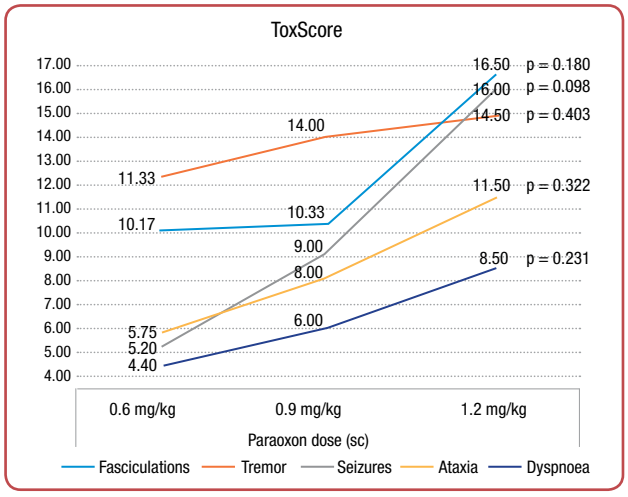

Figure 3: Dose-dependent ToxScore (paraoxon with atropine protection)

** Kruskal-Wallis test; ToxScore: the sum of the values of each sign in each point of time*

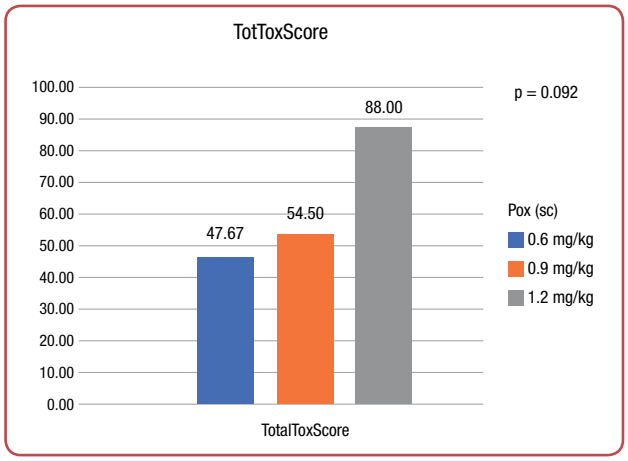

Figure 4: Mean TotToxScore relative to dose of paraoxon with atropine protection

**TotToxScore: the sum of the values of all sign in each point of time; Kruskal-Wallis test*

b) Survival

There was no significant difference in the frequency of symptoms in relation to whether the rat survived or not. It was only observed that ataxia was more common in survivors than in non-survivors (70.00 % vs 12.50 %, respectively; χ^2 = 5,591, p = 0.025) as well as fasciculations (100.00 % vs 50.00 %, respectively; χ^2 = 6,429, p = 0.023).

Table 7: Onset rate of clinical signs of paraoxon poisoning with atropine administration in relation to whether rats survived or not

Sign (ToO)	Survived		p-value
	Yes	No	
Fasciculations	21.70 ± 24.76	6.25 ± 0.96	0.004*
Tremor	10.50 ± 5.58	6.50 ± 2.50	0.083*
Seizures	14.67 ± 11.72	8.38 ± 3.42	0.117**
Ataxia	50.43 ± 69.43	10.00 ± 0.00	-
Piloerection	5.00 ± 1.63	5.50 ± 4.95	-
Exophthalmos	4.22 ± 1.86	4.75 ± 2.71	0.673*
Lacrimation	157.50 ± 65.76	6.00 ± 0.00	0.333*
Dyspnoea	60.50 ± 39.27	7.00 ± 1.41	0.098**

*ToO – time of occurrence (minutes after application of paraoxon). Red colour – statistically significant; *Man-Whitney U-test, ** Student t-test; Values: mean ± standard deviation*

Table 8: Signs intensity at 15 min after paraoxon administration with atropine protection in relation to whether the rat survived or not

Sign (ToO)	Survived		p-value*
	Yes	No	
Fasciculations	1.10 ± 0.74	0.50 ± 0.55	0.147
Tremor	1.50 ± 0.53	1.83 ± 0.41	0.313
Seizures	1.30 ± 0.95	2.00 ± 0.00	0.220
Ataxia	0.40 ± 0.52	0.17 ± 0.41	0.492
Piloerection	0.10 ± 0.32	0.17 ± 0.41	0.875
Exophthalmos	0.80 ± 0.42	1.00 ± 0.63	0.635
Dyspnoea	0.30 ± 0.67	0.17 ± 0.41	0.875

** Man-Whitney U-test; Values: mean ± standard deviation (Intensity: 0 - absent, 1 - mild/moderate, 2 - severe*

A significant difference in the onset of symptoms relative to whether or not a rat survived was observed only regarding fasciculations. The onset rate of poisoning clinical signs relative to whether or not the animals survived is shown in Table 7. Most signs of poisoning occurred earlier in non-survivors, but statistical significance was found only regarding fasciculations (p = 0.004). The intensity of any signs of poisoning did not differ significantly in survivors and non-survivors (Table 8).

Discussion

Paraoxon is one of the most toxic OPIs and is therefore banned from use as an insecticide in most countries. The obtained LD_{50} of paraoxon was 0.33 mg/kg sc, which roughly corresponds to the data from the literature.[17] Death in almost all animals occurs in the first two hours of OP

CONTENT

poisoning, which is consistent with the data obtained in this study.[18]

The LD_{50} of paraoxon, when 10 mg/kg of atropine is administered after 1 minute, was 0.91, so the PR of atropine was 2.73, similar to the data in the literature.[19] Atropine is used as an antidote for poisoning with AChE inhibitors. It is much more effective in carbamate poisoning than in those caused by OPCs.[19] It is logical that PR will be significantly higher in carbamates than OP poisoning given that carbamates are reversible AChE inhibitors, so the spontaneous reactivation of AChE makes the task of atropine as an antidote less demanding.[21-23] Considering the dose of atropine, 10 mg/kg im was selected because it is the most frequently used dose; therefore the results obtained in this experiments are comparable to the results of other researchers.[24] Krutak-Kol and Domino tried different doses of atropine against paraoxon poisoning: 10, 33 and 100 mg/kg and found that higher doses did not increase survival. Moreover, a dose of 100 mg/kg was significantly less effective than the dose of 10 mg/kg.[25] Repeated lower doses of atropine over time might potentially give better results.[26]

The presence, time of occurrence and intensity of paraoxon poisoning signs were examined. The same parameters of poisoning were also observed at high lethal doses of paraoxon, in animals given atropine. Those signs that can be noticed only by observation without manipulation of the animal were analysed.

Lacrimation occurs by excessive stimulation of muscarinic receptors. Although not life-threatening, it can serve as a good indicator of muscarinic excitation, as well as sign of adequate atropinisation during treatment.[3] Neither the intensity nor the rate of lacrimation occurred statistically significantly at different doses of paraoxon and in relation to the survival rate. However, when atropine was administered after paraoxon, lacrimation was significantly less common and occurred later, although significantly higher doses of paraoxon were administered (only 22 % vs 55 % when atropine was not administered). Regardless of the dose, the results obtained were expected since it is well known that atropine is an anti-muscarinic drug. The existence of lacrimation can indirectly indicate that there are other effects of muscarinic excitation (hypotension, bradycardia).

Muscarinic effects that are directly life-threatening are bronchoconstriction and bronchorrhoea. Dyspnoea was monitored as a parameter indicating respiratory failure, which in studies by other researchers proved to be the main cause of lethality in OPI poisoning.[27-29] The disadvantage of this study is that dyspnoea was only subjectively observed, without objective measuring of respiratory failure degree. Sometimes, in severe seizures dyspnoea could be noticed only when the intensity of seizures subsided. Nevertheless, a clear link was found in the intensity and speed of occurrence of dyspnoea in relation to whether the animal survived or not. In animals treated with atropine after paraoxon administration, atropine delayed and reduced the intensity of dyspnoea, although these were significantly higher doses of paraoxon. Houze et al analysed whether respiratory failure in OP poisoning was due to central or peripheral muscarinic effects.[30] He showed that respiratory failure was almost completely corrected by atropine and not corrected at all by 100 times the equimolar dose of N-methylatropine and concluded that respiratory failure is due to central muscarinic effects. On the other hand, Villa et al found that 50 % and 75 % of LD_{50} paraoxon caused alterations in ventilation, but did not cause respiratory failure.[31]

Fasciculations were a sign that, along with seizures, proved to be the most consistent parameter of the poisoning severity. Fasciculations occurred faster and were of higher intensity when the dose of paraoxon as well as survival of rats was observed. This can be explained by the fact that among the examined signs, fasciculations are those that follow overstimulation of nicotinic receptors on the neuromuscular junction. If it is assumed that according to the Poison Severity Score (PSS) in humans, any occurrence of nicotine sings of poisoning is considered at minimum as moderate poisoning, then it can be concluded that fasciculations, as a parameter of nicotine effects of poisoning, directly indicates the severity of poisoning.[32] An additional problem is lack of effective antidote that would function as a nicotine antagonist, without serious side effects.[33, 34] As expected, atropine administration did not affect the rate and intensity of fasciculations - they were of higher intensity and occurred earlier, because the dose of paraoxon was higher.

Muscle tremor was also a sign of inappropriate stimulation of nicotinic receptors on the neuro-

CONTENT

muscular joint, ie weakness of skeletal muscles. Tremor has been more often described in the literature as part of the delayed onset of extrapyramidal syndrome, rather than acute OP poisoning sign.[35] Since the monitoring of poisoning signs was based on observation, tremor served as a good indicator of muscle weakness. A strong relationship was found between both the speed of onset and the intensity of tremor, and the dose of paraoxon or the lethal outcome in rats. As expected, the results did not change when atropine was given after paraoxon, but the tremor occurred earlier and was of stronger intensity at higher doses and in non-survivors.

ACh is also found in the preganglionic fibres of the sympathetic nervous system. Piloerection is a sign that occurs as a consequence of excessive stimulation of preganglionic nicotinic receptors of the sympathetic nervous system.[36] Therefore, piloerection can be considered as potential predictor of sympathetic stimulation. The results of this study showed a strong relationship between the dose of paraoxon and the frequency of piloerection. Studies from other researchers show that the incidence of tachycardia in OP poisoning is 35-60 %, making it more common than bradycardia.[37] The alpha-1-adrenergic receptor, in addition to piloerection, in rats is also involved in retraction of the eyelids, leading to exophthalmos.[38] Unlike piloerection, exophthalmos remained most persistent throughout the observed period and had no statistical significance in the intensity and rate of exophthalmos in relation to paraoxon dose and rat mortality. The same results were obtained when atropine was administered after high doses of paraoxon.

Seizures initially occur due to stimulation of cholinergic receptors in the CNS and at this stage can be reversed by atropine. Prolonged seizure activity is due to excessive release of glutamate, when atropine is not effective.[39] For now, seizures are treated with combinations of anticholinergics and benzodiazepines but, given the role of glutamate in seizures, future therapies should include N-methyl-D-aspartate receptor (NMDA) antagonists.[40] The rate and intensity of seizures were clearly correlated to dose and especially to death in paraoxon poisoning. Seizures occur significantly less frequently in OPI than in nerve agents.[41] In this study, seizures occurred in 83 % of rats and 100 % of non-survivors, which additionally contribute to the characteristics of paraoxon as a nerve agents. Administration of atropine somewhat postponed and decreased the intensity of seizures. There was still a clear relationship between these parameters and paraoxon dose as well as fatal outcome, but seizures occurred later and were less intense than would be expected, given the paraoxon doses administered.

Ataxia is caused by stimulation of muscarinic receptors in the CNS. In addition, in acute OP poisoning, ataxia occurs as a consequence of muscle fatigue, but ataxia is more intense than would be expected from muscle fatigue alone.[42] The results of this study show that ataxia occurred earlier at higher doses of paraoxon and in non-surviving rats.

