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Address:
Serbian Journal of Experimental and Clinical Research, Faculty of Medical Sciences, University of Kragujevac
Svetozara Markovića 69, 34000 Kragujevac, PO Box 124
Serbia
izdavacka@medf.kg.ac.rs
www.medf.kg.ac.rs/sjecr

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PREDICTORS OF PRESSURE ULCERS IN PATIENTS WITH SPINAL CORD INJURIES

Sasa Milicevic[1], Zoran Bukumiric[2], Aleksandra Karadzov Nikolic[3], Rade Babovic[1], Aleksandra Sekulic[1], Srbislav Stevanovic[1], Slobodan Jankovic[4]
[1]Clinic for Rehabilitation "Dr M. Zotović", Sokobanjska 13, Belgrade
[2]Medical Faculty in Pristina, Institute of Medical Statistics and Informatics, Kosovska Mitrovica
[3]Institute of Rheumatology, Resavska 69, Belgrade
[4]Medical Faculty in Kragujevac, Institute of Pharmacology, Kragujevac

FAKTORI KOJI DOPRINOSE NASTANKU DEKUBITUSA KOD PACIJENATA SA POVREDAMA KIČMENE MOŽDINE

Saša Milićević[1], Zoran Bukumirić[2], Aleksandra Karadžov Nikolić[3], Rade Babović[1], Aleksandra Sekulić[1], Srbislav Stevanović[1], Slobodan Janković[4]
[1] Klinika za rehabilitaciju „Dr M. Zotović", Sokobanjaska 13 Beograd
[2] Medicinski fakultet u Prištini, Institut za medicinsku statistiku i informatiku, Kosovska Mitrovica
[3] Institut za reumatologiju, Resavska 69 Beograd
[4] Medicinski fakultet u Kragujevcu, Katedra za farmakologiju, Kragujevac

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ABSTRACT

Introduction: *Pressure ulcers (PUs) are often secondary complications in spinal cord injury (SCI) patients.*

Purpose: *To investigate the presence and possible factors associated with pressure ulcers in SCI patients undergoing acute and functional rehabilitation.*

Methods: *This was a retrospective study of 453 patients with SCI treated at the Clinic for Rehabilitation "Dr M. Zotovic", Belgrade, Serbia, between January 2000 and December 2009. Factors that were tested for their influence on pressure ulcers in spinal cord injury patients included age, sex, mechanism of injury, neurological level of injury, completeness of injury, spasticity and length of stay. The presence and location of pressure ulcers were recorded on admission, during acute and functional rehabilitation and at discharge. The level of statistical significance in our study was set to 0.05.*

Results: *The study included 453 patients:* 383 (*84.5%) did not have a pressure ulcer during rehabilitation, and 70 (15.5%) patients had a pressure ulcer during rehabilitation. Of the total number of patients, 333 (73.5%) were male, and 120 (26.5%) were female. The average age of patients enrolled in the study was 51.8 ± 17.2 years. In a multiple logistic regression model, one statistically significant predictor of pressure ulcers during rehabilitation was pressure ulcer before rehabilitation (B = 1420, p <0.001), with an odds ratio (OR) = 4.1. This result shows that patients who had pressure ulcers on admission are 4 times more likely to regain pressure ulcers during rehabilitation after controlling for all of the factors in the model. Another statistically significant predictor of pressure ulcers during rehabilitation was FIM score on admission (B = -0036, p = 0.015).*

Conclusion: *The prevention of pressure ulcers in acute and functional rehabilitation increases functional outcomes in patients with SCI.*

Keywords: *spinal cord injury, pressure ulcer, predictors, rehabilitation*

SAŽETAK

Uvod: *dekubitalni ulkus je česta komplikacije kod pacijenata sa povredom kičmene moždine.*

Cilj: *utvrditi učestalost i faktore rizika dekubitalnih ulkusa kod pacijenata sa povedom kičmene moždine u toku rehabilitacije.*

Materijal i metode: *ovaj rad predstavlja retrospektivnu studiju koja je obuhvatila 453 pacijenta sa povredom kičmene moždine koji su rehabilitovani u Klinic za rehabilitaciju Dr M. Zotović, Beograd, Srbija u periodu od Januara 2000. do Decembra 2009. godine. Faktori rizika dekubitlanih ulkusa kod pacijenata sa povredom kičmene moždine koji su ispitivani u ovoj studiji su: starost, pol, način povređivanja, neurološki nivo povrede, kmpletnost lezije, spasticitet i dužina boravka. Postojanje lokalizacija dekubitalnih ulkusa je bila registrovana na prijemu, u toku rehabilitacije i na otpustu. Nivo statističke značajnosti je testiran na nivou od 0.05.*

Rezultati: *u studiju je uključeno 453 pacijenata od kojih 383 (84.5%) nije imalo dekubitalni ulkus u toku rehabilitacije i 70 (15.5%) pacijenata sa dekubitalnim ulkusom u toku rehabilitacije. Od ukupnog broja pacijenata 333 (73.5%) na bilo muškog i 120 (26.5%) je bilo ženskog pola. Prosečna starost pacijenata koji su uključeni u studiju je bila 51.8±17.2 godina. U multiplo regresionom modelu statiskički značajan prediktor dekubitalnog ulkusa u toku rehabiltiacije je bio postojanje ulkusa pre rehabilitacije (B=1420, p<0.001), sa odnosom šansi OR=4.1. Ovo na pokazuje da pacijenti koji su imali dekubitalni ulkus na prijemu imaju četiri puta veću šansu da ponovo dobiju ulkus u toku rehabilitacije. Takođe statistički značajan predikto dekubitlanih ulkusa u toku rehabilitacije je bio FIM skot na prijemu (B= -0036, p<0.015).*

Zaključak: *prevencija dekubitlanih ulkusa skraćuje vreme rehabilitacije i povećava funkcionalni oporavak kod pacijenata sa povredom kičmene moždine.*

Ključne reči: *povrede kičmene moždine, dekubitalni ulkusi, prediktori, rehabilitacija*

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Correspondence to: Dr Saša Milićević, Clinic for rehabilitation "Dr M. Zotović", Sokobanjska 13, Belgrade, Serbia,
Home adrress: Bulevar Despota Stefana 110/43, Belgrade, Serbia; phone: +381655006070, e-mail: rsmilicevic@gmail.com

INTRODUCTION

A pressure ulcer (PU) is defined as a localised area of cellular necrosis and vascular destruction created as a result of prolonged exposure to pressure, shearing or friction (1). Pressure ulcers occur at a high incidence in spinal cord injury (SCI) patients (2). The epidemiology of PU varies considerably in patients with SCI by clinical setting, with incidence rates ranging from 0.4 to 38% in acute care, 2.2 to 23.9% in long-term care, and 0 to 17% in home care (3,4). The annual incidence and prevalence rates of PUs range from 20 to 31% and 10.2 to 30%, respectively (4). Yarkony and Heinemann reported a prevalence of 8% during initial rehabilitation, 9% during the 2-year follow-up after rehabilitation, and 32% 20 years after rehabilitation (5). In a previous study, over two hundred risk factors for the development of pressure ulcers were described. The most important factors are inactivity, incontinence, neurological level of injury, completeness of injury, autonomic dysreflexia, secondary complications, such as spasticity, and nutritional, physical and socioeconomic status (5,6). Secondary conditions, such as smoking, age and some conditions or diseases, such as gastrointestinal, cardiopulmonary and renal diseases, diabetes mellitus, reduced cognitive abilities, malnutrition, anaemia, hypoalbuminaemia (<3.4 g/dl) and low haemoglobin (<12 g/dl) (8), play an important role in the development of pressure ulcers. PUs may have a significant impact on patients with SCI. PUs significantly decrease the quality of life, and if the PUs cannot be treated conservatively, they may lead to interrupted rehabilitation, extended length of stay, increased cost of care and increased hospital readmission (9,10).

The first aim of our study was to investigate occurrence of pressure ulcers in patients with spinal cord injuries. The second aim of this study was to identify factors that influenced the development of PUs during rehabilitation.

MATERIALS AND METHODS

This was a retrospective study of 453 patients with spinal cord injury treated at the Clinic for Rehabilitation "Dr M. Zotovic", Belgrade, Serbia, between January 2000 and December 2009. For all of the patients, a detailed hospital history with respect to sex, age, mode of trauma, and clinical and radiological examination was obtained. The influential factors of pressure ulcers in SCI patients included age, gender, mechanism of injury, pressure ulcer in the acute phase of rehabilitation, neurological level of injury, completeness of injury, spasticity, FIM score on admission and length of stay. The criteria for conducting the study were the following: 1st, all of the patients were diagnosed with spinal cord injuries; and 2nd, all of the patients had spinal cord injury that showed signs of neurological lesions of the spinal cord. The criteria for exclusion from the study were the following: 1st, any type of underlying deterioration that resulted in termination of rehabilitation; 2nd, patients younger than 18 years; and 3rd, all patients with an injury below the L_1 level of the spinal cord.

The presence and location of pressure ulcers were recorded on admission, during acute and functional rehabilitation and at discharge. During hospitalisation, the patients were assessed by tests aimed at measuring the degree of their functional recovery and the presence of neurological sequelae after spinal injury: (1) the FIM test (Functional Independence Measure) was used to assess the functional status of patients, (2) the ASIA scale (American Spinal Injury Association impairment scale) was used to assess motor and sensory levels and completeness of the injury and (3) the MAS score (Modified Ashworth Score) was used to determine the level of spasticity (11,12,13,14).

Statistical analysis: For the analysis of primary data, descriptive statistical methods and hypothesis-testing methods were used. Among the descriptive statistical methods, we used central tendencies (arithmetic mean and median), measures of variability (standard deviation) and relative numbers. To test the hypothesis of the difference in frequency, a Chi-squared test was used. A t-test of exact probability was used for testing hypotheses of the difference in arithmetic means. The relationships between binary outcomes and potential predictors were analysed by logistic regression. The level of statistical significance in our study was set to 0.05.

RESULTS

The study included 453 patients, of whom 383 (84.5%) did not have a pressure ulcer and 70 (15.5%) had a pressure ulcer during rehabilitation.

The average age of patients in the study was 51.8 ± 17.2 years. The youngest patient was 19, and the oldest was 91 years old. The average age of patients without PUs was 52.1 ± 17 years, while for patients with PUs, the average age was 50.2 ± 14.6 years. Between the two groups, there was no significant difference (p = 0382).

Of the total number of patients, 333 (73.5%) were male, and 120 (26.5%) were female. The gender frequency between patients with or without PUs was statistically insignificant (p = 0297).

Of the total number of patients, 292 (64.5%) had traumatic and 161 (35.5%) had non-traumatic spinal cord injury. Patients with pressure ulcers had significantly more traumatic injuries compared with patients without pressure ulcers (77.1% vs. 62.1%, respectively) (p = 0.016).

Of the total number of patients, 253 (55.8%) had an incomplete lesion, and 200 (44.2%) had a complete spinal cord lesion. Patients with pressure ulcers had significantly more complete lesions than patients without pressure ulcers (58.6% vs. 41.5%, respectively) (p = 0.008).

At admission, in relation to the neurological level of injury, injuries of the thoracic spinal cord (43.9%) were most frequent, followed by cervical injuries (40.8%) and lumbar

	Without pressure ulcer (n=383)	With pressure ulcer (n=70)	p
Age, $\bar{x}$±SD	52.1±17.6	50.2±14.6	0.382
Gender, n (%) male female	 278 (72.6%) 105 (27.4%)	 55 (78.6%) 15 (21.4%)	0.297
Mode of trauma, n (%) traumatic non-traumatic	 238 (62.1%) 145 (37.9%)	 54 (77.1%) 16 (22.9)	**0.016**
Completeness of lesion, n (%) incomplete complete	 224 (58.5%) 159 (41.5%)	 29 (41.4%) 41 (58.6%)	**0.008**
Level of injury, n (%) cervical thoracic lumbar	 158 (41.3%) 163 (42.6%) 62 (16.2%)	 27 (38.6%) 36 (51.4%) 7 (10.0%)	0.266
Spasticity on admission, n (%)	105 (27.4%)	17 (24.3%)	0.587
Pressure ulcer in acute phase of rehabilitation, n (%)	20 (5.2%)	15 (21.4%)	**<0.001**
FIM score on admission, $\bar{x}$±SD	80.9±11.9	75.3±10.3	**<0.001**

Table 1. Characteristics of patients with spinal cord injury

injuries (15.2%). In patients without pressure ulcers, thoracic spine injuries were the most common (42.6%), followed by injuries of the cervical spine (41.3%) and lumbar spine (16.2%). In patients with pressure ulcers, thoracic spinal cord injuries occurred in 51.4% of patients, followed by cervical spine (38.6%) and lumbar spine injuries (10.0%). The incidence of the neurological level of injury between patients with or without PUs was statistically insignificant (p = 0266).

At admission, 27.4% of patients without ulcers had spasticity, and 24.3% of patients had bedsores, which was not a statistically significant difference (p = 0587).

A total of 35 (7.7%) patients had pressure ulcers at admission. Patients with pressure ulcers during rehabilitation were significantly more likely to have had pressure ulcers at admission than patients without pressure ulcers (21.4% vs. 5.2%, respectively) (p < 0.001). Of the total number of patients with pressure ulcers, 54 (77.1%) had a pressure ulcer on the sacrum, 8 (11.4%) on the trochanter, 6 (8.5%) on the heels, and two (2.8%) on the ischium.

The average FIM score at admission for all patients was 80.1 ± 11.9. The average FIM score in patients who had no pressure ulcers was 80.9 ± 11.9, while the average FIM in patients with pressure ulcers was 75.3 ± 10.3, which was a statistically significant difference (p <0.001).

The average length of stay for all patients was 153.1 ± 92.9 days. The average length of stay for patients without pressure ulcers was 149.9 ± 91.4 days, and 170.6 ± 100.0 days for patients with pressure ulcers, which is not a statistically significant difference (p = 0.085).

A multiple logistic regression model was constructed by including predictors that were statistically significant in simple regression models at a 0.05 level of significance. The model also included the neurological level of the lesion, which was expected to be a significant predictor of pressure ulcers based on previous research. The results of the multiple logistic regression analyses are shown in Table 2.

The model contained five predictors listed in Table 1, which were compared in 453 patients. The entire model (all of the predictors) was statistically significant (Chi-square = 30.260, DF = 5, p <0.001).