ToxScore and TotToxScore are parameters that were introduced in order to see the persistence of symptoms related to the dose. Thus, the values of each sign in each point of time were summed, which made up the ToxScore, and the values of all ToxScores were added the calculate the TotToxScore for each animal. The pre-condition is that the animal survived the observation period. These parameters can also be used in future studies, when the protective effect of antidotes (either individually or in combination) can be compared, not only as a measure of survival, but also their ability to alleviate the signs of poisoning. ToxScore showed that the same parameters (seizure, fasciculation, tremor) that were significant indicators of the severity of poisoning lasted longer at higher doses. The TotToxScore had higher value at higher dose and, although the difference was not significant, showed a clear trend.

Conclusion

Seizures and fasciculations followed by tremor were strong prognostic parameters of the probability of lethal outcome of paraoxon poisoning. Also, the mentioned signs of poisoning were with their intensity and speed of occurrence in a clear correlation with the dose of paraoxon. Even at high doses of paraoxon, atropine blocked the muscarinic (but not nicotinic) effects, and somewhat mitigated the CNS effects.

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Conflict of interest

None.

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CONTENT

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HISTORY OF MEDICINE

Impact of Gerard van Swieten on the Development of Austrian Medicine Throughout the 18th Century

Boro Bronza[1]

Abstract

Arrival of Doctor Gerard van Swieten in Vienna, in 1745, as new personal physician of the Holy Roman Empress Maria Theresa, was starting point of a huge wave of transformation in the scope of Austrian medicine. Scientific and methodological experience which doctor from Leiden brought in Habsburg capital was so overwhelming that whole structure of medical science was shattered and reconstructed in a much more efficient way. Impact of Van Swieten was a splendid example of dominance of scientific method in the Netherlands, where modern European science gained more ground than anywhere else during the classical era of baroque, throughout the 17th and first half of the 18th century. On the other hand, internal reforms and transformation of Austria, from the mid-18th century, helped a lot in the process of successful reception of new structural ideas. Through this kind of merging, inside of only several decades, Vienna managed to grow into one of leading centres of medical science in Europe and the world.

Keywords: The Netherlands; Medicine; Leiden; Gerard van Swieten; Austria; Vienna.

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Consequences of "the Dutch Golden Age"

The complex political processes of the second half of the 16th century at the plains of the Netherlands, imbued with aspects of the consequences of the Reformation after a long war for liberation from Spanish domination (1568-1648), resulted in the emergence and rapid development of the United Provinces of the Netherlands (officially called the Republic of the Seven United Netherlands and usually called the Dutch Republic). Politically and economically, the most important of seven provinces was Holland, and because of that the whole state system was usually set in the same manner as in the strongest province.[1] Almost the entire 17th century represented the golden age of the Dutch state, because in that period it developed into the most economically prosperous and socially sophisticated European region. Such social and economic development was accompanied by adequate development in the field of science and one of the most developed scientific fields was medicine.[2]

Already during the 17th century, the Dutch medical school was established on new scientific empirical bases, and increasingly distanced itself from the European medieval medical heritage domiated at that time still mainly by alchemy. Many famous Dutch masters of painting of that

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CONTENT

Figure 1: Rembrandt van Rijn -The Anatomy Lesson of Dr Nicolaes Tulp (1632)

time displayed scenes on their canvases, that witnessed the progressiveness of medical science in the Dutch provinces. Well-known examples of such an approach were, among others, paintings by Rembrandt van Rijn (1606-1669) "The Anatomy Lesson of Dr Nicolaes Tulp" (*De anatomische les van Dr. Nicolaes Tulp*), in 1632 (Figure 1) and "The Anatomy Lesson of Dr Deijman" (*De anatomische les van Dr. Deijman*) in 1656.

With the intellectual and cultural surrounding enriched by the presence of persons like René Descartes (1596-1650), Christiaan Huygens (1629-1695) or Baruch Spinoza (1632-1677) and with genius pioneering inventions of Antoni van Leeuwenhoek (1632-1723) in the field of microscopy and his further revolutionary approach in the establishment of microbiology as new branch of science, scene was completely set in the Netherlands for another wave of breakthrough in medicine at the turn of 18th century.

Amsterdam was unquestionable centre of oeconomic life and trade of all Seven Provinces of the Netherlands and a leading world port, which was especially underlined with the founding of huge colonial corporations the Dutch East India Company (*Veerenigde Oostindische Compagnie – VOC*), since 1602, and the Dutch West India Company (*Geoctrooieerde Westindische Compagnie – GWC*), since 1621 and first world modern stock exchange as subsequence of mentioned companies.[3] On the other hand, The Hague, in South Holland, was political centre of Provinces, with the permanent seat of the "stadtholder" and government there since 1588.[1] At the same time, City of Leiden, geographically situated between Amsterdam and The Hague, became a crucial scientific centre, especially with the role of the first university in Dutch Republic opened there in 1575.

University of Leiden grew pretty fast into one of the leading institutions of European science, mainly because of special conditions of intellectual tolerance that ruled in the Netherlands, and in Holland more than anywhere else. Throughout the 17th century University of Leiden managed to build excellent reputation at the continent and was starting point for careers of all already mentioned carriers of "the Dutch Golden Age". During this time, Leiden also became the home for leading persons in the field of medical science.

One of superb products of medical science developed at University of Leiden was Herman Boerhaave (1668-1738). He had a chair at the Institute of Medicine in Leiden since 1701. Boerhave was leading European physician of era at the turn of the 18th century. Besides of that he was outstanding botanist and chemist, as connections and borders between medicine, biology and chemistry were still blurry at that time. Famous doctor from Leiden is often regarded as the father of clinical teaching and inventor of the quantitative approach into medicine. Since 1714 he was appointed as rector of the leading Dutch university.

CONTENT

Van Swieten between Leiden and Vienna

It was exactly in this exceptional scientific surrounding of Leiden that Gerard van Swieten was born, on 7 May 1700. He was offspring of a prominent Catholic family in Leiden. Although majority of Dutch provinces, and Holland especially, were predominantly inhabited with Reformed Protestants (Calvinists), Catholics have been mainly pretty fairly tolerated. Still, some restrictions have been in place even further, as a consequence of past times and conflicts with catholic Spain, and Van Swieten was about to learn some rough sides of those hurdles in the upcoming decades of his development in Leiden. Start of the life itself was already harsh for little Gerard, for he lost both of his parents, father Thomas (1662–1712) and mother Elisabeth (d. 1708), early in the childhood.[4]

Without any sibling, growing with two friends of his late father, who were appointed as his guardians, Van Swieten made first steps in his education and managed to excel from the very start. After finishing the Latin school at the age of 12, under careful tutelage of Jesuits, he continued fast further up to the first enrolment at the Leiden University, when he was only 14.[5] His first intention was to study philosophy, but later on he moved to also very famous University of Leuven, in the Austrian Netherlands (today Belgium) and because of special interest in pharmacy he developed there, he decided to pursue training at one of the leading pharmacists in Amsterdam, Laurens Tatum. After some two years in Leuven and Amsterdam he returned to Leiden, finishing his education in the field of pharmacy at the Leiden University first, and then studied medicine only. His crucial professors have been rector Herman Boerhaave himself and Bernhard Siegfried Albinus (1697-1770), prominent lecturer in Anatomy and Surgery, originally from the German city of Frankfurt on the Oder, brought to Leiden from Paris, by personal invitation from the rector Boerhaave.[6] Van Swieten's studies have been crowned with obtaining of his medical doctorate in 1725. His dissertation with the title *De arteriae fabrica et efficacia in corpore humano*, that concentrated on the functioning of arteries, was finished under the mentorship of Albinus, who will later also hold the position of rector at the Leiden University.[4]

Although Van Swieten was outstanding student throughout his complete education, he was not in a position to become professor at the University of Leiden, because religious restrictions still prevented Catholics to gain such level at one of the leading institutions of the protestant Netherlands. Therefore, he was only granted with possibility to start a private medical practice in Leiden.[5] Even that kind of activity brought him in some conflict situations with the University and sometimes even with the rector Boerhaave. Nevertheless, after his promotion he has continued to attend the Boerhaave's classes all the way until the death of this leading professor at the University in 1738.[6] In this way, Van Swieten managed to gather best possible medical knowledge and his ever-broader practice enabled him even broader scope of worthy experience. Besides scientific career, Van Swieten also managed to be a successful family man. He married Maria van Coesfelt (1711–1784) in 1729 and they would have six children between 1731 and 1746. Among them was the oldest son, Gottfried van Swieten (1733-1809), who will later continue father's glory in the role of one of leading Austrian diplomats.[5]

New turn of events came for Van Swieten at beginning of the 1740's, when his medical glory in Leiden was already well-known fact around Europe. Immediately after the death of the previous personal physician of the Holy Roman Empress Maria Theresa, Joannes Baptista baron Bassand, in November 1742, Austrian diplomatic network was set in motion to find a suitable replacement. In that frame Gerard van Swieten was contacted by the Austrian ambassador in The Hague, Judas Thaddäus baron Reischach (1696-1782), with the offer to come to Vienna and to take over the role of new personal doctor in the service of the empress.[4] Joannes Baptista Bassand was also former student of Boerhaave, so the choice of Van Swieten as a Catholic, was a very logical move of the Vienna Court. The offer for position of personal physician of the Empress was even combined with the role of director of the court library.[5]

At first, he decided to decline that splendid offer. The idea of leaving Leiden did not easily fall to Van Swieten. When, in 1744, he received another invitation from the Austrian Secretary of State, Koch, to move to a new post in Vienna, he replied with a lot of melancholy: "The past has taught me that my heart is too delicate to bear the thought of separating from my homeland, from my family and from my friends." (*Het verleden heeft mij geleerd dat mijn hart te teer is om aleen al de gedachte te verdragen dat ik zou moeten scheiden van mijn vaderland, van mijn familie and van mijn vrienden.*).[4]

CONTENT

In his approach regarding the future career Van Swieten was more and more advised by his friend from the studies in Leiden and another pupil of Boerhaave, Portugese doctor António Nunes Ribeiro Sanches (1699-1783). At that time Sanches was the personal physician of the Russian Empress Elizabeth Petrovna (ruled 1741-1762).[7] Up until October 1744 Van Swieten changed his mind and accepted the Austrian offer and by May 1745 he arrived in Vienna together with the family. His earlier convictions that he, as convinced republican cannot be turned into "monarchist" have been overturned, so he made another huge step in his transformation (*Van republikein tot monarchist*),[4] which actually was another testimony of maturity and pragmatism in his development.

Wave of reforms

During the first half of the 18th century, Austria has made only small steps regarding the internal stabilisation and centralisation of the state power. Reign of the Habsburg Emperor Charles VI (1711-1740) was mainly filled with failures and defeats in the international arena. For Charles VI was without a male heir, his oldest daughter Maria Theresa succeeded him as Empress in 1740, as young woman of only 23. Lack of experience in almost every aspect of leadership at the state level was very obvious in Austria, through the 1740's. That was one of key explanations why Van Swieten was granted with so much freedom to implement reforms in education from the very start.

When arrived in Vienna, Van Swieten faced the social system with much less democracy and liberty than in his homeland. Although even provinces of the Netherlands were in decline around the middle of the 18th century in comparison with the standards of Golden Age from earlier decades, they were still far ahead of level of Habsburg hereditary lands. The shape of his personal relations with the Empress was indeed crucial for the position of Van Swieten and his maneuverable capabilities from the very start. For he was very able to secure her intensive inclination pretty fast, therefore he was granted with huge prerogatives in the scientific field. First of all, he aimed to implement huge transformation inside of the system of medical education in Austria and also to completely reform the health service.

One of the first measures was concentration on sanitary reform in Austrian lands. This reform was closely connected with the organisation of sanitary cordon at the border of Habsburg Monarchy towards the Ottoman Empire. Institutional development of a system to prevent the spread of plague epidemics from the Ottoman Empire to Austria ran in certain way through the whole first half of the 18th century. As early as in 1710 Vienna Court issued the first official regulatory document about the border behaviour in the case of imminent danger of plague epidemic spreading, known as "the Plague patent" (*Pestpatent*).[8] Also, special governmental institution was formed in Vienna already in 1718, under the name Court Sanitary Commission (*Sanitäts Hofkomission*).[9]

But it was with the arrival of Gerard van Swieten that sanitary cordon gained new ground and system of quarantine was so efficiently introduced that total elimination of the disease at the area of Habsburg lands was achieved in the timeframe of only few decades. In the period up until 1763 the Austrian government was mainly occupied with major wars led against Prussia in the northern parts of the country. Because of that the creation of a dense quarantine system in the true sense began only after 1763. Among others, special regulations have been made in Vienna to schedule the obligations of border guards who were in service at the quarantine stations.[10] Special sanitary commissions were established, with headquarters throughout border region and with lazarettes, as institutions for the accommodation of patients directly related to quarantines.[11] Van Swieten was main proposer of new measures in the system of structure buildings at the border and he collected and published all necessary knowledge in patent called *Generale Normativum in Re Sanitatis*. Empress officially implemented this patent in 1770.[12]

The Austrian protection system established under the guidance of the scientist from the Netherlands has proven to be very effective. Success in dealing with the plague epidemics has strengthened the Vienna court in its orientation towards further implementation of modern scientific measures in facing with the challenges of medicine and therefore helped significantly to Van Swieten in his efforts to overcome aspects of Jesuit influences still very present in education in the Habsburg lands.[13] Although he alone was Catholic and in the childhood under tutelage of Jesuits, he was a loud opponent of the Order who was a dominant force in the education of all Catholic lands in Europe throughout previous two centuries. Like

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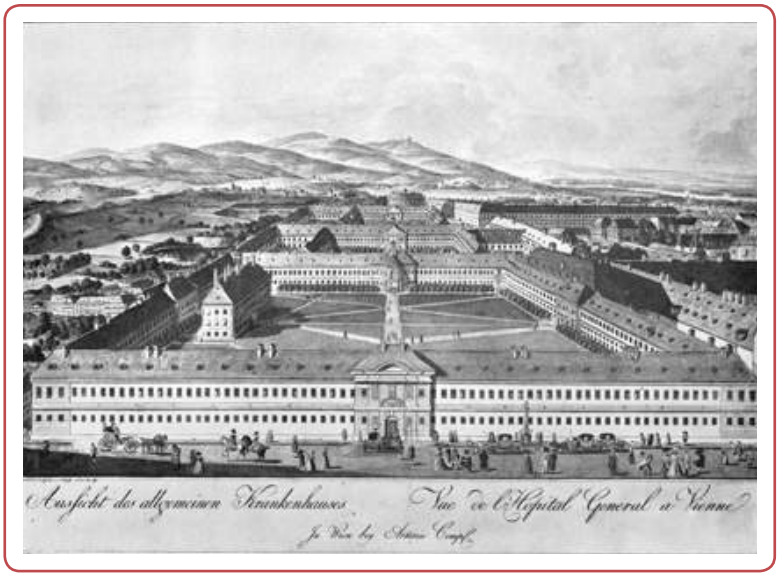

Figure 2: Vienna General Hospital in 1784

in the case of famous Austrian diplomat and later internuncio in Istanbul, Baron Peter Philipp von Herbert-Rathkeal (1735-1802), who was a member of Jesuit Order during his early years and later fierce opponent to his former guardians,[14] Van Swieten was one of several personalities in Austria with Jesuit educational background who actually mostly helped in defeating the Jesuit dominance and consequently advised Empress in approach to finally suppress and expel the Society of Jesus from Austrian lands in 1767.

Already during first several years in Vienna, Gerard van Swieten pushed in very different fields with implementation of reforms, not only inside of medical science, but also in the social system in general. Structurally, for medicine it was of huge importance that he has implemented separation of medical training and administration of system of public health in 1749. In addition, from the very start in Vienna in 1745, he was entitled as librarian for Empress Maria Theresa in what at that time still was the Imperial Library.[4] Position in the library and close relations with the Empress enabled his dominance in the questions regarding the censorship. He broke with the middle age practice of public burning of the books but was very strict in the sense of purging of science especially of influences that came from alchemy and superstitions.[5] With this modern and direct approach he has managed to remove Jesuits from the control of censorship. Jesuit control over this institution actually enabled them to keep all aspects of science in Austrian lands inside of their sphere of power. Similar approach that was in use by Van Swieten in the world of medical science was immediately after widened to the prosecution of literature in general. He constantly insisted only on logic and rational and scientific aspects in his judgements regarding the publication of books and pamphlets.

Still, his primary concentration on medicine was unquestionable. Therefore, he mainly paved the way for further institutional development of the Medical Faculty at the University of Vienna. He has advised Empress to hastily issue patents for organisation of separate courses at the faculty. In that way he has prescribed formation of courses for chemistry, botany, surgery and, as a special innovation in Austria and direct consequence of influences by his mentor Herman Boerhaave, he has founded separate courses for clinical medicine, where the approach in treating of the patients was actually taught to students directly next to patient's bedside.[6] Practice of direct teaching in hospitals was a quantum leap for medicine in Austria.

Placement of Van Swieten on the position of the director of studies was unavoidably a decision in the context of his dominant role. At the same time, young dean of the Medical Faculty Anton Störck (1731-1803) was his close collaborator and former pupil.[4] Van Swieten was also mainly responsible for creating a new curriculum, where medical theories were strongly interlinked with practice. Instructions and supervisions in the curriculum have been specifically separated also for education and guidance of specific medical professions like surgeons and barbers (Figure 2). This system was quickly implemented at all medical

CONTENT

institutions of Austrian lands, so what started in Vienna was very soon new reality also in Prague, Buda, Lemberg and Padua and all other universities under control of the Habsburg Monarchy.[5]

All mentioned merits and credits he has earned in Vienna during first several years were closely followed from the side of leading European academic institutions. It was not surprising at all that very soon he was included as member in some of most prestigious scientific academies. It was in 1749 that he was elected a Fellow of the Royal Society in London and two years later he was also elected a foreign member of the Royal Swedish Academy of Sciences in Stockholm.[4] Finally, in 1754, when he was already made baron (*Freiherr*) by the Empress, Van Swieten was accepted as a member in the Leopoldina, German Academy of Sciences in Halle.[5]

Fight against barbarism

A very illustrative episode of the changing influence that Van Swieten has brought with him in Vienna was his clash with remnants of superstition, in the frame of fight against the perception of vampires and vampirism. Myths about vampires were one of the crucial points he wanted to tackle in his battle against "barbarism of ignorance" and total eradication of such kind of folklore misconceptions was his loudly proclaimed goal. It was actually very convenient for him that throughout the first half of 18th century stories about vampires in Austria were in circulation even more than before. One of the geographic regions where the concept of vampires was predominantly concentrated was the Balkan Peninsula.[15]

With the victorious end of war against the Ottomans (1716-1718) and following the undersigning of Peace Treaty of Požarevac (Treaty of Passarowitz, in August 1718), Austria managed to gain significant parts of territories in Northern Serbia and also the northernmost strip of the land of Bosnia, around rivers Una and Sava. Therefore, Austrian officials have been in position to encounter a growing number of stories connected with vampirism and particularly illustrative episodes have been reported from several villages in Northern Serbia in 1725, and then again in 1732 about local robbers-hero freedom fighters (hajduks), who "came back from the dead".[16] Those reports actually were a starting point for transfer of the "knowledge" about vampires to the German speaking regions and even for the beginning of "scientific" explanations about possibility of vampire existence (*Die europaweit erscheinenden Berichte über die Heiducken-Vampyren von Serbien von 1725 und 1732 lösen eine Flut von 'Facharbeiten' über Vampyre aus...*).[17]

Allegedlly strong evidence about the "vampire activity" have been in circulation in Moravia, so Empress Maria Theresa decided to send Van Swieten to the research *in situ*. Arriving in Moravia, to the north from the City of Brno (Brünn), in 1755, Van Swieten carefully investigated all testimonies.[18] After concluding the investigation, he made a detailed report with the title "Treatise on the Existence of Wraiths" (*Abhandlung des Daseyns der Gespenster*).[17] This report was published more than a decade later, in German for the first time in 1768.

In his report Van Swieten concentrated elaboration strictly on the scientific ground. In the usual stories about vampires of that time main intention was always description of presence of almost "intact dead bodies" in graves. So, such bodies have been dug out from graves and beheaded or even burnt in the attempt to finally get rid of the continuous "danger". Such shape of "defence" against vampires was shocking for Van Swieten. He insisted that imperfect putrefaction was the crucial criterion that all alleged vampires had in common. From personal experiences with grave and coffin openings he recognised, a good 20 years before the discovery of oxygen, that such phenomena occur especially when the corpse was buried under particularly strong air exclusion, which actually prevented usual rate of body decomposition.[18] In his statement he underlined: "...that all the noise came from nothing else than a vain fear, a superstitious gullibility, a dark and agitated phantasy, simplicity and ignorance among that people." (...*daß der ganze Lärm von nichts andern herkömme, als von einer eitlen Furcht, von einer aberglaubischen Leichtglaubigkeit, von einer dunklen und bewegten Phantasey, Einfalt und Unwissenheit bei jenem Volke*.).[17] Even more, Van Swieten was very willing to add some ironic remarks in his final review of the stories connected with the testimonies about development around the graves at cemeteries: "Others believed they saw or heard a dog, a calf, a pig, a calf's head. Was it then necessary for the devil to give life to a dead human body, to appear in such a dog or calf shape? There is not the slightest connection between the cause and the given effect." (*Andere*

CONTENT

haben geglaubt, sie sehen oder hören einen Hund, ein Kalb, ein Schwein, ein Kalbskopf. Hatte denn der Teufel nöthig, einen menschlichen todten Körper lebendig zu machen, in einer solchen Hundes- oder Kalbesgestalt zu erscheinen? Es ist ja zwischen der Ursache, und der vorgegebenen Wirkung nicht die geringste Verbindung).[17] Final consequence of Van Swieten's mission to Moravia was immediate ban on all activities connected with the desecration of graves, through the so called "Vampire Proclamation" (*Vampir-Erlass*), from March 1755.[18] Myths about vampires have been efficiently exterminated from Austrian lands. Nevertheless, longstanding impact of Van Swieten in his "fight against vampires" was so strong, that even some 150 years later he was one of crucial inspirations for Irish author Bram Stoker (1847-1912) in creation of the character Abraham Van Helsing, a vampire-hunter, for his famous novel "Dracula" from 1897.[19]

First Vienna Medical School

Concept of the First Vienna Medical School was rooted inside of the complete system that Van Swieten was able to set in motion. It was his personal suggestion to the court that inspired foundation of first modern clinic in Vienna in 1754. Dealing with human resources in upcoming years and decades was another testimony of Van Swieten's efficiency, for all of his choices have been among the best possible in Europe of that time. First of all, he has brought from Leiden Doctor Anton de Haen (1704-1776). De Haen studied together with Van Swieten and he was another successor of methods earlier applied by Herman Boerhaave in Leiden.[20] Under the instructions of Van Swieten, De Haen was appointed as first director of clinic in Vienna placed in Bürgerspital. His approach was full triumph of Bierhaave's practical instruction "Away from the textbook, towards the patient!". With De Haen, first Vienna clinic not just became place of lecture, it grew as well into significant place of research.[21]