In the multiple logistic regression model, a statistically significant predictor of pressure ulcers during rehabilitation was pressure ulcers before rehabilitation (B = 1420, p <0.001), with an odds ratio (OR) = 4.1. This result shows

The independent variable	B	p	OR	95% confidence interval	
				Lower limit	Upper limit
Mode of trauma	-0.209	0.536	0.8	0.4	1.6
Completeness of lesion	0.302	0.304	1.4	0.8	2.4
Level of injury	0.082	0.709	1.1	0.7	1.7
Pressure ulcer in acute phase of rehabilitation	1.420	<0.001	4.1	1.9	8.7
FIM score on admission	-0.036	0.015	0.9	0.94	0.99

Table 2. Multiple logistic regression with pressure ulcers during rehabilitation as a dependent variable.

that patients who had pressure ulcers at admission are 4 times more likely to regain pressure ulcers during rehabilitation after controlling for all of the factors in the model. Another statistically significant predictor of pressure ulcers during rehabilitation was FIM score at admission (B = -0036, p = 0.015).

DISCUSION

In our study, PU was present in 15.5% of the sample during a 10-year study period. Garber and Rintala found PU in 36% of their mail-based survey and 39% of 553 veterans in the Houston VA SCI registry over a 3-year period (15). Age of SCI onset, SCI duration, presence of depression, and faecal/urinary incontinence showed no significant association with the presence or development of PUs. Similar to the findings of Salzberg et al., Mawson et al. and Rodriguez and Garber found diabetes mellitus, smoking, and depression to influence PU development (15,16). Many risk factors are associated with the development of pressure ulcers in SCI patients. All of these factors were associated with PUs; it is not known whether they increase the risk of PU development or are the result of PUs.

In our study, the most commonly reported location of pressure ulcers was at the sacrum (77.1%), followed by the trochanter (11.4%), ischium (8.5%) and heel (2.8%). In other studies, the sacrum was the most commonly reported location (39–52%), followed by the ischium (8–59%) and heel (13–31%) (17).

Using a simple logistic regression, we found that statistically significant predictors of PUs were mode of trauma, completeness of injury, PUs in acute phase of rehabilitation and FIM score at admission. Age, gender, duration of rehabilitation and neurological level of injury were statistically insignificant for the development of PUs during functional rehabilitation. In our study, the neurological level of injury was statistically insignificant as a PU predictor. Therefore, PUs occur more frequently in paraplegic patients than tetraplegic patients. Previous studies have reported similar findings (7,18,19).

Using the multiple logistic regression model, we found that statistically significant predictors of PUs were pressure ulcers in the acute phase of rehabilitation and FIM score at admission. A pressure ulcer in the acute phase of rehabilitation was a strong predictor of PUs during functional rehabilitation, with OR = 4.1. This result shows that patients who had a pressure ulcer at admission had a four-time greater probability of regaining a pressure ulcer during functional rehabilitation. Similar findings have been reported in a previous study. Verschueren et al. showed that a pressure ulcer in the acute phase of rehabilitation was a strong predictor of PUs, with OR=5.1 (19). In our study, FIM score at admission was a strong predictor for the development of pressure ulcers. This finding is in accordance with previous studies (19). This association is because increased immobilisation (due to absent motor function) promotes the development of PUs.

CONCLUSION

Significant risk factors for developing pressure ulcers during acute and functional rehabilitation are pressure ulcers during the acute phase of rehabilitation and FIM score at admission. Because PUs have a significant impact on rehabilitation and functional outcomes in patients with SCI, it is necessary to construct a predictive model for the development of pressure ulcers. Developing a model for the prediction of PUs allows us to recognise risk categories of patients and react in terms of prevention or treatment of PUs. This study emphasises the need to continue educating patients with SCI about the importance of effective regular healthy skin care in preventing PU development.

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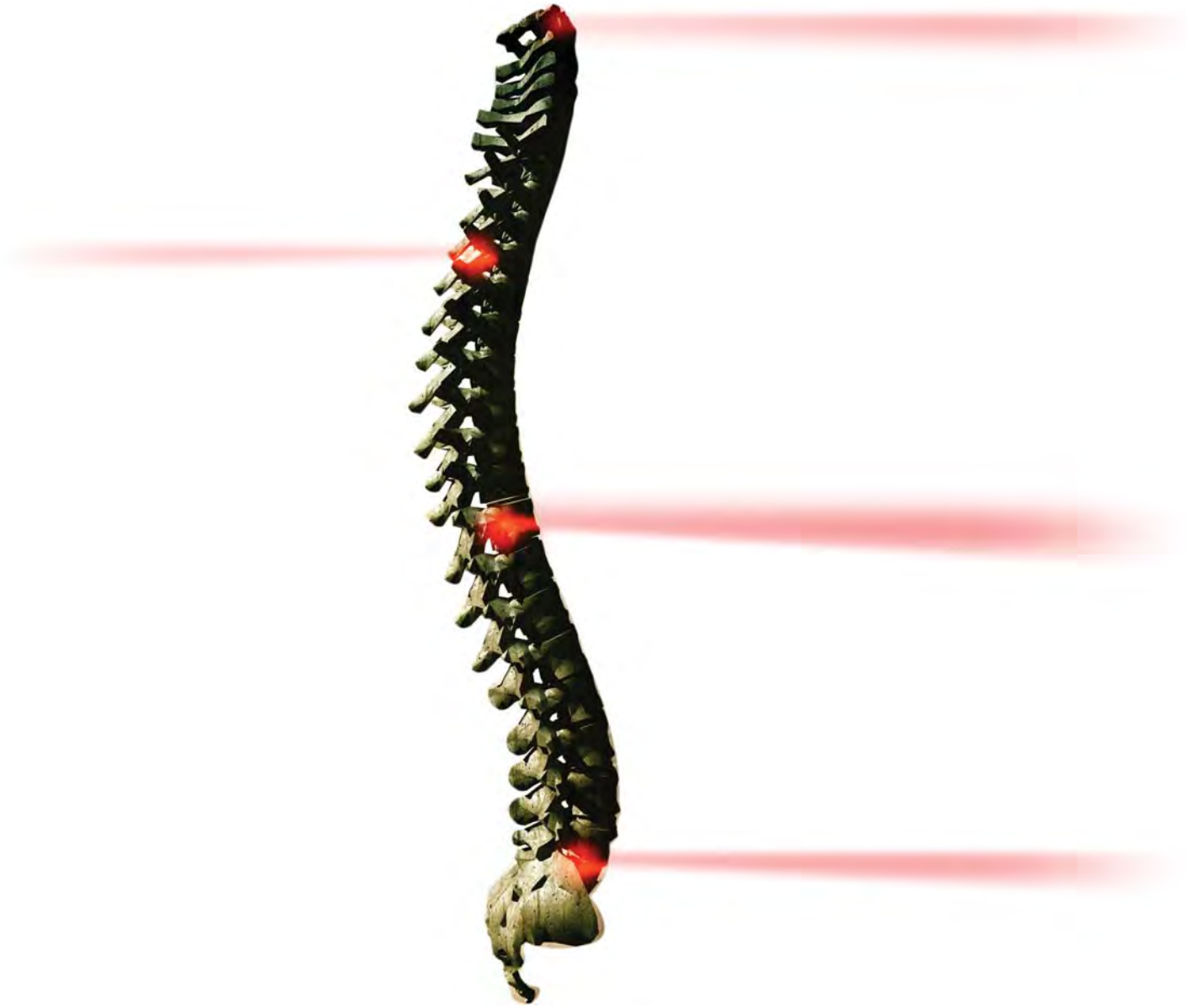

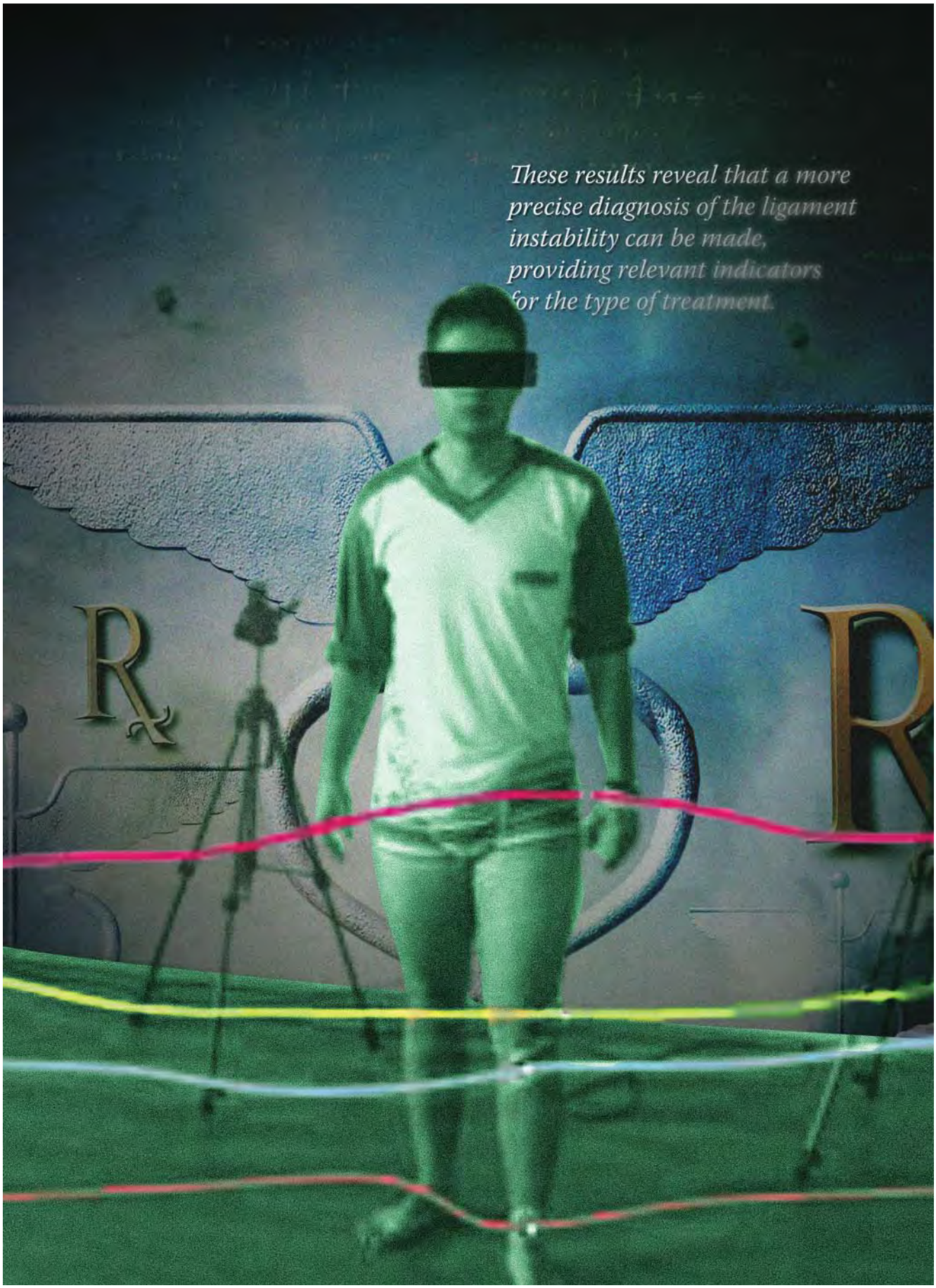
These results reveal that a more precise diagnosis of the ligament instability can be made, providing relevant indicators for the type of treatment.

GAIT ANALYSIS IN PATIENTS WITH CHRONIC ANTERIOR CRUCIATE LIGAMENT INJURY

Aleksandar Matic[1,2], Branko Ristic[1,2], Goran Devedzic[3], Nenad Filipovic[3], Suzana Petrovic[3], Nikola Mijailovic[3], Sasa Cukovic[3]
[1] Faculty of Medicine, Svetozara Markovića 69, Kragujevac, Serbia
[2] Clinical Centre Kragujevac, Clinic for Orthopedics and Traumatology, Zmaj Jovina 30, Kragujevac, Serbia
[3] Faculty of Engineering, Sestre Janjić 6, Kragujevac, Serbia

ANALIZA HODA KOD BOLESNIKA SA HRONIČNOM POVREDOM PREDNJIH UKRŠTENIH LIGAMENATA

Aleksandar Matić[1,2], Branko Ristić[1,2], Goran Devedžić[3], Nenad Filipović[3], Suzana Petrović[3], Nikola Mijailović[3], Saša Ćuković[3]
[1] Fakultet medicinskih nauka, Svetozara Markovića 69, Kragujevac, Srbija
[2] Klinički centar Kragujevac, Klinika za ortopediju I traumatologiju, Zmaj Jovina 30, Kragujevac, Srbija
[3] Fakultet inženjerskih nauka, Sestre Janjić 6, Kragujevac, Srbija

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ABSTRACT

Anterior cruciate ligament (ACL) injuries are relatively common in young athletes and quite often require surgical reconstruction. The purpose of the ACL reconstruction is to achieve stability in the entire range of motion of the knee and to re-establish a normal gait pattern.

For this study, we examined nineteen adult men. Subjects walked along a pathway at their own speed. Motion curves were obtained based on the kinematic data collected using the OptiTrack system with six infrared cameras. Anterior-posterior tibia translation, as the leading ACL pathological parameter, was indirectly determined by monitoring the difference in the length of the distance between markers positioned at the femoral lateral epicondyle and at the tuberosity of the tibia in space and in the sagittal plane. Additionally, the angle of the internal-external rotation was monitored using the gradient of the tangent line of the motion curve.

Anterior-posterior tibia translation and internal-external rotation were significantly different after reconstruction surgery compared with preoperational measurements. Preoperational measurements included the maximal values of the AP translation and IE rotation in the early stance phase of the gait cycle. An increase of the AP translation and IE rotation values may cause degeneration of the cartilage.

These results reveal that a more precise diagnosis of the ligament instability can be made, providing relevant indicators for the type of treatment.

Keywords: *Gait analysis, ACL injuries, AP shift, IE rotation*

SAŽETAK

U ovoj studiji ispitano je 19 odraslih muškaraca. Pacijenti su se kretali duž putanje kretanja sopstvenom brzinom. Krive kretanja su dobijene na osnovu kinematskih podataka skupljenih korišæenjem OptiTrack sistema sa šest infracrvenih kamera. Anteriorno posteriorna translacija tibie, kao vodeæi patološki parametar, je indirektno određena praæenjem razlike u dužini rastojanja između markera pozicioniranih na latelarnoj epikondili femura i na tuberozitosu tibie, u prostoru i u sagitalnoj ravni. Takođe, ugao interno eksterne rotacije je praæen korišæenjem gradijenta tangente krive kretanja.

Anteriorno posteriorna translacija tibie i interno eksterna rotacija se znaèajno razlikuju nakon rekonstruktivne operacije prednjeg ukrštenog ligamenta upoređujuæi sa preoperativnim merenjima. Preoperativna merenja uključuju maksimalne vrednosti AP translacije i IE rotacije u ranoj fazi ciklusa hoda. Poveæanje vrednosti AP translacije i IE rotacije mogu dovesti do degenerativnog procesa na hrskavici.