Successor of De Haen at the position of director of clinic in Vienna was Maximilian Stoll (1742-1787). Stoll was another close follower of medical traditions established by Boerhaave and Van Swieten. His results at the field of epidemiology have been especially remarkable. As a doctor he was one of first practitioners in keeping of daily progress records of patients and also of percussion methodology. As young physicians Leopold von Auenbrugger (1722-1809) and already mentioned Anton Störck started their careers at the clinic as assistants in the 1750's. In upcoming decades, they had main role in further widening of initial Van Swieten's visions regarding the transformations of medicine. Von Auenbrugger was real inventor of percussion. He was the first physician ever able to understand differences in sound that arise during the tapping on the surface of the patient's chest wall at different points. Therefore, he was in position to fairly assess the status of texture in underlying organs and tissues deeper in body. His research was published in 1761 under the title *Inventum novum ex percussione thoracis humani ut signo abstrusos interni pectoris morbos detegendi*.[22]

Anton Störck was indeed a sublimation of First Vienna Medical School. He came to Vienna from the German region of Baden-Württemberg as an orphan. Since 1752 he studied medicine under personal guidance of Van Swieten. Under his mentorship Störck was promoted in 1757. Next years have been very fruitful for Störck. His university career was crowned by his appointment as dean of the Medical Faculty already in 1766 and as R*ector magnificus* of the Vienna University in 1768. Maria Theresa chose him as the treating doctor when she contracted smallpox in 1767. After a successful healing, she appointed Störck as her personal physician and he was made a Baron in 1775.[22] Career of Anton Störck was most similar to the fast rising of Van Swieten few decades earlier. Cooperation with his famous mentor was also of huge significance in the field of experimental pharmacology. Störck made first breakthrough in the clinical research of various healing herbs. In this frame he has also established clinical trials in modern medicine, for he experimented with the dosage of quantities, initially on animals, and later by a personal trial.[21]

Development of pharmacology in Vienna was closely bound with old Van Swieten's connection with pharmacy and botany. Van Swieten founded a botanical garden in Vienna and a chemical laboratory next to it. He also introduced clinical instruction in the field of appliance of new methods of pharmacy and chemistry. In this frame of utter importance was another research initiative started by the Dutch doctor.[4] Nikolaus Joseph von Jacquin (1727-1817) was yet another pupil from Leiden brought to Vienna by Van Swieten. In Vienna he attended lectures by the famous Dutch professor. For his inclination towards botany and

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Figure 3: Austrian National Library

chemistry was exceptional, he was chosen by Van Swieten to lead the first Austrian expedition to the West Indies and Central America with the goal to collect plants for the Schönbrunn Palace.[23] Between 1755 and 1759 Jacquin amassed a very large collection of animal, plant and mineral samples and after return of those specimens to Vienna Van Swieten was in position to further organise botanic and pharmaceutical studies. As further reward, in 1768, Jacquin was appointed Professor of Botany and Chemistry at the University of Vienna and he also became director of the botanical garden within the University.[5] Expedition led by Jacquin was forerunner of wave of expeditions that Austria was about to organise in upcoming decades towards Americas, Africa, Asia and Pacific, both in the context of economy and trade and in intention of further bolstering of science, like in the case of botanical expedition led by Franz Joseph Märter (1753-1827), towards southern parts of newly formed United States of America, Caribbean Islands, northern parts of South America, South Africa and Madagascar, between 1783 and 1788.[23]

Figure 4: Statue of Gerard van Swieten in Vienna

Numerous breakthroughs made by Vienna Medical School during 1780's were not led and followed anymore by Gerard van Swieten. He died in 1772. His son Gottfried at that time already became famous on his own, primarily as an Austrian diplomat. He was posted in the embassies to Brussels, Paris and Warsaw, but his mission as ambassador in Berlin (1770–77), at the court of Frederick the Great, King of Prussia, was most important. Like his father, Gottfried van Swieten also held position of the Prefect of the Imperial Library (Figure 3). During the decade of the rule of Joseph II (1780-1790), younger Van Swieten was at the height of his political power, in the positions of Councillor of State and Director of the State Education Commission and also later on as Director of a Censorship Commission, which was another footprint his father already made decades earlier. Gottfried van Swieten was also known as supporter and patron of some famous classical composers, like Mozart, Haydn and young Ludwig van Beethoven.[5] In that way the contribution of the Van Swieten family to the development of Vienna

CONTENT

in the second half of 18th century was even more rounded (Figure 4).

Unfortunately, some aspects of contributions of the First Vienna Medical School have been long neglected in the general history of medicine. It is only in last several decades that growing research is showing vast affluence of this system and institutions that were produced in the course of its development. Rightly so, for foundations that were set up by Gerard van Swieten in Vienna, in the 18th century, are pretty much foundations of modern system in medical science, especially in the field od clinical development and pharmaceutical research.

Conclusion

Activities of Gerard van Swieten in Vienna during the era 1745-1772 are hard testimony about possibilities of one person to implement dramatic set of reforms in the field of science in the case when that particular person is in possess of superb foundation in educational and scientific frame. Coming from Leiden, Netherlands, with outstanding tradition of medical science, Van Swieten embodied all the liberal qualities developed in most progressive part of Europe during previous century. Influences of Herman Boerhaave, as crucial carrier of new systematic approach in Leiden medical practice have been deeply rooted in Van Swieten throughout all decades of his transformative attitude in Vienna.

Van Swieten was lucky that he has arrived in Vienna in situation where structures of Habsburg dynasty were much more flexible and readier to open the field od medicine and science in general to modern influx of liberal tendencies. Decadent baroque heritage of previous decades of rule of Charles VI, father of Empress Maria Theresa, was fairly quickly left behind and foundations for First Vienna Medical School were strong in rolling already in the 1750's. But it was the spirit of Van Swieten with his general modesty and complete readiness for cooperation that enabled such huge step forward and almost instant transformation of Vienna into one of the leading medical centers.

Acknowledgements

None.

Conflict of interest

None.

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PROFESSIONAL ARTICLE

Impact of Body Mass Index on the Initial In-Brace Correction in Patients With Idiopathic Scoliosis

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Abstract

Background/Aim: Many factors affect initial in-brace correction and treatment outcome in patients with idiopathic scoliosis. Previous studies have observed contradictory results on the role of BMI in orthotic treatment. The aim of this study was to examine whether BMI impacts in-brace correction, isolated and in relation to other predictive factors (curve magnitude, curve location and Risser sign).
Methods: A retrospective study has been conducted on patients with idiopathic scoliosis treated with Cheneau-Sobernheim brace, that had no prior treatment. The collected and analysed data included patient demographics, BMI percentile and radiological parameters (curve magnitude in Cobb angle, curve location, Risser sign). The initial in-brace correction was expressed as a percentage of Cobb angle reduction in the brace as opposed to Cobb angle out of brace. Patients were categorised into groups according to their BMI, expressed in percentiles, ie: low BMI (< 5 percentiles), normal BMI (5-85 percentiles) and high BMI (> 85 percentiles). To determine the significant difference and correlation relationship between the examined variables, variance tests, t-test with unequal variance, and Pearson correlation coefficient have been used.
Results: The cohort study comprised 213 patients (170 females and 43 males) with a mean age of 13.5 years at brace prescription. Low BMI has been detected in 10 % patients, normal BMI in 78 % and high BMI in 10 % patients. No significant difference in in-brace correction has been found between BMI groups, nor has there been any significant correlation between BMI and in-brace correction. Regarding other factors, significant difference within BMI groups was found between in-brace correction and curve location, as well as in-brace correction and Risser sign. Lumbar curves had significantly better in-brace correction than thoracic curves. Significant correlations between in-brace correction and curve magnitude, curve location and Risser sign were detected.
Conclusion: The results of the present study show that, as an independent factor, BMI does not impact in-brace correction. Other factors, such as curve magnitude, curve location and Risser sign, play a more significant role in the orthotic treatment of patients with idiopathic scoliosis.

Keywords: Scoliosis; Body mass index; Brace; Correction.

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Introduction

Idiopathic structural scoliosis is a three-dimensional spine deformity with a lateral deviation of the spine in the frontal plane, a vertebral rotation in the transversal plane and an impaired sagittal profile. The aetiopathogenesis is as yet unclear and the cause is unknown. However, it is pre-

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CONTENT

sumed to be multifactorial[1] and manifests solely in healthy children. It can occur at any age, especially during the growth spurt, where most progression is expected at the beginning of puberty.[2] The diagnosis of scoliosis is based on anamnesis, clinical and radiological assessment. Conservative treatment of scoliosis includes observation, physical therapy and bracing. The aim of the treatment is to stop the curve progression – possibly even reducing it – and to avoid surgery. Recent studies have shown that it is also possible to obtain some amount of curve correction.[3] Factors that have been suggested as possible determinants of a higher risk of scoliosis progression include positive family history, laxity of skin and joints (connective tissue defect), flattening of physiological thoracic kyphosis, angle of trunk rotation exceeding 10°, and growth spurt.[4] Brace treatment is indicated for curves from 20 to 45 ± 5 degrees of Cobb angle in children that are skeletally immature and have growth potential. Initial in-brace correction represents a reliable parameter for brace quality[5] and a prognostic factor for short-term results,[6] as well as long-term results of orthotic treatment.[7] In addition to well-defined prognostic factors – which affect initial in-brace correction[8] and therefore treatment results – various studies explored the influence of Body Mass Index (BMI) on the initial in-brace correction and treatment outcome, with varying results.

The aim of this study was to examine whether BMI impacts initial in-brace correction in children with idiopathic scoliosis isolated and in relation to other prognostic factors (curve magnitude, curve location and Risser sign).

Methods

This was a retrospective study that included patients from the Institute for physical and rehabilitation medicine „Dr Miroslav Zotovic“ in Banja Luka, Republic of Srpska, Bosnia & Herzegovina. The study has been approved by the Institute's Ethics Committee. All patients were diagnosed with idiopathic scoliosis and have been treated by the Team for scoliosis, which includes specialists for spinal deformities, orthotists and therapists. The treatment of these patients is based on the SOSORT guidelines for conservative treatment of scoliosis (2016)[1] and standardised treatment protocols. In this facility, the type of brace being prescribed and manufactured is Cheneau-Sobernheim. This study only included patients who had no prior brace treatment. In patients with one or more compensatory curves, the focus was on the primary scoliotic curve.

The analysed data was obtained from the internal database, the hospital information system KIS, including patient demographics, BMI in percentiles and radiological parameters (on the X-ray image at brace prescription and on in-brace X-ray, one month after the final brace application). Radiological parameters include magnitude of the primary curve (measured by Cobb's angle at out- and in-brace X-ray), location of the primary curve, and Risser stage. The location of the primary curve is classified into the following categories: thoracic, thoracolumbar and lumbar.

The initial in-brace correction is calculated as a reduction of Cobb angle of the primary curve in brace and out of brace, expressed as a percentage of the Cobb angle reduction in brace.

The BMI is evaluated differently in children then in adults, given that children's bodies change rapidly. This is why the standardised formula for children, ages 5 through 18 years, is based on height, weight and the patient's age. In this study, the authors looked at these parameters at brace application.

Patients have been categorised into groups according to their BMI, expressed in percentiles: low BMI (< 5 percentiles), normal BMI (5-85 percentiles) and high BMI (> 85 percentiles).

To examine the effect of the BMI on in-brace correction, the correlation between BMI and initial in-brace correction in total and according to BMI groups has been tested. Other relevant factors, which affect in-brace correction, have also been taken into consideration, such as: curve magnitude of the primary curve measured by Cobb angle, location of the primary curve and Risser sign.

For the statistical analysis, the program SPSS Statistics for Windows 21.0 has been used. To test whether significant differences exist between the examined variables and BMI groups, variance tests (ANOVA) and t-test with unequal variance have been performed. To test the correlations between BMI and other variables, Pearson correlation coefficient was used. The level of significance has been set to $p < 0.05$.

Results

Out of 213 patients, 170 were females (79.8 %) and 43 males (20.2 %). The mean age at brace prescription was 13.5 (SD ± 2.4, range 5 to 17.5) years. The mean BMI was 44.2 percentiles (SD ± 29.74). The results, according to groups, were as follows: low BMI has been detected in 21 patients (10 %), mid-BMI in 167 patients (78 %) and high BMI in 25 patients (12 %). The mean magnitude of the primary curve was 29.53° of Cobb angle (SD ± 9.2, range 13-59°). The mean Risser sign was 1.65 (SD ± 1.4, range 0 to 4). The mean initial in-brace correction was 53.5 % (SD ± 24.6, 0-100 %).

Table 1 shows the number of patients according to BMI groups, as well as mean values of their age at brace prescription, radiological parameters (curve magnitude and Risser sign) and in-brace correction.

Table 1: Patients' mean values (± SD) of age, radiological parameters and in-brace correction according to BMI groups

BMI groups*	Number of patients	%	Age at brace prescription (in years)	Risser sign	Curve magnitude (°)	In-brace correction (%)
Low BMI	21	10	14.5 (± 2.43)	1.7 (± 1.56)	32.4 (± 9.72)	46.7 (± 21.2)
Mid BMI	167	78	13.8 (± 2.24)	1.8 (± 1.44)	29.2 (± 8.96)	54.8 (± 26.2)
High BMI	25	12	11.5 (± 2.78)	0.8 (± 1.22)	29.5 (± 10.45)	50.8 (± 26.8)

**Low BMI < 5 percentiles; Mid BMI 5-85 percentiles; High BMI > 85 percentiles*

BMI and in-brace correction

Figure 1 illustrates the mean values (%) of the initial in-brace correction in total and according to BMI groups. Even though the greatest in-brace correction has been detected in the mid-BMI group (54.79 %), and the least in the low-BMI group (46.73 %), the difference is not significant (F = 1.18, p = 0.31).

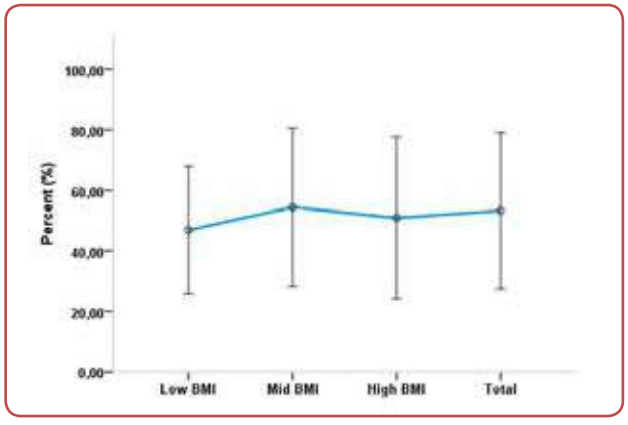

Figure 1: Mean values (±SD) of in-brace correction (%) in total and according to BMI groups

Figure 2 shows the results of the correlation between BMI, expressed in percentiles, and in-brace correction. It depicts a negative correlation that is not statistically significant (r = -0.061, p = 0.38).

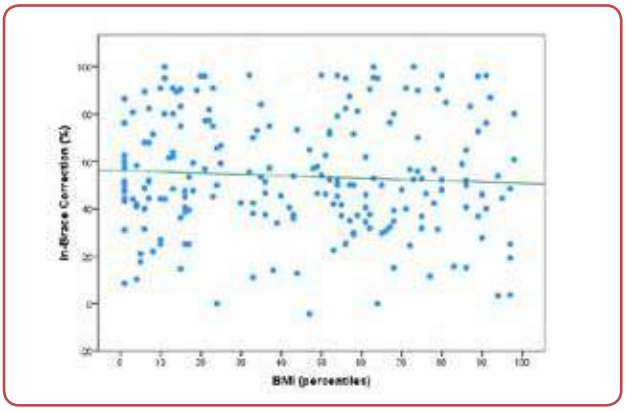

Figure 2: Correlation between BMI and in-brace correction

Curve magnitude and in-brace correction

Figure 3 displays the mean values of curve magnitude (°) and in-brace correction in total and according to BMI groups.

There was no significant difference in curve magnitude between BMI groups (F = 1.13; p = 0.33). There is a significant negative correlation between curve magnitude and in-brace correction (r = -0.399, p = 0.00). There is a better correlation in high- (r = -0.622, p = 0.001) and low-BMI groups (r = -0.615, p = 0.003), compared to the mid-BMI group (r = -0,402, p = 0,00).

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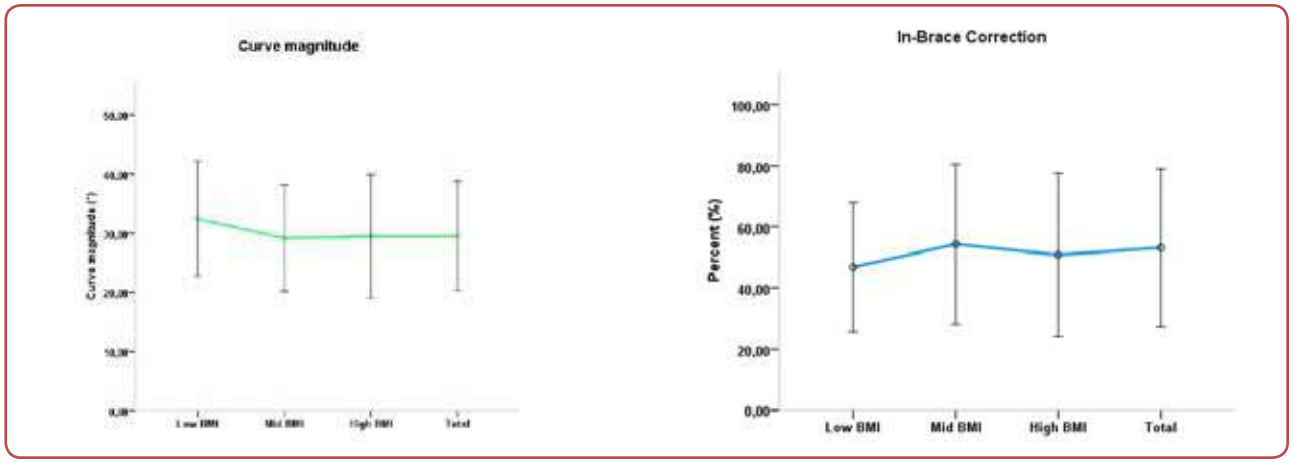

Figure 3: Mean values (±SD) of curve magnitude and in-brace correction in total and according to BMI groups

Curve location and in-brace correction

Figure 4 shows the in-brace correction in BMI groups according to the location of the primary curve.

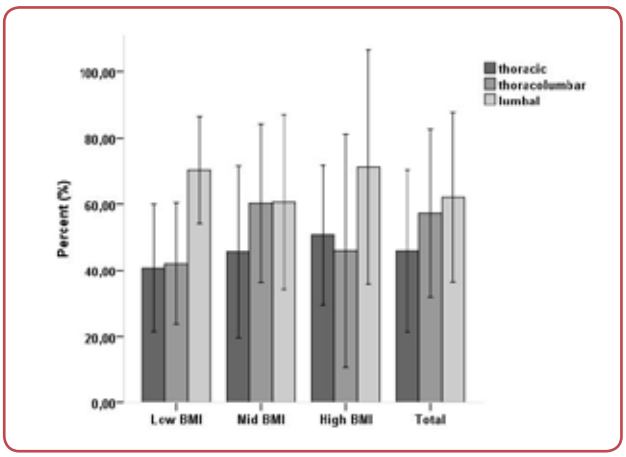

Figure 4: Mean values (± SD) of in-brace correction for different curve locations in total and according to BMI groups

The location of the primary curve is thoracic in 94 patients (44.1 %), thoracolumbar in 76 patients (35.7 %) and lumbar in 43 patients (20.2 %). There was a significant difference in the in-brace correction between groups that are categorised according to the location of the primary curve ($F = 7.59$, $p = 0.001$).

A better in-brace correction was observed in patients with primary lumbar curves (62 %), as opposed to patients with primary thoracic curves (46 %) in the low- ($p = 0.035$) and mid-BMI group ($p = 0.012$). There were no significant differences in the correction between thoracolumbar and lumbar curves ($p = 0.588$).

A significant correlation can be observed between in-brace correction and location within the low- ($F = 4.006$, $p = 0.036$) and mid-BMI group ($F = 6.653$, $p = 0.002$). The correlation in the high-BMI group was not significant ($F = 0.698$, $p = 0.508$).

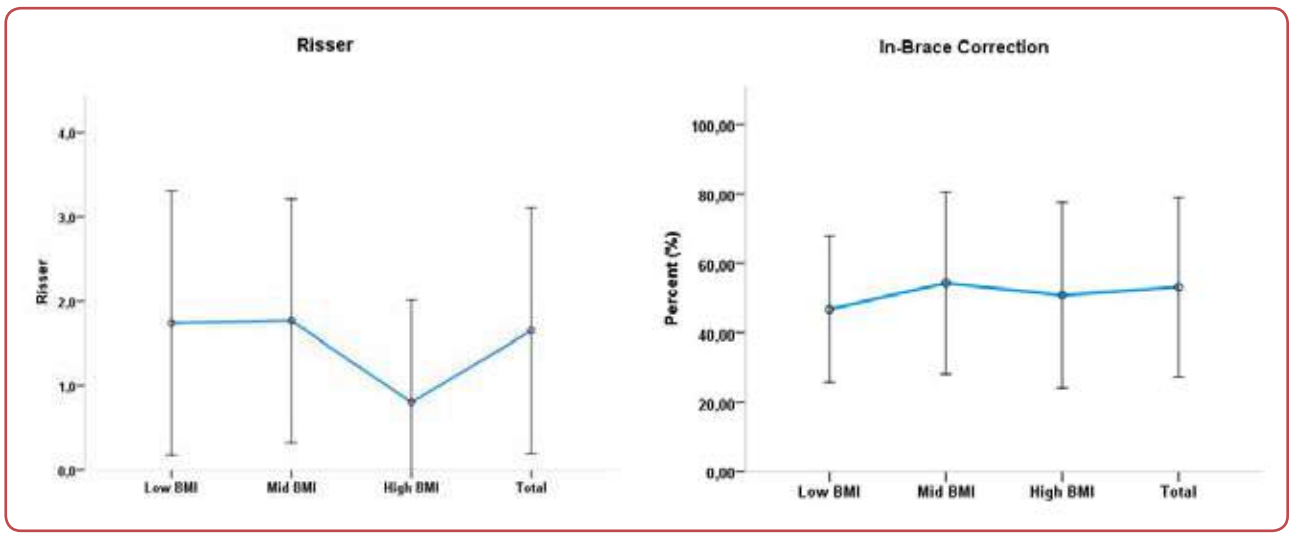

Figure 5: Mean values (± SD) of in-brace correction for different curve locations in total and according to BMI groups

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Risser sign and in-brace correction

Figure 5 depicts mean values of the Risser sign and in-brace correction (%) in total and according to BMI groups.

A significant difference in the Risser sign between BMI groups (F = 5,001, p = 0.008) has been identified, especially between mid- and high-BMI groups (F = 5.01; p = 0.01).

A lower Risser sign in the high-BMI group can be explained by the patients' age, who were significantly younger compared to the other two groups (F = 11.45; p = 0.00).

There was a significant negative correlation between in-brace correction and Risser sign (r = -0.203, p = 0.003). Within group, this correlation was only significant for mid-BMI group (r = -0.250, p = 0.001).