Rezultati dobijeni u ovom istraživanju omoguæavaju precizniju dijagnozu ligamentarne nestabilnosti kolena pružajuæi relevantne pokazatelje za tip leèenja.

Ključne reči: *analiza hoda, ACL povrede, AP translacija, IE rotacija*

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Correspondence to: Aleksandar Matic, Clinical Centre Kragujevac, Clinic for Orthopedics and Traumatology, Zmaj Jovina 30, Kragujevac, Serbia, aleksandar.matic@gmail.com

INTRODUCTION

The primary function of the anterior cruciate ligament (ACL) is to control the anterior dislocation of the tibia, preventing hyperextension of the lower leg and disabling excessive axial rotation of the knee during extension [1]. Anterior cruciate ligament injuries are a relatively common in young athletes [2]. The typical orthopaedic treatment involves the surgical reconstruction of the ACL. In the U.S., more than 100 000 reconstructions of the ACL are performed per year. The purpose of the ACL reconstruction is to achieve stability in the entire range of motion of the knee, enabling the patient to perform everyday activities and sports-related activities, and to prevent new chondral and meniscoligamental injuries [3] and early arthritis. Additionally, ACL reconstruction should re-establish the normal gait pattern, which is distorted in patients with chronic ACL rupture. The gait pattern of patients with ACL injuries is changed due to a significant increase in the anterior-posterior (AP) translation of the tibia relative to the femur and internal-external (IE) rotation during specific phases of the gait cycle.

The aim of the study is to present a more precise and objective method of gait analysis before and after surgery in patients with ACL rupture.

MATERIALS AND METHODS

Nineteen adult men volunteered to perform the gait analysis test. The mean height of the subjects was 183.33±2.24 cm, mean weight 86±3.48 kg, and mean age 29.89±1.73. The subjects were recreational or professional athletes with a history of arthroscopic reconstruction of the ACL after a severe knee injury; the reconstruction involved the use of the semitendinosus and gracilis muscle tendons as nthe autograftdiagnosis . Test analysis and surgery were performed at the Kragujevac Clinical Centre (Clinic for Orthopedics and Traumatology).

The Shelbourne Knee Center rehabilitation protocol was used for postoperative rehabilitation.

Kinematic data were collected using an OptiTrack (Natural Point, Inc., Oregon, www.naturalpoint.com) system with six infrared cameras (V100:R2) and a resolution of 640×480 pixels and ARENA software (Natural Point, Inc., Oregon, www.naturalpoint.com) [4]. Cameras were placed along the pathway, and the positions of markers that were placed at characteristic landmarks on subjects' lower limbs were recorded (Fig. 1a). Four markers, which were placed at the great trochanter region (RVT), femoral lateral epicondyle (LEF), tuberosity of the tibia (TT) and the region of the centre of the ankle joint (CSZ) (Fig. 1b), were used.

Subjects walked along the pathway for approximately 5.00 m at their own speed. The signal at the knee with the deficient AC ligament was recorded first, and then the procedure was repeated for the healthy knee. Every subject was asked to perform this task four times.

To determine preoperative tibia translation along the AP direction, gait analysis was performed the day before surgery. Relevant motion curves were registered for fluorescent markers attached at the femoral lateral epicondyle (LEF) and tuberosity of the tibia (TT) for both the deficient and healthy knee. The test was repeated 15 days later and then after 6 weeks. The results used in this study are the results obtained after 6 weeks.

The subjects' motion was visualised as 3D curves, which were exported from the ARENA software in standard VICON .c3d format and further processed in Matlab (MathWorks, Inc., USA, www.mathworks.com) [5] and Catia V5 (Dassault Systemes, France, www.3ds.com) [6]. The LEF motion curve represents distal femoral movements, and the TT motion curve provides data for tibial shift and IE rotation.

The key functional gait phases were identified using the motion curve of the ankle joint centre in sagittal plane (Fig. 2). From the beginning of the curve, there is a descent to the local minimum, labelled as the heel strike, followed by

Motion curve of the great trochanter region
Motion curve of the femoral lateral epycondile
Motion curve of the tuberosity of the tibia
Motion curve of the center of the ankle joint
RVT *region of the great trochanter,*
LEF *lateral epycondil of the femur,*
TT *tuberosity of the tibia, and*
CSZ *centre of the ankle joint,*

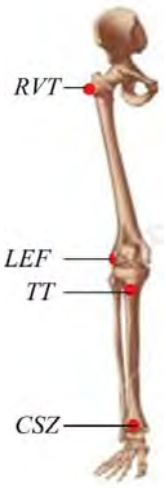


Figure 1. Experimental set-up

a.) **b.)**

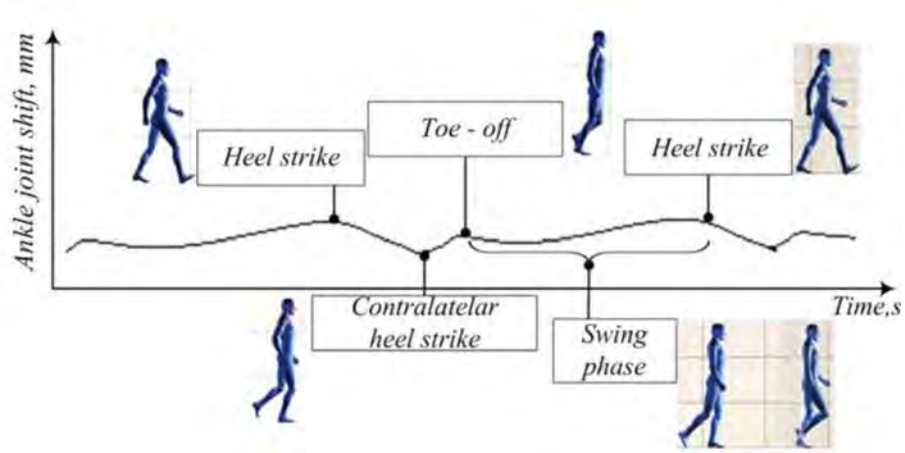


Figure 2. The key functional gait phases of the motion curve of the ankle joint centre

the contralateral heel strike. This is followed by a continuous curve increase, marked as the toe-off point in the next maximum. From the local maximum, the motion curve extends almost horizontally, denoting the swing phase, after which the decline to the next local minimum begins, labelled as the new heel strike of the same leg. The values that define shift of the tibia relative to the femur are given s a function of time, e.g., defined as a percentage of the gait cycle relative to time.

For indirect determination of the AP-tibia translation, we monitored the differences in the distance between LEF and TT points (Fig. 3) in space and in the sagittal plane, as well as the tibia shift along the AP axis. The shortest distance between LEF and TT points was determined using collected data for markers positions in space from .c3d file, such as:

- the spatial distance between LEF and TT

$$PDP = \sqrt{(x_{TT} - x_{LEF})^2 + (y_{TT} - y_{LEF})^2 + (z_{TT} - z_{LEF})^2}, \quad (1)$$

and

- the distance between LEF and TT in the sagittal plane

$$DPSR = \sqrt{(y_{TT} - y_{LEF})^2 + (z_{TT} - z_{LEF})^2}, \quad (2)$$

where x is the AP direction and *z* is the superior–inferior direction.

Based on the definitions of the distance between LEF and TT in space and in the relevant planes, differences in length are determined as:

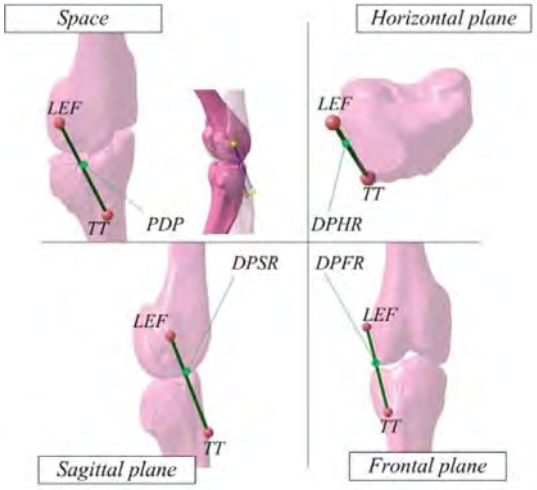
- the spatial difference in the lengths of the distance between LEF and PP

$$d_{PDP} = (PDP)_i - \min(PDP)_i, \quad (3)$$

and

- the difference in lengths of the distance between LEF and PP in the sagittal plane

$$d_{DPSR} = (DPSR)_i - \min(DPSR)_i. \quad (4)$$



LEF *-lateral epycondil of the femur,*
TT*- tuberosity of the tibia,*
PDP *- distance between LEF and TT points in space,*
DPHR *- distance between LEF and TT points in horizontal plane,*
DPSR *- distance between LEF and TT points in sagittal plane, and*
DPFR *- distance between LEF and TT points in frontal plane.*

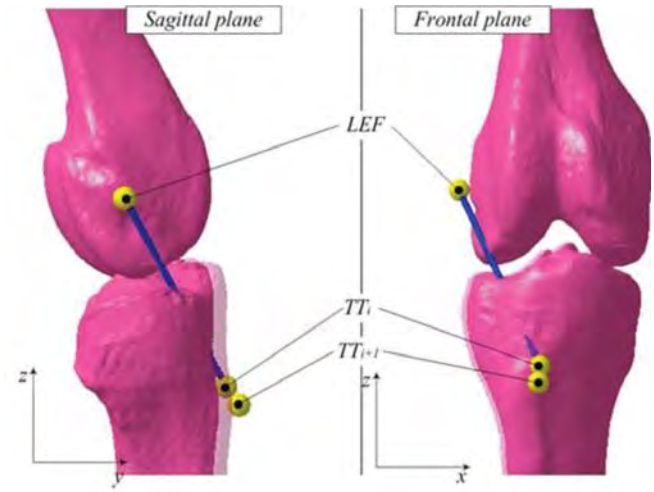
Figure 3. Identification of the distances between LEF and TT in space and in the horizontal, frontal and sagittal planes

The tibia shift along the AP direction (Fig. 4) is determined by successive calculations of the affine coordinates along these directions:

$$d_{TTAP} = (TTAP)_{i+1} - (TTAP)_i. \quad (5)$$

The angle of the IE rotation is determined based on definition of the tangent line coefficient of the movement curve and on the definition of the angle between the tangent line and AP axis of the femoral coordinate system (Fig. 5) [7].

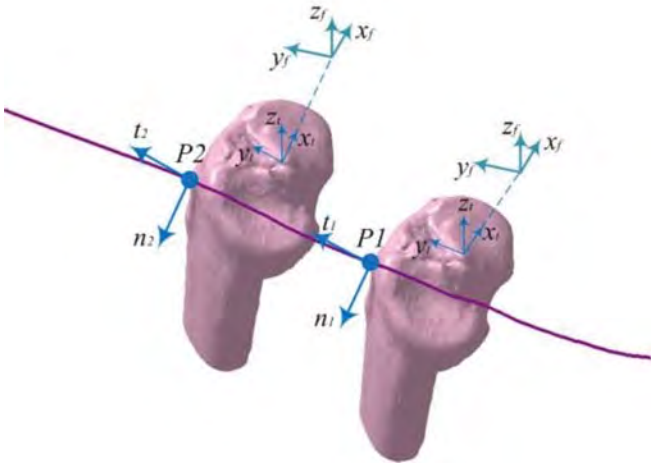
During the motion, the femoral coordinate system is considered the referent coordinate system that does not change its orientation. On the other side, IE tibia rotation occurs during motion. During the motion, i.e., within the stride (point *P1*) (Fig. 5), the tibia rotates at a certain angle relative to femoral coordinate system. On the tibial motion curve at point P1, in this particular moment within the gait cycle, it is possible to define



LEF *- lateral epycondil of the femur,*
TT$_i$*- tuberosity of the tibia in i-th moment, and*
TT$_{i+1}$*- tuberosity of the tibia in i+l-th moment.*

Figure 4. Tibial translation along the AP and mediolateral (ML) axes

the tangent axis on the curve *t1* and corresponding normal axis *n1*. In the next moment (point *P2*), the tibia rotates at some other angle according to the referent femoral coordinate system. Accordingly, on the tibial motion curve at the point *P2*, we define tangent axis on the curve *t2* and corresponding normal axis *n2*. These axes, together with their corresponding binormals, form the tibial frame of reference. At the point P1:



xf*- mediolateral axis of the femur,*
yf*- anteroposterior axis of the femur,*
zf*- superior - inferior axis of the femur.*
xt *- mediolateral axis of the tibia.*
yt *- anteroposterior axis of the tibia.*
zt *- superior - inferior axis of the tibia,*
ti, t2 *- tangent line of the curve at the point PI, e.g. P2, and*
n1n2 *- normal line of the curve at the point PI, e.g. P2.*

Figure 5. Internal-external rotation of the tibia

$$x_{f1} \perp y_{f1} \text{ and } t_1 \perp n_1 \Rightarrow x_{f1} \parallel n_1 \text{ and } y_{f1} \parallel t_1 \quad (6)$$

Analogous to the previous expression, for point P2:

$$x_{f2} \perp y_{f2} \text{ and } t_2 \perp n_2 \Rightarrow x_{f2} \parallel n_2 \text{ and } y_{f2} \parallel t_2 \quad (7)$$

The previous two expressions show that for any point *i*:

$$x_{fi} \perp y_{fi} \text{ and } t_i \perp n_i \Rightarrow x_{fi} \parallel n_i \text{ and } y_{fi} \parallel t_i \quad (8)$$

By defining the position of the tangent to the curve, it is possible to define the normal position and consequently the orientation of the coordinate system of the tibia. Given the curve of the real function *f*, the direction of the tangent *t* at point $P(x_i, f(x_i))$ has the same gradient as the curve function *f*, defined as:

$$t = f'(x_i) = \frac{dy_i}{dx_i} \quad (9)$$

The angle between the tangent to the tibial motion curve and the AP femoral axis is identical to the angle between the normal to the curve and ML femoral axes:

$$angle(t, y_f) = angle(n, x_f) \quad (10)$$

This angle defines IE tibia rotation and represents one of the key parameters for the diagnosis of ACL rupture.

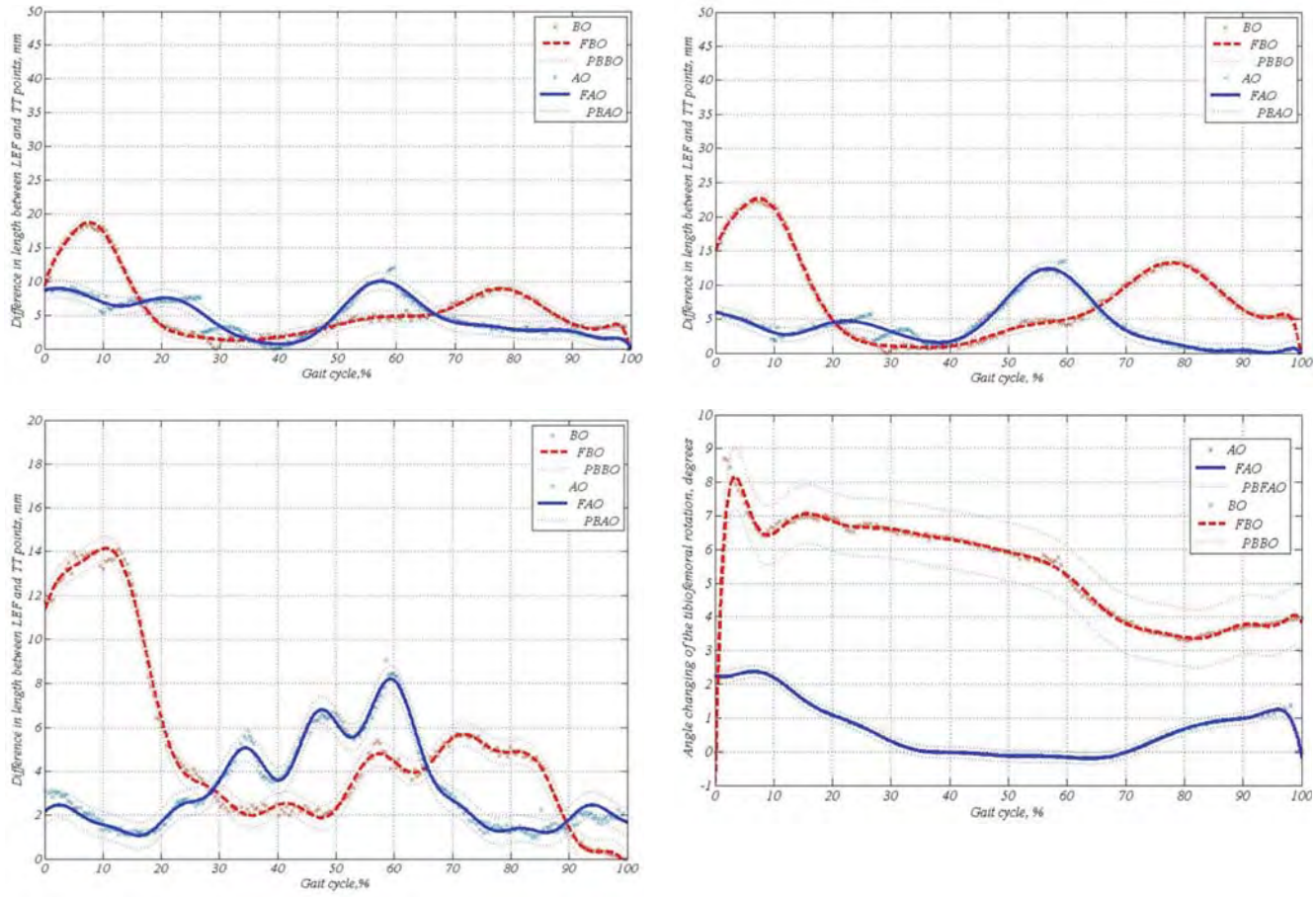
RESULTS

The horizontal axis represents the percentage distribution of the gait cycle, and the vertical axis represents the difference in lengths between LEF and TT in millimetres for tibia shift (Fig. 6a-c) and the difference of the change in IE angle rotation (Fig. 6d).

The curves on the diagrams obtained from the preoperational measurement results indicate that a large difference in lengths between the LEF and TT points along the AP axis occurs at the beginning of the gait cycle, just before the heel strike (Fig. 6a-c). The mean spatial value and its standard deviation of LF-TT lengths was 7.378±1.673 mm (Tab. 1). In the sagittal plane, this value is 11.064±1.961 mm, and along the AP axis, it is 6.619±1.447 mm (Tab. 1).

Curves on diagrams show that the patients' gait patterns have lower amplitudes after surgery, and the intensity of the LEF-TT length changes is decreased. The maximal spatial length change in our investigation corresponds to a heel strike of 4.179±0.886 mm (Tab.1). Additionally, the maximal shifts in sagittal plane occur in this part of the gait cycle, with approximate values of 5.748±0.859 mm, and an approximate value of 3.0901±0.511 mm along the AP direction (Tab. 1).

A diagram of the change in angle of the tibiofemoral rotation (Fig .6d) shows maximal rotation at the beginning of the gait cycle, when maximal tibial translation along the AP axis occurs, with a mean value and standard deviation of 6.169±0.711°. After surgery, this value decreases to 2.382±0.477° (Tab. 1).



(BO: Changes in lengths (or changes of the IE angle) between LEF and TT points before surgery, FBO: Fitted curve of the changes in lengths (or changes of the IE angle) between LEF and TT points before surgery, PFBO: Confidence boundary of the fitted curve of the changes in lengths (or changes of the IE angle) between LEF and TT points before surgery, AO: Changes in lengths (or changes of the IE angle) between LEF and TT points after surgery, FAO: Fitted curve of the changes in lengths (or changes of the IE angle) between LEF and TT points after surgery, and PFAO: Confidence boundary to the fitted curve of the changes in lengths (or change of the IE angle) between LEF and TT points before surgery)

Figure 6. Changes in lengths between LEF and TT: a) spatial, b) in the sagittal plane, c) along the AP axis, and d) changes in the angle of the IE rotation

	Before operation	*After operation*
Spatial distance between LEF and TT points	*7.378±1.673 mm*	*4.179±0.886 mm*
Distance between LEF and TT points in the sagittal plane	*11.064±1.961 mm*	*5.748±0.859 mm*
Tibia shift along the AP axis	*6.619±1.447 mm*	*3.0901±0.511 mm*
IE tibia rotation	*6.169 °±0.711*	*2.382±0.477°*

Table 1. Mean value and standard deviation

DISCUSSION

We used a Sstudent's t-test to demonstrate the statistical significance of the difference of the related patterns between the preoperational and post-operational period. The test shows that there is a significant difference in the spatial distance between LEF and PP, the distance between LEF and PP in the sagittal plane, the tibial shift along the AP axis, and in IE rotation before and after operation. The threshold of the significance was computed as $p<0.01$ for a certainty of $P>99\%$. The character of the change is non-random and was the result of differences between pathological preoperational and corrected post-operational gait patterns.

The results of this study show that the reconstruction of the AC ligament reduces tibial translation to an acceptable level in space, and consequently in each plane and along all directions, simultaneously reducing the IE rotation of the tibia. Static knee stability tests (such as the Lachman test) are considered standard for determination of AC ligament

deficiency, and these are based on the surgeon's personal impression. In contrast, in vivo studies of the knee joint stability are much more suitable for diagnosing clinical AC ligament deficiency as well as for the estimation of the positive effects of the surgery because they are based on calculations of the real shifts [8].

To achieve the functional role of the AC ligament, it is critical to determine the gait cycle phases that induce pathological kinematics of the knee joint with a deficient AC ligament. Assuming that the AC ligament and posterior cruciate ligament are rigid bodies, knee motion combines two different motions, gliding and rolling [9]. The gliding phase is very important for the determination of the rotation and translation values. During this phase, in patients with chronic ACL rupture, tibial translation is increased in the AP direction, as is IE rotation, which is clearly observed in diagrams (Fig. 6).

The restoration of tibiofemoral kinematics is based on decreasing tibial IE rotation and AP translation. In other words, at the beginning of the gait cycle (heel strike), in the gliding phase, tibial rotation and tibial translation along the AP direction achieve their maximal values. A stable knee joint assumes small values of IE rotation and AP translation of the tibia. An increase in those values that exceeds usual (physiological) measurements serves as a clinically clear symptom of AC ligament deficiency, whose further deterioration may cause cartilage degeneration [10,11,12,13].

Using in vitro and in vivo experiments, numerous researchers have recorded tibial translation along the AP direction, as well as an increased angle of IE rotation, which are related to a deficiency of the AC ligament [8,9,14,15]. The results of the studies published so far [7], [10], [16] confirm the findings in our study, shown in Figure 6. The maximal values of AP tibial translation and IE rotation occurred in the early stance phase. After ligament reconstruction, translation and rotation values were decreased, e.g., the amplitudes of the curves were smaller.

The limitations of this study are related to measurement errors and data noise coming from the skin and soft tissue motion. This problem is widely recognised [9], [16] and is common for non-invasive approaches in which IC cameras and fluorescent (reflective) markers are used. However, the clinical benefits of the methodology far exceed the imprecision of the measured values. Using the same clinical position for the leg markers may minimise these errors, providing a more precise clinical picture [8].

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SUPPRESSED INNATE IMMUNE RESPONSE AGAINST MAMMARY CARCINOMA IN BALB/C MICE

Ivan Jovanovic, Gordana Radosavljevic, Marija Milovanovic, Katerina Martinova, Nada Pejnovic, Nebojsa Arsenijevic, Miodrag L. Lukic
Centre for Molecular Medicine and Stem Cell Research, Faculty of Medicine, University of Kragujevac, Serbia

SUPRIMIRANI UROĐENI IMUNSKI ODGOVOR TUMORA DOJKE KOD BALB/C MIŠEVA

Ivan Jovanović, Gordana Radosavljević, Marija Milovanović, Katerina Martinova, Nada Pejnović, Nebojša Arsenijević, Miodrag L. Lukić
Centar za molekulsku medicinu I istraživanje matičkih ćelija, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Srbija

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ABSTRACT

Breast carcinoma is one of the leading causes of deaths among women worldwide. The immune response in breast cancer is mediated by innate and adaptive immune cells, including natural killer (NK) cells, dendritic cells (DCs) and T lymphocytes. The 4T1 mammary carcinoma line derived from BALB/c mice shares many characteristics with naturally occurring human breast cancer. We aimed to investigate the mechanisms of anti-tumour immunity using the experimental 4T1 breast cancer model in syngeneic BALB/c mice. After 12 days of tumour inoculation, mammary carcinoma-bearing mice had significantly decreased numbers of NKp46⁺ NK cells compared with healthy mice and lower cytotoxic activity of total splenocytes and NK cells in vitro. Additionally, significantly higher numbers of CD11c⁺ DCs were detected in the spleens of tumour-bearing mice, but the number of activated CD80⁺CD86⁺ dendritic cells was entwithsimilar to that in healthy mice, indicating an increased number of immature DCs in tumour-bearing mice. The data indicate that 4T1 mammary carcinoma progression in BALB/c mice is associated with suppressed innate anti-tumour immunity.

Keywords: *4T1 mammary carcinoma, BALB/c mice, NK cells, dendritic cells.*

APSTRAKT

Rak dojke je jedan od najčešćih uzroka smrti žena, širom sveta. Imunski odgovor na tumor dojke posredovan je ćelijama urođene i stečene imunosti, uključujući ćelije ubice (NK), dendritske ćelije (DCs) i T limfocite. 4T1 mišji karcinom dojke, dobijen iz BALB/C miša, deli mnoge karakteristike sa spontano nastalim humanim karcinomom dojke. Cilj istraživanja je bio ispitati mehanizme anti-tumorske imunosti koristeći 4T1 eksperimentalni model tumora dojke singen sa BALB/c miševima. Dvanaest dana nakon inokulacije tumora, miševi sa tumorom imali su značajno manji broj NKp46+ NK ćelija, u poređenju sa zdravim miševima kao i manju citotoksičnost ukupnih splenocita i NK ćelija, in vitro. Takođe, detektovan je značajno veći broj CD11c+ dendritskih ćelija u slezini miševa sa tumorom, dok se broj aktiviranih CD80+CD86+ dendritskih ćelija nije značajno razlikovao u poređenju sa zdravim miševima, ukazujući na povećan broj nezrelih dendritskih ćelija u miševa sa tumorom. Rezultati ukazuju da je progresija 4T1 karcinoma dojke povezana sa suprimiranim urođenim anti-tumorskim odgovorom.

Ključne reči: *4T1 karcinom dojke, BALB/c miševi, NK ćelije, dendritske ćelije.*

INTRODUCTION

Breast cancer is characterised by the development of metastasis in distant organs, such as the lungs, bones, liver and brain, and it is one of the leading causes of cancer deaths among women (1, 2). The role of innate immunity in breast cancer growth and progression remains unknown, but the role of the specific immune response has been extensively studied (3-4). The role of NK cells in immune surveillance as a first line of antitumor defence is well established (5-8). NK cell activity is variable during tumour progression and is related to clinical stage and disease outcome (4, 8-11). T cells are important effector cells against tumours, according to many studies on tumour models in mice (12-16). Cytotoxic CD8⁺ T cells kill tumour cells, while the anti-tumour immune response of CD4⁺ T cells can be polarised towards Th1, Th2 or Th17 type. The type-1 immune response is characterised by the secretion of interferon-gamma (IFN-γ), which contributes to tumour rejection by stimulating the cytotoxic activity of CD8⁺ T and NK cells (17-20). In contrast, in the type-2 anti-tumour immune response, *interleukin-4 (*IL-4), *interleukin-5 (*IL-5) and *interleukin-10 (*IL-10) suppress cellular immunity and therefore facilitate tumour growth and metastases (21-22). The role of the type-17 anti-tumour immune response has not been clarified. Interleukin-17 (IL-17), a hallmark Th17

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Correspondence to: Ivan Jovanovic, M.D., PhD Centre for Molecular Medicine and Stem Cell Research, Faculty of Medicine, University of Kragujevac
Svetozara Markovica 69, 34000 Kragujevac, Serbia; Tel: +38134306800, Mob: +381648776741, Fax: +38134306800112; Email: ivanjovanovic77@gmail.com

cell cytokine, is a potent mediator of inflammation in autoimmune diseases (23-24), but it has dual roles in antitumor immunity: not only can it promote tumour growth and metastasis by stimulating neoangiogenesis (25-27), IL-17 can also induce the cellular immunity responsible for tumour rejection (28-30). Dendritic cells (DCs) are part of the innate immune system involved in the activation and proliferation of tumour-specific T cells (31-33) and in enhancing the tumoricidal activity of NK cells (34-35). Immature DCs induce immunosuppressive $CD4^+$ T cells (36-39), and tumour cells produce factors that could prevent DC maturation (40-41). Immature DCs are characterised by the weak expression of MHC II and co-stimulatory molecules with low IL-12 production and therefore have limited capacities to stimulate T cells (36-39). B cells contribute to antitumor immunity by secreting tumour-specific antibodies that facilitate the killing of tumour cells (42), but they can also induce a pro-angiogenic and pro-tumorigenic microenvironment that supports tumour growth (42).