Discussion

Bracing proved to be effective in the treatment of idiopathic scoliosis.[9] In-brace correction is considered to be one of the most important parameters for brace quality assessment, but it can also be a predictor for short-term and long-term treatment outcomes in patients with idiopathic scoliosis. In the literature, currently reported percentages of in-brace correction vary from 20-25 % to 40-50 %. This depends on various factors, such as curve magnitude and location, Risser sign, curves exceeding 30°, vertebral rotation, compliance and BMI.[10] It is suggested that the in-brace correction must be at least 30-50 % in order to prevent significant curve progression.[11] In his study, Landauer reported that a good initial correction, of over 40 % in a Cheneau type brace, had significant impact on the final outcome.[12] According to another study[13] insufficient in-brace correction is associated with poor treatment outcome. For a successful orthotic treatment of patients with idiopathic scoliosis, it is significant to understand how different factors affect in-brace correction.[14]

The primary objective of the present study has been to determine whether the BMI significantly impacts in-brace correction in patients with idiopathic scoliosis, as well as whether it can be considered one of the predictive factors for treatment outcome. Previous studies on the influence of BMI on in-brace correction and outcome have showed contradictory results. High BMI is considered a negative predictive factor for brace effectiveness. It has been reported that patients with a high BMI have a 3.1-fold greater likelihood of unsuccessful bracing than normal-weight patients.[15] This is explained by the reduced action of corrective forces on the spine over the ribs due to excessive adipose tissue. These findings have been confirmed by Goodbody and colleagues[16] where such poor treatment outcome in high-BMI patients is associated with insufficient in-brace correction and compliance. Furthermore, it has been found that a low BMI is an independent risk factor for poor outcome. It should be noted that in this study different range was set in low-BMI category (< 20 percentiles). Similar results were observed in the study of Weixiang and colleagues[17] who concluded that low BMI could predict brace failure. Lori and colleagues[18] likewise concluded that a low BMI has the highest progression and the greatest surgical risk, although they were more complaint than high-BMI patients.

The results of present study showed that the BMI has no significant impact on in-brace correction in patients with idiopathic scoliosis. Therefore, it cannot be considered a predictive factor for the final outcome of bracing treatment, given the significance of immediate in-brace correction in predicting long-term results.[19]

The present findings are in accordance with those of Zaina and colleagues.[20] They reported that high BMI cannot be considered a negative predictive factor for treatment outcome, due to insignificant differences in in-brace correction between mid- and high-BMI group. Their study concluded that type of treatment, brace type and efficiency play a key role in stopping the curve progression.

Even though a negative correlation between BMI and in-brace correction can be observed, it is not significant. The mean in-brace correction in these patients can be considered sufficient for successful outcome by the current standards (53 %). The lowest in-brace correction has been identified in the low-BMI group, which can be explained by older age, higher curve magnitude and Risser sign, with significant difference compared to other groups. This group also included more thoracic curves that show lower in-brace correction than

CONTENT

lumbar curves. Nevertheless, there is no significant difference in in-brace correction between the low-BMI group compared to the other two groups.

In this study, other factors, such as curve magnitude and location, play a more critical role in the in-brace correction. There is a significant negative correlation between curve magnitude and in-brace correction, especially in the low- and high-BMI groups. There is also a significant correlation between the location of the primary curve and in-brace correction in the low- and mid-BMI group. In both groups, better correction was achieved in lumbar curves, compared to thoracic curves, while no difference has been identified between thoracolumbar and lumbar curves.

Even though a significant negative correlation can be observed between in-brace correction and Risser sign, it only occurs in the mid-BMI group. Limitations of this study include its retrospective nature and inability to take into consideration other important factors associated with in-brace correction and treatment outcome – such as adherence to treatment and compliance – due to lack of appropriate objective monitoring. Dose response to bracing has been earlier confirmed[21] concluding that wearing a brace a greater number of hours correlated with lack of curve progression and vice versa. Hours of bracing is associated with successful orthotic treatment.[22]

In conclusion, the results of this study show that the BMI, as an independent factor, does not impact initial in-brace correction. Other factors, such as curve magnitude, curve location and Risser sign, play a more significant role in the orthotic treatment of patients with idiopathic scoliosis. There are also other important factors that affect in-brace correction, such as compliance, that authors were not able to monitor. Even though some studies report that the BMI plays a significant role in treatment outcome, from these results it can be concluded that BMI cannot be considered as relevant prognostic factor for in-brace correction and treatment outcome.

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None.

Conflict of interest

None.

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CONTENT

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PROFESSIONAL ARTICLE

Body Scrub Containing Virgin Coconut Oil, Coffee Grounds (*Coffea arabica* Linn) and Carbon Active Coconut Shell (Activated Carbon *Cocos nucifera* L) as a Moisturiser and a Skin Brightener

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Abstract

Introduction: Virgin Coconut Oil (VCO) contains a lot of medium chain fatty acids. VCO combined with coffee grounds (*Coffea arabica* Linn) and activated carbon (Activated carbon *Cocos nucifera* L) has the potential to form a preparation that can moisturise and brighten the skin. The purpose of this study was to make cosmetic cleansing preparations containing three natural ingredients.
Methods: This study evaluated the organoleptic body scrub preparations, homogeneity, dispensability and pH for three different formulations. *In vivo* test for irritation (oedema and erythema) was carried out on albino rabbits (n = 3) for each treatment group. Clinical irritation testing was performed on the forearm of healthy volunteers, 17 - 45 years of age with no history of allergies (n = 30). Determination of skin moisture content and melanin index was carried out as a measure of effectiveness.
Results: Organoleptic and homogeneity tests showed that preparations had dark black colour, the distinctive smell of coffee, it was homogeneous, spread ability was in the range of 4 cm with the pH at a safe pH for the skin. Irritation results also did not show any oedema and erythema in *in vivo* testing. In clinical testing no irritation occurred by testing the body scrub samples. Body scrubs routinely used by volunteers for 2 months increased moisture on the skin as well as brightness ($p < 0.05$).
Conclusion: Body scrub creams containing VCO, coffee grounds and activated carbon are preparations that have the potential to be cosmetic cleansers.

Keywords: Body scrub; Virgin coconut oil; Coffee grounds; Activated carbon.

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Introduction

Body scrub is a body care product that can be used to maintain healthy skin. The basic ingredients for body scrubs are always the same, namely as a cleanser.[1] The basic ingredients for body scrubs are usually made from synthetics as well as from natural ingredients derived from herbal plants.[2] Herbal plants are one that can be used as safe pharmaceutical preparations and as cosmetics that can maintain healthy skin. One of the Indonesian herbal plants that can be used is Virgin Coconut Oil (VCO). The content of saturated fatty acids in VCO can be used as a cosmetic form because it can moisturise the skin and VCO also has a high sun protection factor (SPF).[3] In addition, other natural ingredients such as coffee grounds can also be used as cosmetic ingredients in skin

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CONTENT

care, because coffee has a high antioxidant content.[4] Coffee grounds also have a distinctive aroma and rough texture so they can be used to remove dead skin cells.[5] Coffee grounds also have a long life shelf for 9 months.[6] Another natural ingredient that can be used is activated charcoal which has detoxification activities.[7] The body scrubs made in this study were in the form of creams. The cream form has physical stability which can increase the effectiveness of active ingredients on the skin, it is easy to use and to distribute, thus consumers prefer to use cream body scrubs rather than other forms.

Material and Methods

Materials

VCO and activated charcoal were obtained from Galenika (Central Java, Indonesia). Coffee grounds were obtained from several coffee shops in the Balaraja area (Tangerang, Indonesia). Cetearyl alcohol and catearate (Cera lanae), phenoxy ethanol, propylene glycol, liquid paraffin, cetyl alcohol, corn starch, polyethoxylated sorbitan and oleic acid (Tween 80), distilled water (Aquadest) were obtained from Brataco (Jakarta, Indonesia).

Methods

The procedure carried out has been approved by the Health Research Ethics Committee, Muhammadiyah University Prof. Dr. Hamka, with ethical approval number 01 / 19.11 / 0240.

Preparation of Body Scrub

Body scrubs were made by using the basic ingredient of Cera de lano, using additives phenoxy ethanol, propylene glycol, liquid paraffin, cetyl alcohol, tween 80.

The procedure for making a body scrub was: Cera de lano was mixed over a water bath (70 °C) and mixed until it was homogeneous with other ingredients. For each formula (Formula I, Formula II and Formula III) VCO with distilled water (70 °C) were added and mixed until a white cream base was formed. Coffee grounds, activated charcoal and corn starch were added, then stirred using a homogenizer for about 5 minutes until a body scrub was formed. The composition of the formulas is shown in Table 1.

Table 1: Body scrub formulations containing Virgin coconut oil, ground coffee and activated carbon*

Formulation	Body Scrub Formula		
	F1	F2	F3
Virgin coconut oil (VCO)	5	15	25
Activated carbon	1	3	5
Coffee grounds	9	7	5
Cera lanae (mix of cetearyl alcohol and catearat 33)	14	14	14
Phenoxy ethanol	0.8	0.8	0.8
Propylene glycol	10	10	10
Liquid paraffin	5	5	5
Cetyl alcohol	2	2	2
Corn starch	6	6	6
Tween 80	3	3	3
Distilled water ad	100	100	100

** Virgin coconut oil (VCO) is coconut oil that is extracted. VCO is made with copra or dried coconut meat that is removed from the shell and pressed to extract natural oils. Tween 80 it is made from polyethoxylated sorbitan (chemical compounds derived from the dehydration of sugar alcohol) and oleic acid, a fatty acid found in animal and vegetable fats.*

Evaluation of Body Scrub

Organoleptic Test

Organoleptic testing was done visually with the changes in shape, colour and smell of body scrub preparations. The test was carried out for 8 weeks with examination time intervals, namely at week 0, 2, 4, 6 and 8 at 25 °C.

Homogeneity Test

To clarify the homogeneity of body scrub preparations, homogeneity testing was carried out using a microscope (Olympus CX23) with magnification (magnification of 40 times). A number of samples were smeared on the glass preparation then the top was covered with a glass preparation. Sample testing was done by taking 3 parts of the body scrub preparation, namely top, middle, bottom.[8]

Spread ability

Samples (0.5 g) of each formula were placed in the centre of the Petri plate, then the Petri plate was placed on top. A load of 150 g was given for 1 minute at 25 °C, after which the diameter of the spread was measured. The diameter of the dispersive power should be in the range 3 - 5 cm.[9]

pH Evaluation

The pH evaluation was carried out using a pH meter (Mettler Toledo S220). The sample was made as 1 % solution, ie 1 g sample was dissolved in 100 mL of distilled water. The acceptable pH was considered in the range 4.5-6.5.[9]

CONTENT

Effectiveness of Body Scrub

The humidity tester used the Dermalab combo (Cortex) tool. Volunteers with the inclusion criteria were 17 - 45 years old, with healthy skin (n = 30). Non-inclusion criteria were: pregnant and breastfeeding women and/or those that at that time were using drugs that can affect the condition of the skin. It was recommended that volunteers apply the body scrub sample twice a week. Determination of skin moisture content and melanin index was carried out at 0, 1 and 2 months after usage.