In this study, using the 4T1 metastatic breast cancer model in BALB/c mice, we aimed to investigate anti-tumour innate immune mechanisms during the progression of primary tumours.

MATERIALS AND METHODS

Mice

In all of the experiments, we used female BALB/c mice that were 10-11 weeks old. The experiments were approved by the Animal Ethics Board of the Faculty of Medicine, University of Kragujevac, Serbia.

4T1 tumour cells

The mouse breast cancer cell line 4T1, which is syngeneic to the BALB/c background, was purchased from the American Type Culture Collection (ATCC, USA). 4T1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 2 mmol/l L-glutamine, 1 mmol/l penicillin-streptomycin, and 1 mmol/l mixed nonessential amino acids (Sigma, USA) (complete growth medium) and cultured under standard conditions as previously described (43). The number of viable tumour cells was determined by trypan blue exclusion, and only cell suspensions with ≥95% viable cells were used. Mice were orthotopically injected with 5×10^4 4T1 cells in the fourth mammary fat pad, as previously described (44).

Cellular analysis of the spleen

Mice were sacrificed on day 12 after tumour inoculation, and their spleens were removed. Single-cell suspensions were obtained from the spleens by mechanical dispersion through a cell strainer (BD Pharmingen, USA) in complete growth medium. Additionally, erythrocytes were removed from the splenocyte cell suspension by lysing solution (BD Pharmingen). After three washes, cells were resuspended in complete growth medium.

Cell stimulation

For analysis of CD107a expression, splenocytes were activated with phorbol 12-myristate 13-acetate (PMA, Sigma) (50 ng/ml) and ionomycin (500 ng/ml, Sigma) with GolgiStop (BD Pharmingen) as previously described (43, 45).

Flow cytometry

Single-cell suspensions from spleens were incubated with mAbs specific for mouse CD3, CD4, CD8, CD19, NKp46, CD107a, CD11c, CD80, and CD86 or isotype-matched controls (BD Pharmingen/BioLegend) and analysed with a FACSCalibur flow cytometer (BD). The gate used for FACS analysis was the mononuclear cell region in FSC/SSC plots (20000 events were acquired). Data were analysed using CELLQUEST (BD) and FlowJo (Tristar) software.

NK cell separation

NK cells were isolated from the spleen by magnetic cell sorting using FlowComp™ Mouse CD49b antibody (Invitrogen, USA), as previously described (43).

$CD8^+$ T cell separation

$CD8^+$ T cells were isolated from spleens by depleting non-$CD8^+$ T cells ($CD4^+$ T cells, B cells, monocytes/macrophages, NK cells, dendritic cells, erythrocytes and granulocytes) using a mixture of monoclonal antibodies against non-$CD8^+$ T cells (Invitrogen), as previously described (43). Isolated cells were highly enriched mouse $CD8^+$ T cells (purity >90%).

Cytotoxicity assay

The cytotoxic activity of splenocytes, enriched NK cells and enriched $CD8^+$ T cells was measured using a 4 h MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay at various target-effector (T:E) ratios, as previously described (45). 4T1 tumour cells were used as targets. Data were expressed as the mean of triplicate wells ± SEM. Cytotoxic activity was also presented by lytic units ($LU_{20}/10^7$ cells), which were calculated from the means of triplicate percentages of killing obtained at four different T:E ratios (46).

Statistical analysis

The data were analysed using the SPSS statistical package, version 13. The two-tailed Student's t test was used. The normality of distribution was tested by the Kolmogorov-Smirnov test. The results were considered significantly different when $p<0.05$ and highly significantly different when $p<0.01$.

RESULTS

Twelve days after tumour inoculation, the percentage and total number of $CD19^+$ B cells were increased while the frequency and number of $NKp46^+$ cells were decreased

We assessed the frequencies and the numbers of major lymphocyte populations in the spleens of naive and tumour-bearing mice at day 12 following tumour chal-

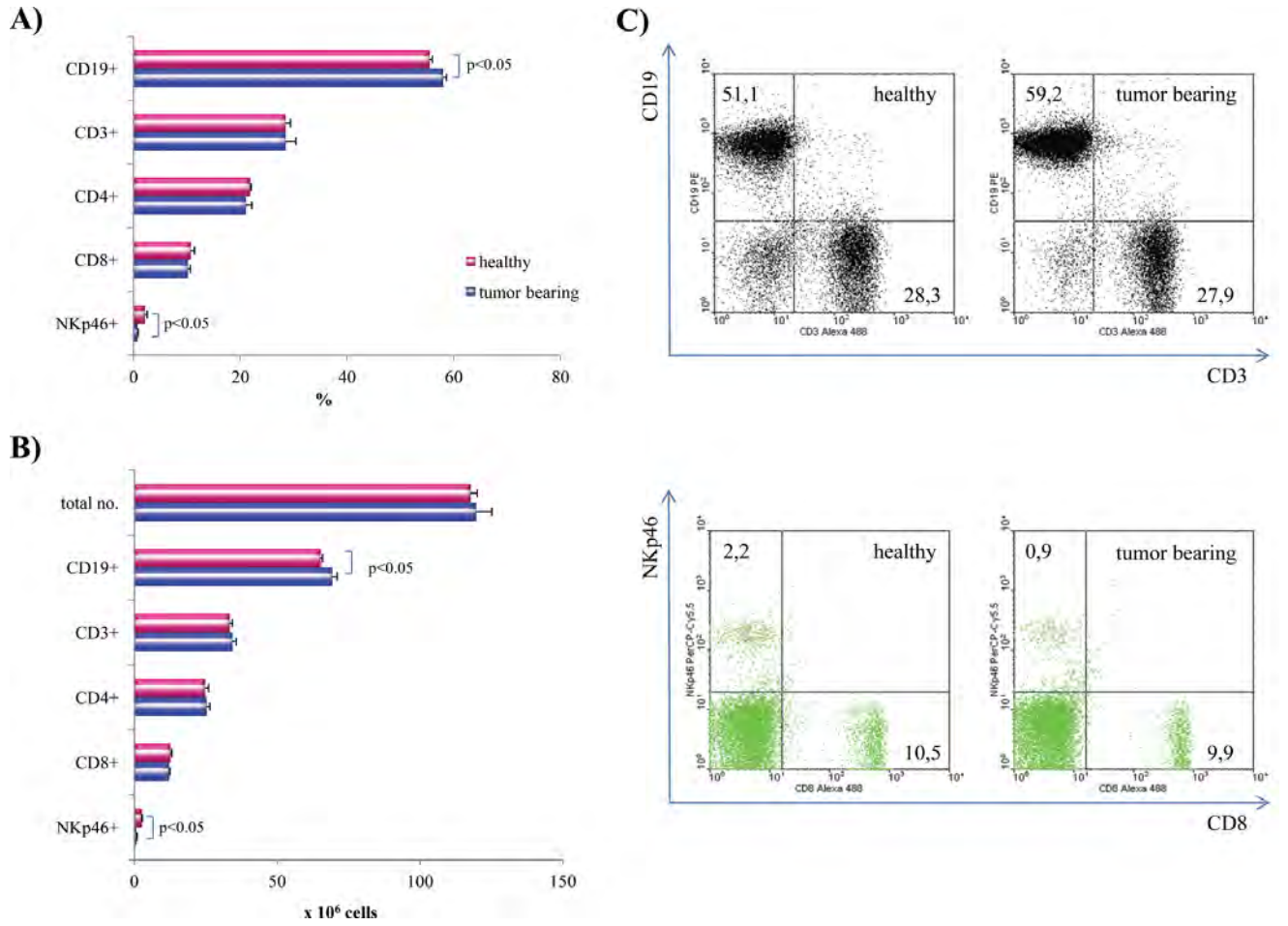

Figure 1. FACS analysis of splenocytes from tumour-bearing versus tumour-naïve mice
A-B) The total cell number of splenocytes was determined in healthy and tumour-bearing mice on day 12 after tumour inoculation. Percentages and total numbers of CD19+, CD3+, CD4+ CD8+ and NKp46+ cells were determined by staining splenocytes with fluorochrome-labelled mAbs and analysing them with a FACSCalibur flow cytometer.
C) Representative flow cytometry dot plots show percentages of CD19+, CD3+, CD8+ and NKp46+ cells in spleens from healthy and tumour-bearing mice. The gate used for analysis was the mononuclear cell region in FSC/SSC plots. Data are presented as the mean ± SEM of two separate experiments, each carried out with four mice per group. Statistical significance was tested by Student's t-test.

lenge. The total number of splenocytes was not significantly changed in tumour-bearing mice compared with healthy mice (Fig. 1B). The frequency and total number of splenic CD19$^+$ B cells were significantly increased after tumour inoculation ($p<0.05$). There was no difference in the percentage or total number of CD3$^+$, CD4$^+$ or CD8$^+$ cells (Fig. 1A and 1B) between tumour-bearing and naïve mice. 4T1 tumour administration markedly reduced the percentages and numbers of NKp46+ cells ($p<0.05$), as shown in Figs. 1A and 1B.

The cytotoxic activity of NK cells but not CD8$^+$ T cells was suppressed after tumour inoculation

On day 12 of tumour progression, the *in vitro* cytotoxic activity of total splenocytes was decreased (Fig. 2). We subsequently isolated NK cells (CD49b$^+$) and CD8$^+$ T cells from spleens and assessed their antitumor cytotoxicity. The cytotoxic activity of CD8$^+$ T cells was higher after tumour inoculation ($p<0.05$; Fig. 2), while the cytotoxicity of NK cells derived from spleens of tumour-bearing mice was significantly lower compared with healthy animals. In addition, activated CD107a$^+$ NK cells were less frequent in tumour-bearing mice, while the percentage of CD107a$^+$CD8$^+$ T cells was higher at the same time point (both $p<0.05$; Fig. 2).

The frequency and total number of CD11c$^+$ dendritic cells were increased after tumour induction

After 12 days of 4T1 tumour inoculation, the percentage and number of CD11c$^+$ DCs were higher in spleens from tumour-bearing mice (both $p<0.05$; Fig. 3). Analyses of the activation status of these cells revealed no significant difference in frequencies or numbers of activated CD80$^+$CD86$^+$ dendritic cells between healthy and tumour-bearing mice (Fig. 3).

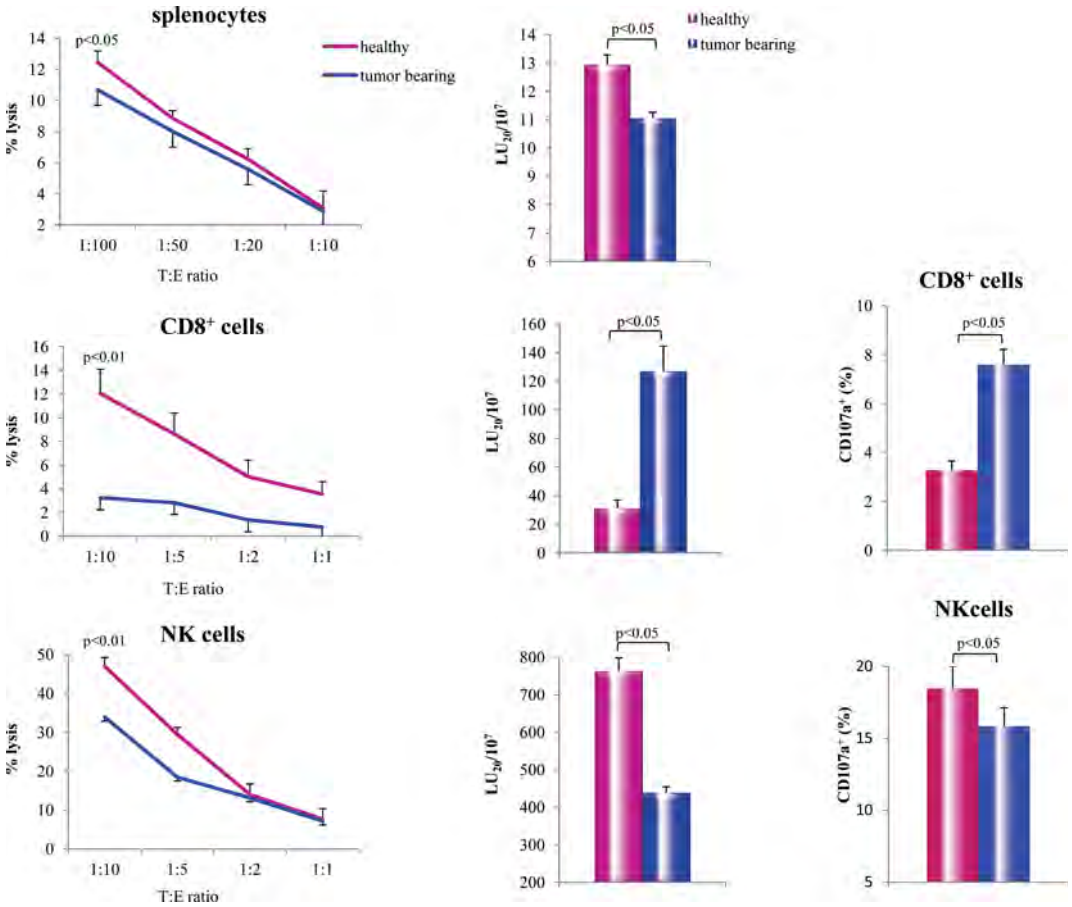

Figure 2. Cytotoxic activity of total splenocytes, CD8+ T cells and NK cells
The cytotoxicity of splenocytes, isolated CD8+ T cells and NK cells was tested with a 4-h MTT assay against 4T1 cell targets at day 12 after tumour inoculation. The data are presented as the mean percentages of specific cytotoxicity and as LU20/107 effector cells, which was calculated on the basis of mean percentages of killing at four different E:T ratios and percentages of effector cells found in the spleen. The cytotoxic capacity of NK and CD8+ T cells was also determined by flow cytometric analysis of CD107a expression on NKp46+ and CD8+ cells. The gate used for analysis was the mononuclear cell region in FSC/SSC plots. Data are means ± SEM of two individual experiments, each carried out with four mice per group. Statistical significance was tested by Student's t-test.

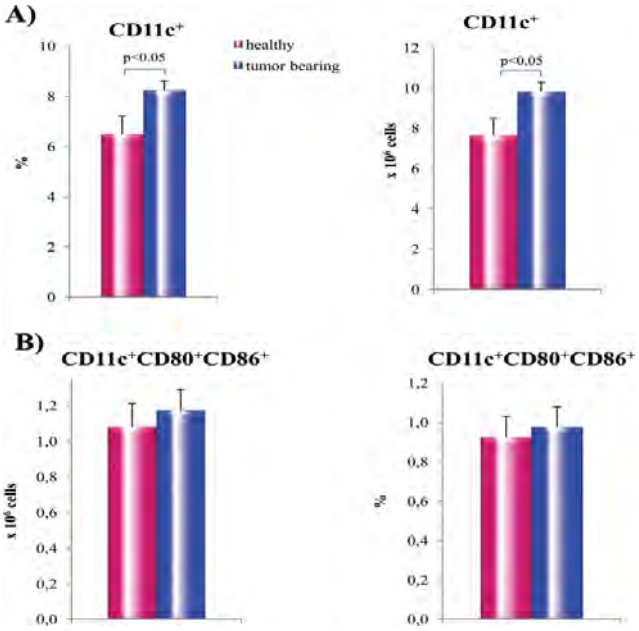

Figure 3. Frequency, number and functional phenotype of CD11c+ dendritic cells derived from the spleen
A) The percentage and total number of CD11c+ dendritic cells were determined with a FACSCalibur flow cytometer before and at day 12 following 4T1 tumour challenge. The results are presented as the mean ± SEM of 8 mice per group. B) The percentage and total number of CD80+CD86+ dendritic cells were also analysed by flow cytometry. The results are presented as the mean±SEM of 4 mice per group. Statistical significance was determined by Student's t-test.

DISCUSSION

In this study, we report that 12 days after the inoculation of 4T1 mammary carcinoma cells, the percentage and number of $CD19^+$ B cells in spleens were significantly increased, while the number and cytotoxic activity of NK cells were decreased (Fig. 1). Additionally, the frequency and number of $CD11c^+$ dendritic cells were higher in spleens from tumour-bearing mice compared with naïve mice, but the frequency of activated dendritic cells in both groups was not significantly different (Fig. 3).

The increased frequency and number of $CD19^+$ B cells in spleens of tumour-bearing BALB/c mice could be the result of Th2 polarisation, which implicates the predominance of the type-2 antitumor immune response (22). However, there is evidence that the type-17 response can also induce B cell proliferation and the formation of germinal centres (47). Several studies have shown that B cell proliferation in lymph organs correlates with tumour progression (48-49). Furthermore, we found no difference in the percentage or number of splenic T cells and $CD4^+$ and $CD8^+$ subpopulations, indicating weak or no activity of adaptive cellular immunity, on day 12 after tumour challenge (Fig 1).

We also detected the decreased cytotoxicity of total splenocytes isolated from tumour-bearing mice (Fig. 2). To determine which cell population was responsible for this phenomenon, we isolated NK and $CD8^+$ T cells and tested their antitumor cytotoxic activity *in vitro* at day 12. We found enhanced cytotoxicity of $CD8^+$ T cells (Fig. 2). However, the cytotoxic activity of NK cells was significantly decreased at the same time point, which contributed to the lower cytotoxic activity of total splenocytes (Fig. 2). During the cytotoxic killing of tumour cells, NK cells and $CD8^+$ T cells rapidly release granules containing perforin and granzymes into the immunological synapse, thereby inducing the death of target cells (50). Lysosomal-associated membrane protein-1 (LAMP-1), also known as CD107a, is a marker of cytotoxic degranulation, as it lines the membrane of these granules (51). CD107a can be used as an indirect indicator of the cytotoxic capacity of NK and $CD8^+$ T cells (52). In line with these findings, we found a higher frequency of activated $CD8^+CD107a^+$ T cells while activated $NKp46^+CD107a^+$ NK cells were less frequent in spleens from tumour-bearing mice (Fig. 2). Several studies have revealed lower cytotoxic activity of NK cells in patients with breast cancer compared with healthy controls, with a negative correlation of NK cell lytic activity with lymph node progression of disease (9).

Functional maturation and the activity of NK and T cells appear to be dependent on the functional phenotype of DCs (53). We showed that tumour inoculation led to an increase in the frequencies and numbers of $CD11c^+$ DCs in spleens (Fig. 3). During maturation/activation, DCs express more MHC class II and CD80 and CD86 co-stimulatory molecules, all of which have T-cell stimulatory capacity. However, there was no difference in the percentage or total number of fully functional $CD80^+CD86^+$ DCs in tumour-bearing mice compared with healthy animals (Fig. 3). Thus, it could be assumed that 12 days after tumour inoculation, the functional maturation of dendritic cells was absent. The interaction of mature DCs with NK and T cells is essential for the tumoricidal activity of these effector cells, while immature DCs can induce immunosuppressive activity of the same effector cells (34-35).

Taken together, we demonstrate that 12 days after inoculation with 4T1 mammary breast carcinoma, NK cell cytotoxicity was markedly reduced, and DCs were not activated after tumour challenge. These findings suggest a suppressed innate anti-tumour immune response in mammary carcinoma-bearing BALB/c mice.

ACKNOWLEDGEMENTS

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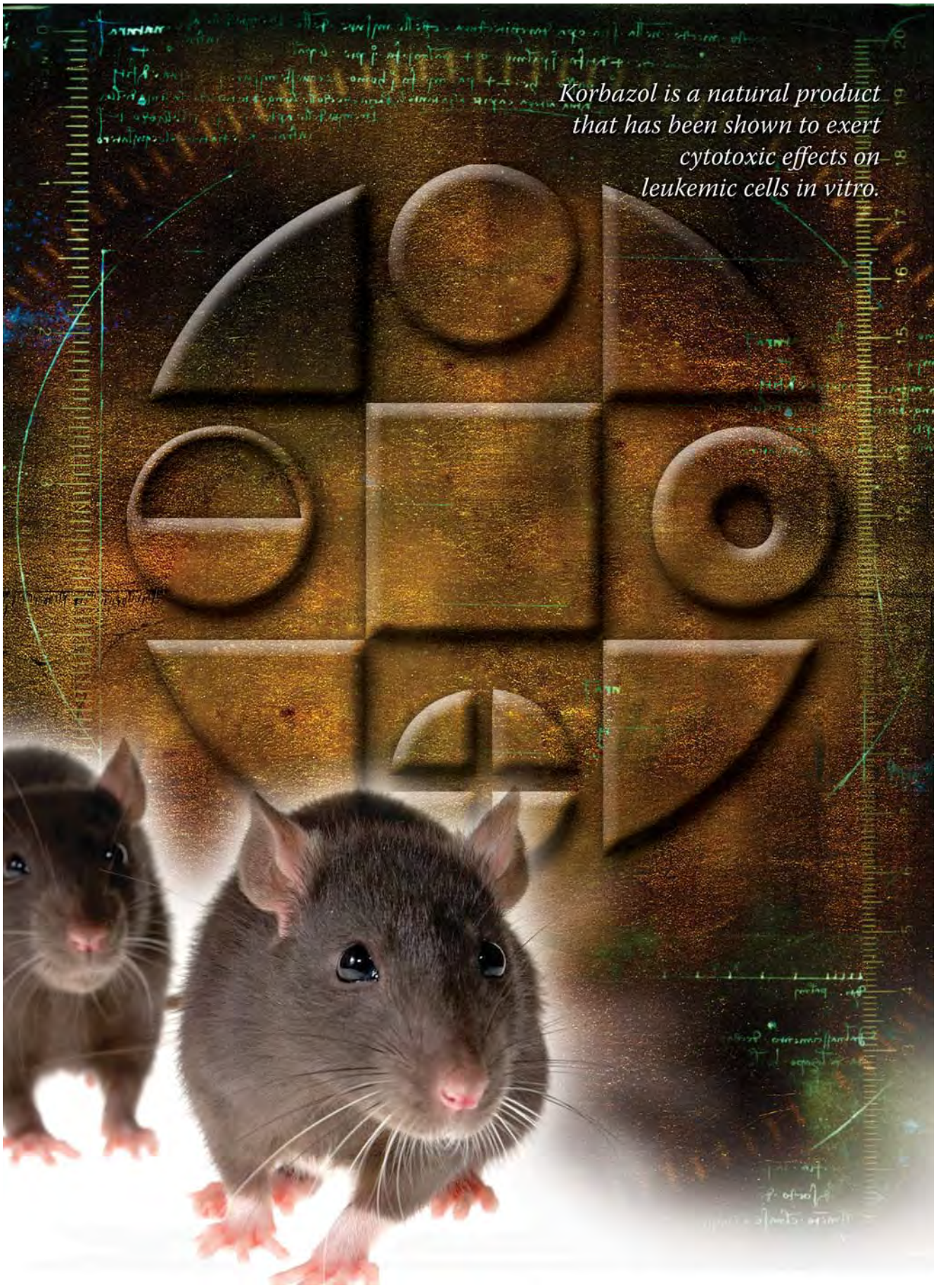
Korbazol is a natural product that has been shown to exert cytotoxic effects on leukemic cells in vitro.

THE CYTOTOXICITY OF KORBAZOL AGAINST MURINE CANCER CELL LINES

Suzana Popovic[1], Dejan Baskic[1], Ivanka Zelen[2], Predrag Djurdjevic[3], Milan Zaric[2], Dusko Avramovic[4], Nebojsa Arsenijevic[1]
[1]Centre for Molecular Medicine and Stem Cell Research,
[2]Department of Biochemistry,
[3]Department of Pathophysiology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia;
[4] Special Hospital for Internal Diseases, Mladenovac, Serbia

CITOTOKSICNO DEJSTVO KORBAZOLA NA ĆELIJE MIŠJIH TUMORSKIH LINIJA

Suzana Popović[1], Dejan Baskić[1], Ivanka Zelen[2], Predrag Đurđević[3], Milan Zarić[2], Duško Avramović[4], Nebojša Arsenijević[1]
[1]Centar za molekulsku medicinu i istraživane matičnih ćelija,
[2]Odsek za biohemiju
[3]Odsek za patofiziologiju, Fakultet medicinskih nauka,Univerzitet u Kragujevcu, Srbija;
[4] Specijalna bolnica za internističke bolesti, Mladenovac, Srbija

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ABSTRACT

Background/aim: *Korbazol is a natural product that has been shown to exert cytotoxic effects on leukemic cells in vitro. The cytotoxicity and biochemical effects induced by Korbazol were investigated in the murine cell lines 4T1, B16 and BCL1.*

Methods: *The cytotoxic activity of the tested compound was assessed using a colorimetric MTT assay. The concentration of the superoxide anion radical ($O_2^{\cdot-}$) and the activity of superoxide dismutase (SOD) in the samples were determined using spectrophotometric methods. The MDA content was determined using a TBA assay.*

Results: *We found that Korbazol induced cell toxicity, an increased the concentration of the lipid peroxidation end product MDA, decreased superoxide dismutase activity and increased superoxide anion formation.*

Conclusions: *Altogether, these results suggest that oxidative stress is involved in Korbazol-induced cytotoxicity in the investigated cell lines.*

Keywords: *Korbazol; 4T1; B16; BCL1; apoptosis; oxidative stress*

SAŽETAK

Uvod. *Korbazol je prirodni proizvod koji u in vitro uslovima ispoljava selektivno citotoksično dejstvo na ćelije izolovane iz krvi obolelih od hronične limfocitne leukemije.*

Cilj. *Ciljevi ovog istraživanja su bili da se ispita dejstvo Korbazola na ćelije mišjih tumorskih linija 4T1, B16 i BCL1 i da se utvrde mehanizmi njegovog dejstva.*

Metode. *Za utvrđivanje citotoksičnog dejstva Korbazola korišćen je MTT test. Koncentracija superoksid anjona i aktivnost superoksid dizmutaze utvrđena je spektrofotometrijskim metodama. Koncentracija MDA, krajnjeg produkta lipidne peroksidacije, određivana je pomoću TBA testa.*

Rezultati. *Korbazol na ispitivane ćelijske linije deluje citotoksično, povećava koncentraciju MDA, smanjuje aktivnost superoksid dizmutaze i povećava stvaranje superoksid anjon radikala.*

Zaključak. *Korbazol u ispitivanim tumorskim linijama izaziva ćelijsku smrt indukujući oksidativni stres.*

Ključne reči. *Korbazol; 4T1; B16; BCL1; apoptoza; oksidativni stres*

INTRODUCTION

Our previous studies have demonstrated that Korbazol selectively kills human leukaemia cells (1) by causing endoplasmic reticulum stress (2), most likely through the inhibition of SOD and GPx activity and the accumulation of reactive oxygen species (ROS) (unpublished data).

Free radicals, including ROS, are produced in the body primarily as a result of aerobic metabolism. At physiologic levels, they serve as signalling and regulatory molecules in diverse cellular processes (3, 4), but when in excess, these highly reactive radicals can damage intracellular macromolecules (i.e., DNA, RNA, proteins, and lipids). Under normal circumstances, the level of ROS is controlled by antioxidant enzymes, and a disturbance of this control results in oxidative stress, which may lead to cell death (5). The major intracellular source of ROS is the mitochondria (6), but the endoplasmic reticulum also contributes to the production of ROS (7). A variety of intrinsic and extrinsic insults can cause ER stress, and persistent or excessive ER stress induces an increase in ROS that promotes lipid peroxidation, the perturbation of calcium homeostasis and the activation of several apoptotic pathways (8, 12). Nevertheless, oxidative stress is not always disadvantageous. Many new therapeutic strategies act through the generation of free radicals or by inhibiting cellular antioxidative defences (9, 10, 11, 13).

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Correspondence: Suzana Popovic, Faculty of Medical Sciences
Svetozara Markovica 69, 34000 Kragujevac; suza_popovic@yahoo.com

Because we found that Korbazol exerts a selective cytotoxic effect on B-cell chronic lymphocytic leukaemia (B-CLL) cells, the objective of the present study was to evaluate the magnitude and mechanism of Korbazol cytotoxicity against solid tumours, specifically murine metastatic breast cancer (4T1), murine melanoma (B16) and murine B cell lymphoma (BCL1). Based on our previous results, the redox status in Korbazol-treated cells was examined.

MATERIALS AND METHODS

Cell culture

The cell lines used in this study were 4T1 (murine metastatic breast cancer), B16 (murine melanoma) and BCL1 (murine B cell lymphoma), all of which were obtained from the American Type Culture Collection (ATCC). The cells were maintained in RPMI 1640 medium containing 10% foetal calf serum, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin. The culture medium for BCLl was supplemented with 5×10^{-5} mmol/L 2-mercaptoethanol. Cells were incubated in humidified atmosphere containing 5% CO_2 at 37°C and were in logarithmic growth phase at the time of analysis. Exponentially growing cells were cultured with or without Korbazol extract for up to 24 hours. To measure MDA concentration and superoxide dismutase activity, cell suspensions were frozen at -70°C and lysed by three freeze-thaw cycles just prior to analysis.