Pre-Clinic and Clinical Evaluation of Body Scrub

***In Vivo* Evaluation of Irritation**

In vivo test for irritation (oedema and erythema) was carried out on albino rabbits (n = 3) for each treatment group. The test animals were shaved and then given body scrub samples with their respective formulas (F1, F2 and F3). The sample given was smeared with non-reactive gauze. The degree of irritation was measured after 1, 24, 48 and 72 hours. Measurements were made by giving a score: (0) negative reactions; (1) a little irritation; (2) bordered edge irritation; (3) moderate drainage (± 1 mm rising edge); (4) severe irritation (rising edge > ± 1 mm and extending beyond the curb). The score depended on the severity of the skin reactions was produced.[10]

Evaluation of Clinical Irritation

Clinical irritation testing was performed on the forearm of volunteers (n = 30), with an area of 2.5 x 2.5 cm. The criteria for volunteers were: 17 - 45 years of age, healthy, with no history of allergies. After the basting was done, it was left closed for 24 hours, then the observation was carried out for 72 hours. The irritation reaction is characterised by redness, itching and swelling. Measurements were made by giving a score: (0) negative reactions; (1) redness; (2) hives; (3) swelling.[11]

Results

Preparation and Evaluation Body Scrub

A body scrub has been successfully made with a cream base. Organoleptic evaluation by conducting visual observations (Figure 1a), showed that the body scrub sample containing VCO, coffee grounds and activated carbon had a dark black colour with a very sharp distinctive smell of coffee. Homogeneity testing has been carried out under a microscope (Figure 1b). The results of the ob-

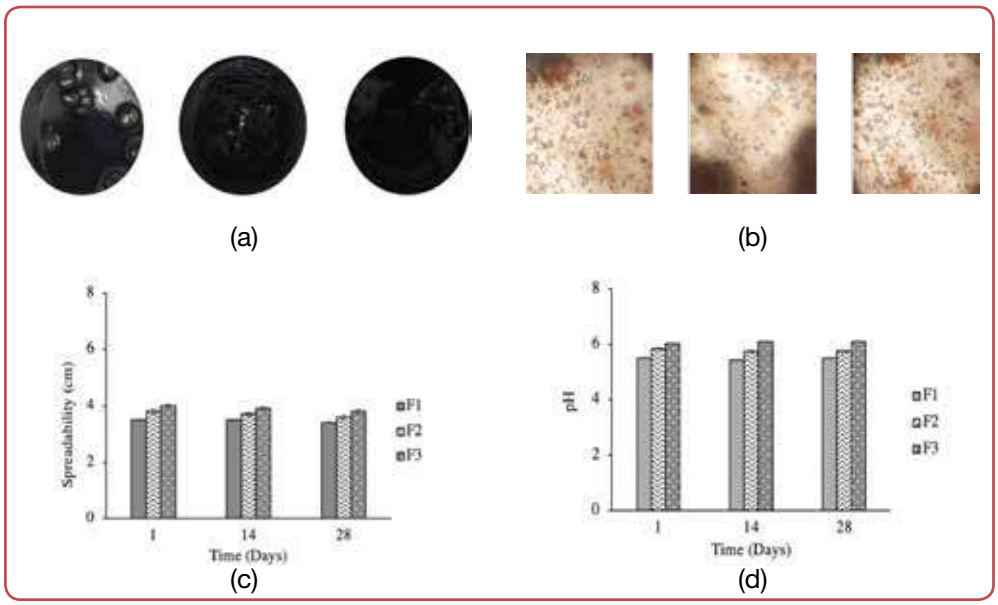

Figure 1: Evaluation of body scrub of VCO, grounded coffee and activated carbon (F1, F2 and F3): (a) Organoleptic test; (b) Homogeneity test; (c) Spreadability test; (d) pH

** F1, F2, F3: different formulation of ingredients*

servations showed that the results of each sample were homogeneous with no lumps. The dispersion test was successfully carried out for 1, 14 and 28 days (Figure 1c). The diameter of the F1, F2, and F3 spread ability tests met the requirements because they were in the 3-5 cm range and there was no significant difference between samples $p > 0.05$. Body scrubs had an acceptable pH and were in a pH range that is safe for the skin (Figure 1d).

Pre-Clinic and Clinical Evaluation of Body Scrub

The rabbits were divided into four test groups (n = 3) (Figure 2a). In testing with groups formulation 1, 2 and 3 and observations made after 1, 24, 28 and 72 hours, it was shown that body scrubs were safe to use and did not cause oedema or erythema in rabbits.[12]

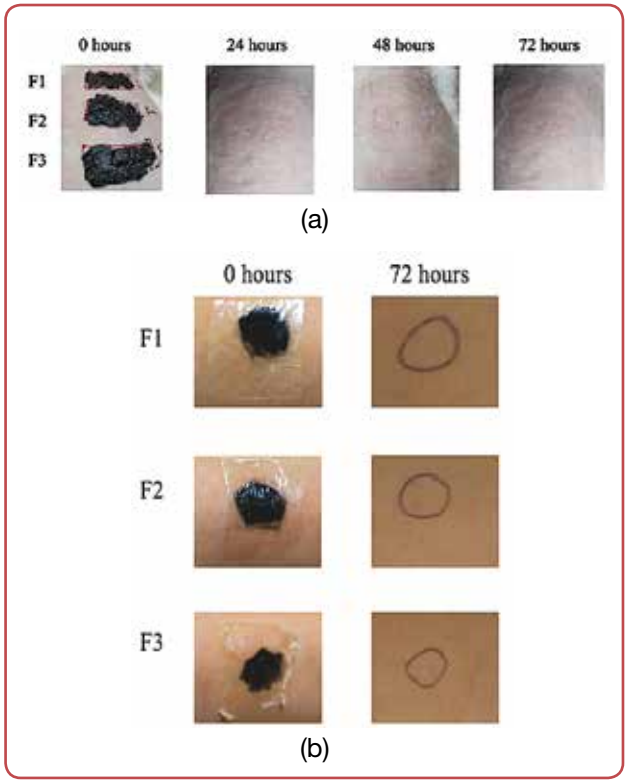

Figure 2: Pre-clinic and clinical evaluation of body scrub of VCO, ground coffee and activated carbon (F1, F2 and F3): (a) Evaluation in vivo of irritation; (b) Evaluation of clinical irritation
** F1, F2, F3: different formulation of ingredients*

Clinical testing on human skin were performed on three test groups (n = 30). Observation on volunteers was carried out for 72 h (Figure 2b). The results of clinical testing using body scrubs F1, F2 and F3 confirmed negative reactions to the skin of the volunteers, meaning that no irritation occurred by testing the body scrub samples.

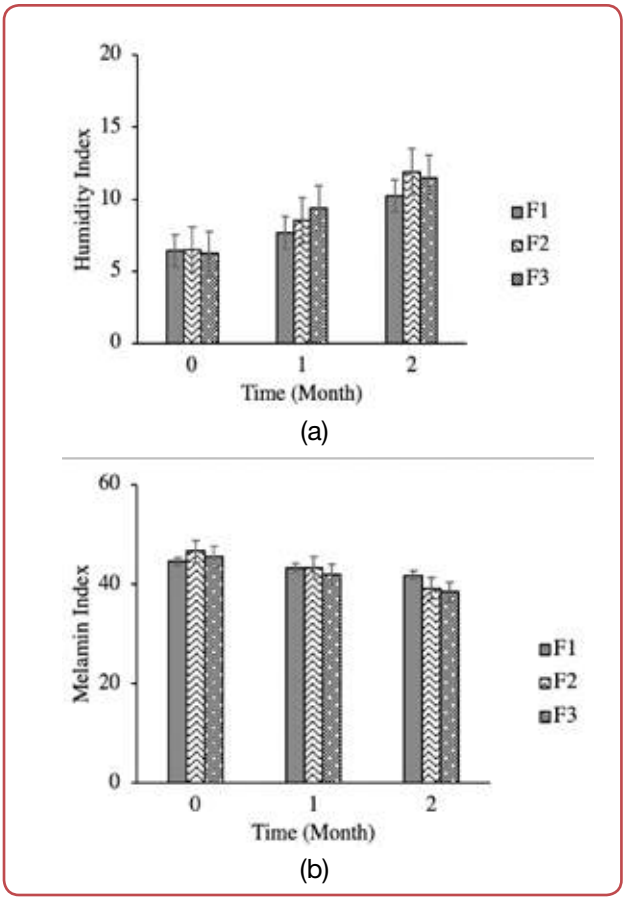

Figure 3: Effectiveness of Virgin coconut oil body scrub, ground coffee and activated carbon (F1, F2 and F3): (a) Humidity index; (b) Melamine index
** F1, F2, F3: different formulation of ingredients*

Effectiveness of Body Scrub

The effectiveness of VCO body scrubs, coffee grounds and activated carbon by performing moisture testing is shown in Figure 3a. Observations were made before being given treatment and after being given treatment for 0, 1 and 2 months. Body scrubs used by volunteers for 2 months routinely increased moisture on the skin with a significance value of $p < 0.05$ without significant difference between formulas (Kruskal-Wallis test, $p > 0.05$). The results of brightness testing on VCO body scrubs, coffee grounds and activated carbon on the skin of volunteers (Figure 3b) showed that there was a significant difference between the time intervals of observation $p < 0.05$.

Discussion

The results of the observations showed that each sample was homogeneous with no lumps. Homogeneity is influenced by the forming ingredients

CONTENT

between the water base and the oil base forming a good cream mass. Good spread ability is obtained because the ingredients that make up cream of cetyl alcohol are fatty alcohols which form a white solid, such as wax, and catereat is an oil/water (O/W) base which can form a dense and oily mass like butter.

In tests on days 1, 14 and 28, it was found that there was no significant change in pH in body scrub samples during the storage period $p > 0.05$. The pH was safe because catereat base material can form a stable pH in cosmetic preparations. In addition, the content of activated carbon has a water content of 15 % which can increase the pH in the skin pH range (4.5 - 6.5). Also, evaluation of body scrub preparations obtained good results because VCO has the stability for 48 days, where this stability affects homogeneity/appearance, changes in colour, odour, consistency, pH and viscosity.[13]

Body scrub preparations with VCO active substances, coffee grounds and activated carbon are safe for use on human skin.[14] The absence of irritation to the skin is also influenced by the physical and chemical properties of VCO which is very good for use as a moisturiser on the skin. Coconut dregs which have the ability as an antioxidants are also able to absorb into the skin layer, where antioxidants can neutralise free radicals.[15, 16]

Comparative data between the three formulas did not have a significant difference in the increase in skin brightness in the volunteers ($p > 0.05$). The effectiveness of body scrub is supported by the presence of antioxidant abilities in coffee grounds which can increase collagen productivity in the skin. The antioxidant content in skin care products, particularly vitamin C, has been shown to be beneficial in reducing melanin pigmentation in the skin.

Conclusion

VCO body scrub with coffee grounds and activated carbon with various formulas 1, 2 and 3 were successfully carried out with several evaluations confirmed. The difference in concentration in each formula did not affect the evaluation results of body scrubs. The pH of each formula was still in the safe range for the skin. The pH results were correlated with *in vivo* testing as well as clinical irritation testing carried out for 72 h without oedema and erythema in the skin of the volunteers. The results of testing the effectiveness of moisture and brightness on VCO body scrubs with coffee grounds and activated carbon found: the longer the scrub was used, the more moisturised and brighter volunteer skin was.

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Conflict of interest

None.

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CASE REPORT

3,4-Methylenedioxymethamphetamine (MDMA) -Induced Macular Haemorrhage: a Case Report

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Abstract

A case of 28-year-old female patient with retinal haemorrhage after taking 3,4-methylenedioxymethamphetamine (ecstasy, MDMA) and having a sexual intercourse is described. Ecstasy is a drug that is often consumed by young people. It leaves various consequences on the human body. Retinal haemorrhage in the eye caused by ecstasy has been described before. Like in this case, the experience in spontaneous resolving of the MDMA-induced retinal haemorrhage is favourable.

Key words: Illicit drugs; 3,4-methylenedioxymethamphetamine; Retinal haemorrhage.

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Introduction

3,4-Methylenedioxymethamphetamine (MDMA), named also ecstasy or molly is a psychoactive synthetic drug, most commonly taken by young people for recreational purposes.[1] MDMA achieves its effects by releasing the neurotransmitters serotonin, dopamine and noradrenaline in the brain. It leads to an increase in energy, amplify sensory perception, pleasure and also empathy.[2] It is usually taken orally, as a tablet or a capsule. The first effects are felt after about 30 minutes and can last for several hours.[3] MDMA users encounter side effects of this drug such as addiction, sweating, palpitations, sleep and memory problems, paranoia, blurred vision.