Tested compound

The natural product Korbazol (Biofarm Group, Serbia, SCG), which has been registered as dietary supplement (Department of Preventive Medicine, MMA, Belgrade, SCG: Korbazol HA108/05), contains plant extracts (*Echinacea purpurea, Paullinia cupana*), micronised zeolite, pollen, and propolis, preserved in honey (Table 1). To produce the extract, 3.2 g of Korbazol was dissolved in 10 ml of water/5% DMSO (Merck), and the water soluble fraction of Korbazol was filtered through a nitrocellulose filter (Millipore, USA). The sterile extract was stored at -20 °C .

MTT cell viability assay

The viability of cultured cells was determined by assaying the reduction of MTT to formazan. In brief, cells were plated at a density of 5×10^3 cells/well into 96-well plates and allowed to grow overnight. Then, cells were treated with different dilutions of Korbazol extract (1:8, 1:16, 1:32 and 1:64) or cultivated in medium alone (control). After 24 h of incubation at 37°C in an atmosphere containing 5% CO_2 and absolute humidity, the culture medium was removed, and MTT (0.5 mg/1 ml of PBS) was added to each well. The cells were then incubated at 37 °C for 4 h, and DMSO (100 µl/well) was added to dissolve the formazan crystals. Absorbance was measured at 590 nm with a multiplate reader (Zenith 3100, Anthos Labtec Instruments GmbH, Austria).

KORBAZOL (250G)
Guarana (liquid extract) 1,19ml
Propolis (dry extract) 595,24mg
Pollen P2 1071,43mg
Echinaceapurpurea 1190,48mg
Honey ad 250g

Table 1. Composition of Korbazol.

MDA determination

The MDA content was determined by the TBA assay as described by Ohkawa et al. (14). Briefly, 4T1, B16 or BCL1 cells (10^6) were incubated with or without Korbazol extract (dilution 1:8) at 37°C in an atmosphere containing 5% CO_2 and absolute humidity. After 24 h, cells were harvested, and the concentration of the lipid peroxidation end product MDA in the cell lysates was measured. The thiobarbituric acid reactants (TBARs) concentration was measured using a spectrophotometer (LKB Biochrom Ultrospec 4050, Cambridge, UK) and expressed as nM/10^6 cells (14).

Determination of superoxide anion production

The concentration of the superoxide anion radical ($O_2^{\cdot-}$) in the sample was determined spectrophotometrically based on the reduction of nitroblue tetrazolium (NBT) to nitroblue-formazan in the presence of $O_2^{\cdot-}$ (15). Briefly, cells were plated at a density of 10^5 cells/well into 96-well plates. After overnight growth, cells were treated with 100 µl of Korbazol extract (dilution 1:8) or cultivated in medium only (control) for 24 h. At the end of the treatment, 100 µl of 0.01% NBT was added to each well and incubated for 1 hour at 37°C in culture hood. Then, 50 µl of 2 M KOH and 50 µl of DMSO were added, and absorbance (A) was read at 590 nm. The superoxide anion concentration was calculated according to relevant mathematic formulas and expressed as nM/ml (15).

Determination of superoxide dismutase activity

Superoxide dismutase (SOD) activity was estimated in cell lysates using Ransod and Ransel kits, supplied by Randox Laboratories, Ardmore, Northern Ireland, UK, according to the manufacturer's instructions. This assay uses xanthine and xanthine oxidase to generate superoxide anions that react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to form a red formazan dye. SOD activity is measured by the degree of inhibition of this reaction. For this assay, 4T1, B16 or BCL1 cells (10^6 cells) were incubated with or without a 1:8 dilution of Korbazol extract for 24 h at 37°C in an atmosphere containing 5% CO_2 and absolute humidity. The activity of SOD was determined in cell lysates and expressed as U/mg of proteins.

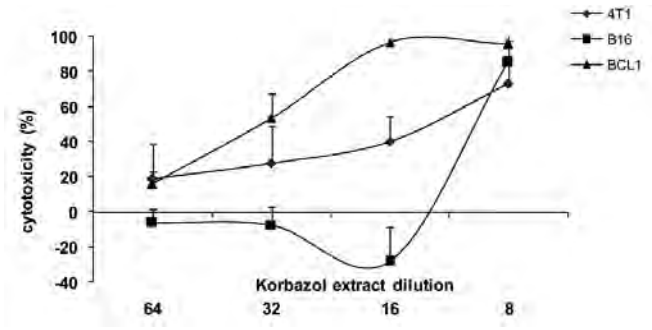

Figure 1. Korbazol-induced cytotoxicity in cancer cell lines. Cells (5×103) were incubated for 24 h with the indicated dilution of Korbazol extract. Cell death was determined by MTT assay. The data are expressed as percentages of cytotoxicity. The values indicated represent the mean (±SD) of triplicate samples from five different experiments.

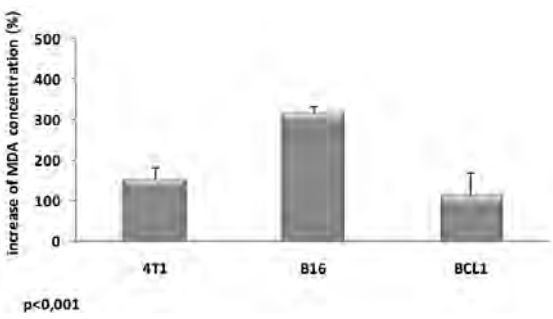

Figure 2. Lipid peroxidation induced by Korbazol. 4T1, B16 and BCL1 cells (106) were incubated with or without Korbazol extract (1:8 dilution) and harvested after 24 h. Cells were lysed by three freeze-thaw cycles. The concentration of the lipid peroxidation end product MDA in cell lysates was determined by thiobarbituric acid assay. The data are expressed as per cent increase in MDA concentration in treated cells relative to the controls. The values represent the mean (±SD) of three different experiments.

Data analysis and statistics

The data were expressed as the mean ± SD. The distributions of data were evaluated for normality using the Kolmogorov-Smirnov test. Quantitative parametric data were compared between two study groups using the unpaired t-test. The Mann-Whitney test was used to compare nonparametric data between two groups. The Kruskal-Wallis test was used for comparisons of nonparametric data between more than two groups. One-way ANOVA was performed to compare parametric data between more than two groups. When ANOVA indicated significant differences, the Bonferroni test was used to identify intergroup differences. All statistical analyses were carried out with commercial statistical software (SPSS version 13.0; SPSS Inc., Chicago, IL). P values less than 0.05 were considered significant.

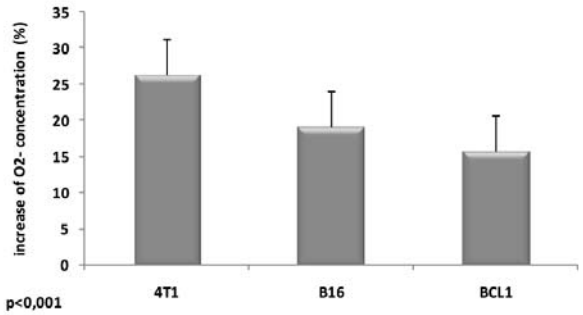

Figure 3. Korbazol induces superoxide anion accumulation. Murine 4T1, B16 and BCL1 cells (105) were incubated with or without Korbazol extract (1:8 dilution) for 24 h. The concentration of the superoxide anion radical (O2.-) in the sample was determined by spectrophotometric method based on the reduction of nitroblue tetrazolium (NBT). The data are expressed as the per cent increase in O2.- concentration in treated cells relative to the controls. The values represent the mean (±SD) of six different experiments.

RESULTS

Korbazol induces cytotoxicity in tumour cell lines

Cell lines were incubated with decreasing dilutions of Korbazol extract (1:64, 1:32, 1:16 and 1:8) or medium alone (control) for 24 h and analysed by the MTT assay. As shown in Figure 1, Korbazol demonstrated a noticeable cytotoxic effect on all three cell lines, enhancing apoptosis in a dose-dependent manner. In the cell line 4T1, all dilutions of Korbazol extracts showed notable cytotoxic effects (73% at 1:8, 40% at 1:16, 28% at 1:32 and 18% at 1:64). Korbazol showed an even stronger effect on BCL1 cells: 96% cytotoxicity at 1:8, 97% at 1:16, 53% at 1:32 and 16% at 1:64. In contrast, only the highest concentration of Korbazol tested (1:8 dilution) induced cell death in cell line B16 (85%); lower concentrations had no cytotoxic effect on this cell line.

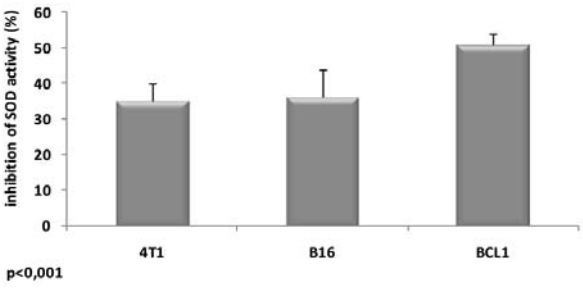

Figure 4. SOD activity is decreased in Korbazol treated cells. Cells (106) were treated with Korbazol extract (1:8 dilution) or cultivated in medium alone (control) for 24 h. SOD activity was estimated in cell lysates using Ransod and Ransel kits. The data are expressed as per cent inhibition of SOD activity in treated cells relative to controls. The values represent the mean (±SD) of three different experiments.

Korbazol treatment induces oxidative stress

To test whether the Korbazol treatment may have caused oxidative stress, the lipid peroxidation end product

malondialdehyde (MDA) was measured. As shown in Fig. 2, a two-fold increase of MDA concentration was noted in Korbazol-treated 4T1 and BCL1 cells, and a three-fold higher MDA concentration was measured in B16 cells after treatment. Specifically, the concentration of MDA (expressed per million cells) increased from 11,3 nM to 28,8 nM in 4T1 cells (155% increase), from 13,3 nM to 28,4 nM in BCL1 cells (114% increase) and from 11,8 nM to 49,1 nM in B16 cells (317% increase). The increase was statistically significant in all cell lines ($p<0,001$).

Korbazol induces superoxide anion production

To investigate the cause of the observed oxidative stress, cellular $O_2^{\cdot-}$ content was determined in untreated control cells and after incubation with Korbazol, and the per cent increase in superoxide anion concentration was calculated. As shown in Figure 3, treatment with Korbazol induced a statistically significant increase ($p<0,001$) in $O_2^{\cdot-}$ formation: 26% in 4T1 cells (from 226,2 nM/ml to 285,4 nM/ml), 19% in B16 cells (from 214,7 to 255,6 nM/ml) and 16% in BCL1 cells (from 219,5 to 237,1 nM/ml).

SOD activity is affected by Korbazol treatment

Because SOD is the key enzyme involved in the metabolic elimination of $O_2^{\cdot-}$, we measured superoxide dismutase activity after treatment with Korbazol and in untreated cells. We noted a 35% inhibition in 4T1 breast cancer cells (activity of SOD declined from 4,720 U/mg proteins in untreated cells to 3,055 U/mg protein after treatment), 36% in B16 melanoma cells (from 5,450 to 4,028 U/mg protein) and 51% in BCL1 cells (from 4,921 to 2,416 U/mg protein).

DISCUSSION

The interest in anticancer agents from natural sources has increased in recent years. Some active constituents have been isolated and used to treat human tumours (16-20). The study of cytotoxic properties of new compounds and selective action is required as a first step. The natural product Korbazol has been shown to strongly and selectively induce apoptosis in CLL cells (1). Therefore, the aim of the present study was to elucidate the effect of Korbazol on other types of cancer.

In this report, we describe the *in vitro* cytotoxic activity of Korbazol against three murine cell lines. Our data showed that Korbazol exerts cytotoxic effects in all three cell lines. The increase in the concentration of MDA markedly increased the percentage of dead cells. The precise mechanism responsible for the cytotoxic effect of Korbazol is not still thoroughly understood, although our most recent results point to oxidative stress as the main mechanism in inducing apoptosis in CLL-lymphocytes (unpublished data). Therefore, we wished to determine whether the same mechanism was engaged in other types of cancer cells. We first measured the concentration of MDA, as the end product of lipid peroxidation and an indicator of oxidative stress. We found a significant Korbazol-induced increase in MDA production for all cell lines ($p<0.001$). Because an increase in reactive oxygen species was found in our previous study on human CLL cells, we also determined the superoxide anion concentration. $O_2^{\cdot-}$ was elevated in Korbazol-treated 4T1, B16 and BCL1 cells ($p<0,001$). In both plant and animal cells, the first line of defence against oxygen-derived free radicals is superoxide dismutase, which catalyses the dismutation of the $O_2^{\cdot-}$, thus contributing to a decrease in oxidative reactions. The inhibition of SOD leads to the accumulation of $O_2^{\cdot-}$. Indeed, we found that treatment with Korbazol significantly ($p<0.001$) inhibited SOD activity in all examined cell lines.

Changes in cellular ROS status have been shown to play an important role in apoptotic cell death. These observations also suggest that it may be possible to damage cancer cells by increasing their free radical contents through the inhibition of antioxidative enzyme activity. The results of our research on different types of cancer cells reveal oxidative stress as a candidate mechanism by which Korbazol exerts its cytotoxic potential. Its activity on other metabolic pathways remains to be elucidated.