Case History

A 28-year-old white female patient was referred to the hospital after a sudden decrease in vision on her left eye that happened ten days ago and which was preceded by a strong headache. After taking a thorough patient's medical history, the patient admitted that she had taken ecstasy and was sexually active after that. The patient was a recreational drug user. On examination her uncorrected visual acuity (UCVA) on the right eye was 1.0 (Snellen), and on the left 0.5 (Snellen) without a possibility for additional spectacle correction. Intraocular pressure was normal (13 mmHg on the right eye, and 12 mmHg on the left eye). Slit-lamp exam had shown no afferent pupillary

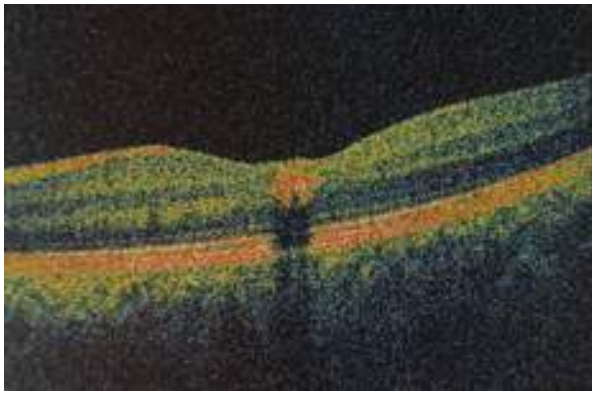

Figure 1: Optical coherence tomography (OCT) of a 28-year-old female patient with 3,4-methylenediohymethamphetamine (MDMA)-induced foveal retinal haemorrhage. OCT taken on 27 March 2020.

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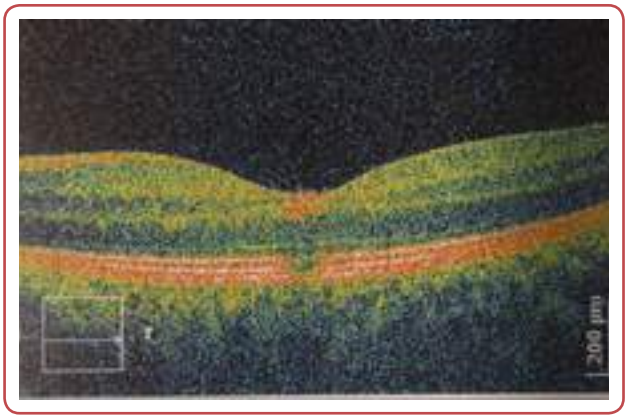

Figure 2: Optical coherence tomography (OCT) of a 28-year-old female patient with 3,4-methylenediohymethamphetamine (MDMA)-induced foveal retinal haemorrhage. OCT taken on 1 April 2020.

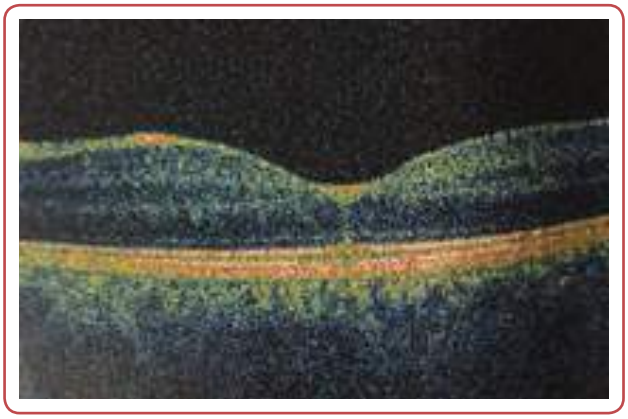

Figure 3: Optical coherence tomography (OCT) of a 28-year-old female patient with 3,4-methylenediohymethamphetamine (MDMA)-induced foveal retinal haemorrhage. OCT taken on 11 April 2020.

defect, nor any other pathological findings in the anterior segment. Furthermore, fundus exam of the right eye was normal and without any pathological findings, while intraretinal haemorrhage was found in the left fovea. The finding was then confirmed by the optical coherence tomography (OCT) of the left eye (Figure 1). As a precaution, the patient was referred for a detailed cardiological and neurological exams, which both turned out normal (blood pressure 110/70 mmHg, heart rate 65 beats/min, no neurological illnesses). No local or systemic ophthalmological therapy was prescribed.

Four days after the initial exam, the patient returned for a regular check-up. Subjectively, her left-eye vision was better. On examination, the right eye retained previous UCVA, while the left eye improved to 0.8 (Snellen) of the UCVA. Fundus exam showed the regression of the haemorrhage, which was also confirmed with the OCT (Figure 2). The improvement in vision was also noticed ten days later when the patient came for the final check-up. The fundus exam showed clear fovea, and the same findings were seen on the OCT (Figure 3). The left eye UCVA was 0.9 (Snellen).

Discussion

In this case report, a female patient with macular haemorrhage after taking ecstasy that led to vision blurring and loss of visual acuity (VA) was presented. Physical strain, increased blood pressure and heart rate could have been additional contributing factors. It would be good if MDMA could have been identified in blood or urine, but the time of elimination of MDMA from the body is about 3-5 days. The patient came to the clinic 10 days after loss of visual acuity. Urinary recovery of MDMA and metabolites over 5 days ranged between 24 % and 52 % of the MDMA dose. Of the total MDMA recovered in urine, 46 % and 36 % were excreted between 0–24 h after the low and high doses, respectively.[4] After 30 minutes, the concentration of MDMA in the blood stream begins to rise[5] and 1, 5 and 3 hours after consumption it reaches its maximum values in the blood.[6] In the next few hours the peak concentrations of MDMA and its metabolites are halved due to their metabolism and excretion.[7] It is possible that Valsalva's phenomenon caused by the sexual intercourse contributed to the bleeding or that the use of MDMA facilitates the mechanism of Valsalva bleeding. In humans who are using low doses of MDMA there are some adverse changes in brain microvasculature.[8–9] Large preretinal haemorrhages can be caused by Valsalva retinopathy.[10] It is not completely clear what is the mechanism of retinal bleeding and vision blurring.[11] Some studies suggest that it may be due to increased blood pressure and heart rate.[11–13] Blood dyscrasias were excluded by laboratory findings.

Ecstasy-induced macular haemorrhage was first described in 1998.[14] Although it is the authors' opinion that bleeding can occur in any part of the retina, macular, foveolar haemorrhage is noticed immediately because blurred vision occurs and such persons seek medical attention. Other drugs, methamphetamine, alcohol, cocaine, marijuana and antiepileptic drugs can induce vascular occlusion and retinal haemorrhage.[15–17] Like in this case, these other reports have also reported spontaneous resorption of macular haemorrhage after a certain period, without permanent consequences.

CONTENT

Drug abusers can sometimes have ocular complications because those substances cause transient increase in blood pressure and can cause a Valsalva retinopathy.[16] Ecstasy can cause ventricular fibrillation, hypertension, hyperpyrexia, tachycardia and intracerebral haemorrhage.[18-20]

If this case is analysed, the cause of retinal haemorrhage could be nothing but the rise in blood pressure. The cause of retinal haemorrhage is the same as the cause of intracerebral haemorrhage.[20] Cocaine and methamphetamine taken nasally can also cause retinal haemorrhage,[21] because they have direct effect on autonomous and vasoactive nerves in choroid but indirect in the retina.[16] This makes the retinal blood vessels susceptible to changes in elevated blood pressure and is a possible mechanism for the occurrence of haemorrhage in this patient. Therefore, ecstasy can be seen as an indirect factor of retinal haemorrhage.

Conclusion

In conclusion, it could be said that this case shows not only the effects that MDMA can have on the retinal pathology, but also that the best treatment of these diseases in many cases is no treatment at all.

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None.

Conflict of interest

None.

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3. Text
The text of the articles includes the following chapters: Introduction, Methods, Results and Discussion. Longer articles may need subheadings within some sections to clarify their content. Case reports should have four sections: Introduction, Case history, Discussion and Conclusion.

Introduction. After the introductory notes, the aim of the article should be stated in brief (the reasons for the study or observation), only significant data from the literature, but not extensive, detailed consideration of the subject, nor data or conclusions from the work being reported.

CONTENT

Methods. Selection of study or experimental subjects (patients or experimental animals, including controls) should be clearly described. Methods, apparatus (manufacturer's name and address in parentheses) and procedures should be identified in sufficient detail to allow other workers to reproduce the results. References should be cited for established methods, including the statistical ones. Identify precisely all drugs and chemicals used, with generic name(s), dose(s), and route(s) of administration. Statements on the study approval by the Ethics Committee for human or animal studies should be included.

Results should be presented in logical sequence in the text, tables and illustrations. Emphasise or summarise only important observations.

Discussion is aimed to emphasise new and significant aspects of the study and conclusions that result from them. Relate the observations to other relevant studies.

Conclusion should be linked with the goals of the study, but avoid unqualified statements and conclusions not completely supported by your data.

Tables
Each table should be typed single-spaced on a separate sheet, numbered in the order of their first citation in the text in the upper right corner and supplied with a brief title each. Explanatory notes are printed under a table. Each table should be mentioned in the text. If data from another source are used, acknowledge them fully. Each table should be named in order to indicate the author's name the number of the table, (eg Johnson_Table 1). The place of the table in the text should be marked as in the following example: "(Table 1 near here)".

Illustrations
Any forms of graphic enclosures are considered to be figures and should be submitted as additional databases in the System of Aseestant. Letters, numbers, and symbols should be clear and uniform, of sufficient size that when reduced for publication, each item will still be legible. Each figure should be named in order to indicate the author's name and the number of the figure (eg Johnson_Figure 1). If a figure has been published, state the original source.

Captions for illustrations are typed on a separate page, with Arabic numbers corresponding to the illustrations. If used to identify parts of the illustrations, the symbols, arrows, numbers, or letters should be identified and explained clearly in the legend. The method of staining and magnification in photomicrographs should be explained. Captions should be detailed enough to allow for understanding of the content of the figure without previous reading of the text. The place of the figure in the text should be marked as in the following example: "(Figure 1 near here)".

Abbreviations and acronyms
Authors are encouraged to use abbreviations and acronyms in the manuscript in the following manner: abbreviations and acronyms must be defined the first time they are used in the text and thereafter must be consistently used throughout the whole manuscript, abbreviations should be used only for terms that appear more than three times in text; abbreviations should be sparingly used. Use of abbreviations in the titles should be avoided. Even if used after its definition in the abstract, the same definition and repetition of the abbreviation should be performed the first time it is used in the text. In order to assure self-explanatory nature of the tables and figures, abbreviations and acronyms should be defined in the captions and then introduced, irrespective of whether it was done earlier in the text.

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References should be superscripted and numerated consecutively in the order of their first mentioning within the text. All the authors should be listed, but if there are more than 6 authors, the first 6 should be quoted, followed by comma and "et al". Abstracts, secondary publications, oral communications, unpublished papers, official and classified documents should not be used. References to papers accepted but not yet published should be cited as "in press". Data from the Internet are cited with the date of citation. Articles from the e-journals should always contain DOI code.

Examples of references:

Gillespie NC, Lewis RJ, Pearn JH, Bourke ATC, Holmes MJ, Bourke JB, et al. Ciguatera in Australia: occurrence, clinical features, pathophysiology and management. Med J Aust 1986;145:584-90.

Hull J, Forton J, Thompson A. Paediatric respiratory medicine. Oxford: Oxford University Press; 2015. Bydder S. Liver metastases. In: Lutz S, Chow E, Hoskin P, editors. Radiation oncology in palliative cancer care. Chichester (UK): John Wiley & Sons, Ltd.; 2013. p. 283-298.

Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. p. 182-91.

Polgreen PM, Diekema DJ, Vandeberg J, Wiblin RT, Chen YY, David S, et al. Risk factors for groin wound infection after femoral artery catheterization: a case-control study. Infect Control Hosp Epidemiol [Internet]. 2006 Jan [cited 2007 Jan 5];27(1):34-7. Available from: http://www.journals.uchicago.edu/ICHE/journal/issues/v27n1/2004069/2004069.web.pdf.

CONTENT