ACKNOWLEDGEMENTS

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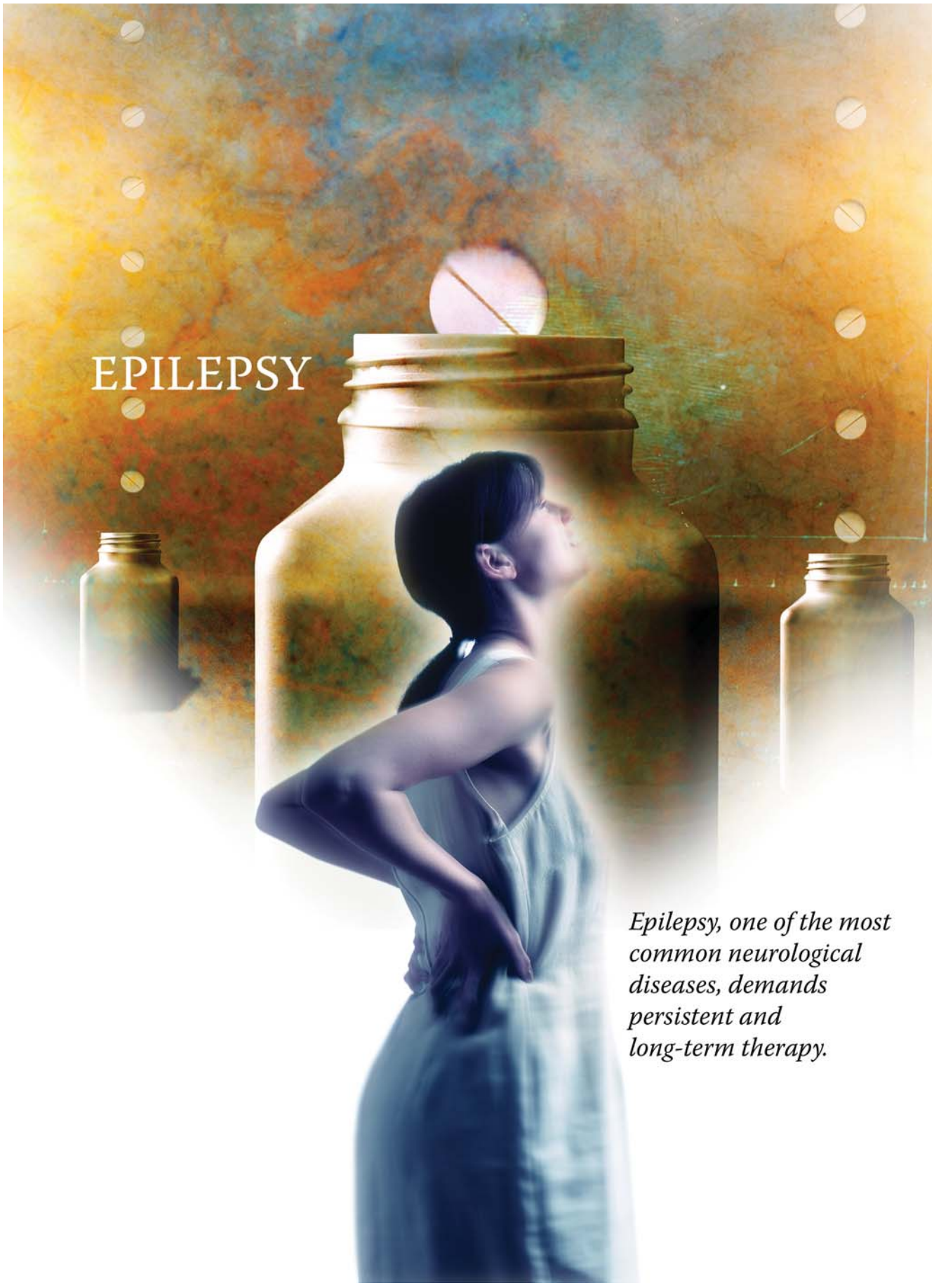

Epilepsy, one of the most common neurological diseases, demands persistent and long-term therapy.

ROLE OF ANTICONVULSANT THERAPEUTIC DRUG MONITORING IN IMPROVING CLINICAL OUTCOMES: AN EXAMPLE OF 12 ADULT EPILEPSY PATIENTS

Ana Rankovic[1], Jasmina Milovanovic[2], Snezana V Jankovic[2], Natalija Todorovic[3], Nemanja Rancic[4], Nikola Jestrovic[5], Iztok Grabnar[6], Slobodan Jankovic[2], Mihajlo Jakovljevic[2]

[1]Radiology Diagnostic Service, The Clinical Centre, Kragujevac, Serbia
[2]Pharmacology and Toxicology Department, The Medical Faculty, Kragujevac, Serbia
[3]The Neurology Department, The Clinical Centre, Kragujevac, Serbia
[4]The Medical Faculty University of Kragujevac, Serbia
[5]Merck Sharp & Dohme Idea Inc., AG Beograd, Serbia
[6]The Faculty of Pharmacy, University of Ljubljana, Slovenia

ULOGA KONTROLISANJA ANTIKONVULZIVNE TERAPIJE U UNAPREDJIVANJU KLINICKIH REZULTATA NA PRIMERU 12 ODRASLIH OBOLELIH OD EPILEPSIJE

Ana Ranković[1], Jasmina Milovanović[2], Snežana V Janković[2], Natalija Todorović[3], Nemanja Rančić[4], Nikola Jestrović[5], Iztok Grabnar[6], Slobodan Janković[2], Mihajlo Jakovljević[2]

[1]Centar za radiološku dijagnostiku , Klinički centar Kragujevac, Srbija
[2]Odsek za farmakologiju I toksikologiju, Fakultet medicinskih nauka, Kragujevac, Srbija
[3]Klinika za neurologiju, Klinički centar Kragujevac, Srbija
[4]Fakultet medicinskih nauka, Univerzitet u Kreagujevcu, Srbija
[5]Merck Sharp & Dohme Idea Inc., AG Beograd, Srbija
[6]Farmaceutski fakultet, Univerzitet u Ljubljani, Slovenija

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Epilepsy, one of the most common neurological diseases, demands persistent and long-term therapy. In spite of the available therapeutic interventions for seizure disorders, the incidence of epilepsy and mortality associated with "status epilepticus" remain significant (1). The goal of seizure management is satisfying seizure control with minimal side effects (2). Quality of life (QoL) studies have suggested that patients who suffer even a single seizure per year exhibit significantly reduced QoL (3). Among the wide range of medications offered in the market, valproic acid is still considered by many to be "a gold standard" to treat many convulsive disorders, including absence , generalised tonic-clonic seizures, myoclonic juvenile seizures, and photosensitive seizures . It also exhibits an acceptable toxicity profile compared with other anticonvulsants (4).

The Serbian health care system is still not capable of systemic therapeutic drug monitoring in clinical practice. The underlying reasons are typical for an upper-middle income transitional market and are attributed to both financial constraints and the lack of skilled, highly educated human resource availability in the field. These reasons influenced the authors to report pilot trial results to provide a small move forward on the issue among local clinicians (5).

The core aim of the trial presented was an exploration of the dose-response relationship in a small group of twelve adults suffering from epilepsy. The authors studied frequencies of drug adverse effects associated with long-term monotherapy and evaluated the appropriateness and clinical value of regular therapeutic drug monitoring (TDM) of valproic acid. The presence of a correlation between drug plasma concentration as an independent variable and frequency of seizures, frequency of adverse events and overall life quality as dependent variables was also tested. The reported results are an unpublished fragment originating from a large-scale collaborative project on pharmacokinetic modelling in juvenile epilepsy treatment (3-6).

This study was performed in 2007 on a small group of 12 adult patients suffering from clinically confirmed epilepsy. An anticonvulsant drug was administered in a full dosing regimen, and steady state was achieved prior to study inclusion. These patients were clinically followed at the Department of Neurology, Clinical Centre, Kragujevac, for three months. Cross-sectional analyses and the determination of clinical outcomes (adverse event frequency and life quality) at both inclusion (zero point) and the end of the study, except for the seizure frequency, were performed . All of the patients were using acid-resistant, film-coated valproate tablets with 2/3 sodium valproate and 1/3 valproic acid content with the brand name "Eftil", which was produced by "Zorka Pharma", Serbia. The determination of valproic acid and its salt concentrations in patient serum samples was performed by fluorescence polarisation immunoassay on an Abbott TDX commercial/FLX device using a set of reagents from the same manufacturer. Valproate was administered two to three times per day in oral form at a daily dose of 250 to 1000 mg. The patients were previously on this drug regimen for 4 to 516 months. The drug concentration in the serum was measured at two points: 2 hours after taking the morning dose (when the peak concentration in blood is expected, which is consistent with known drug kinetics) and 12 hours after ingestion (minimum trough concentration in the blood), e.g., before taking the morning dose.

The effectiveness of treatment outcome in terms of seizure control was estimated by the attending neurologist. This effectiveness was based on the frequency and severity criteria of seizure clinical appearance and regular electroencephalographic examination. A seizure diary was regularly kept, providing evidence of the occurrence, characteristics and frequency of attacks by the patient or a family member. The fact

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Correspondence to: Mihajlo B. Jakovljevic MD, PhD, Department of Pharmacology and Toxicology, Medical Faculty University of Kragujevac
E-mail: jakovljevicm@medf.kg.ac.rs; Tel: +381 34 306 800 EXT 223; Address: Svetozara Markovica 69; 34 000 Kragujevac, Serbia

that most patients lose consciousness during the seizure and have no clear aura but feel recognisable postictal symptoms makes the patient records unreliable. In addition, relatively frequent nocturnal seizures often go unnoticed (7). Because stigmatisation of this disease is common among Balkan nations, it is most often taken seriously by the affected individuals and their family members. Therefore, the expected level of compliance with the treating neurologist is high. The importance of the chronic toxicity of anticonvulsants with a number of cognitive and motor side effects was recognised early (8). In addition, anticonvulsant use is a long-term daily therapy (9). We provided data on side effects through guided interviews with patients and structured questionnaires. The majority of anticonvulsive substances are liposoluble compounds with good penetration through the haematoencephalic barrier and significant impact on emotional life and cognitive processes of the patient. The main clinical outcome, which was epilepsy-related quality of life, was assessed through the QOLIE-31 questionnaire, which has been previously validated and standardised in the Serbian language.

Trial results are presented in Table 1 and Graph 1. A common linear chart shows the arithmetic means of measured values of clinical outcomes in the beginning and at the end of the trial period for each patient observed. Drug plasma concentrations are intentionally provided separately: one measurement represents the arithmetic mean of respective (first and last time point) trough values, and the other one represents the arithmetic mean of peak values per patient. The average valproic acid plasma concentration was 93.98 ± 26.43 mg/l. The average number of seizures during the three months was 25.47 ± 93.52. There was a wide range of drug-related adverse effects. The average number of side effects was 5.06 ± 4.91 per patient. The valproate concentrations measured were in accordance with expected drug pharmacokinetics and within the desired therapeutic range of 40 to 100 μg/l (4). These values confirm that the therapeutic dose was, in most cases, adequately prescribed and individually adjusted.

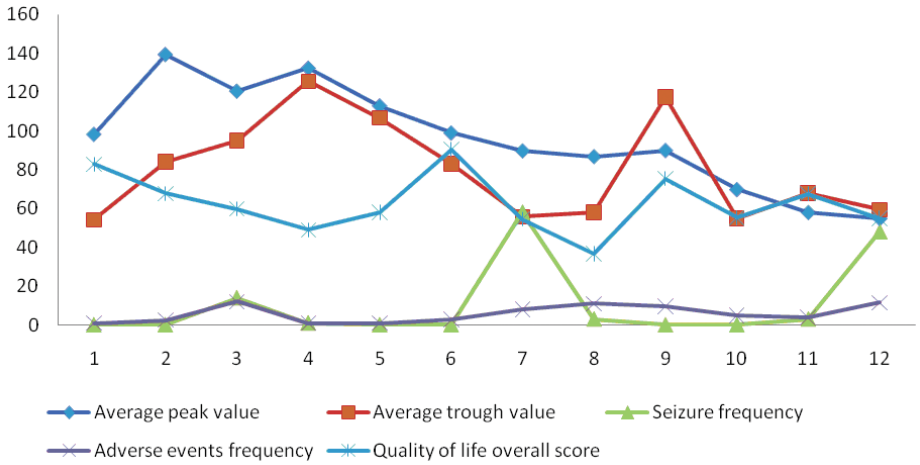
In many patients on prolonged therapy, the frequency and severity of drug side effects are roughly correlated with measured plasma concentrations (9). The consequence is obvious: a higher available fraction of the free drug leads to a higher concentration near the receptors, more extensive binding and a stronger expected biological response. This model is simplified according to what is happening in vivo. Tomson et al. obtained similar results, which showed that TDM had special importance for the new generation of antiepileptic drugs (10). Because of the narrow therapeutic range, the side effect rate was significantly higher, and therefore, performing TDM is invaluable. We noticed an absence of correlation between both peak and through plasma concentration values and the frequency of side effects. In particular, a high frequency of certain adverse events, such as disturbances related to thinking and memory, drowsiness, headache, fatigue, and increased body mass, was observed.

These cognitive- and motor skill-related adverse events are supposed to significantly contribute to quality of life decreases caused by therapy. There were no significant correlations between quality of life scores and peak or trough concentrations in patients. This result was found for both particular domains (seizure concern, overall life quality, emotional well-being, fatigueless/energy, cognitive functions, drug effects, and social activities) and an overall value. We should emphasise that our patient sample consisted mostly of people who had been diagnosed for years prior to study inclusion. There were no psychiatric comorbidities. The adolescent psychosexual personality maturation process was already complete. Most of these patients have also accepted the inevitability and peculiarity of their disease. Considering these issues, we can explain the balanced quality of life scores detected (3). However, the authors still believe that careful adjustment of the dosage regimen could lead to better life quality in patients suffering from epilepsy. To achieve this purpose, measuring plasma drug concentrations and gradually adjusting the dose are necessary.

Case number	Average peak value	Average trough value	Seizure frequency	Adverse event frequency	Quality of life overall score
1	98.295	54.14	0	1	82.85
2	139.38	83.98	0	2.5	67.7
3	120.53	95.035	14	12	59.8
4	132.325	125.49	1	1	48.95
5	112.92	106.675	0	1	58.05
6	98.975	83.06	0	3	90.5
7	89.755	55.785	58	8	54.6
8	86.83	58.145	3	11	36.75
9	89.96	117.355	0	9.5	75.45
10	69.85	54.865	0	5	55.25
11	58.03	67.975	3	4	67.65
12	54.9	59.425	48	11.5	54.8

Table 1. Arithmetic means of plasma concentrations and related clinical outcome values measured at the beginning and the end of clinical follow-up

Graph 1. Arithmetic means of plasma concentrations and related clinical outcome values measured at the beginning and the end of clinical follow-up



No significant correlation was observed between seizure frequency and trough or peak concentrations of valproate. Patients were in an equilibrium state of drug in the body prior to study inclusion. During that time, the choice of drug, time and route of application and the dosage regimen were carefully titrated according to the feedback on clinical effectiveness. Therefore, the absence of such a correlation can partly be interpreted by previously established satisfactory seizure control in many patients (8). Other causes could be natural gradual improvement of most juvenile epilepsies towards the third decade of life and the lack of appropriate sample size availability associated with high co-medication rates of adults in this study.

Because TDM was envisaged in seizure disorder treatment guidelines for a number of years, systematic reviews of decent methodological quality have been written on this topic, with opposing views (10). One of the main practical recommendations from our experiences with the pharmacokinetics of antiepileptic drugs is the important place and utility of TDM procedures. Based on our results, it appears that TDM of valproic acid and its salts is fully justified for optimising the dosage regimen. If these findings were proved in a larger pool of patients, it would mean that by adjusting drug concentration in the blood, we can significantly shape the main target of epilepsy treatment, quality of life. Although among our research population sample, the assumed dose-response relationship was weak, we certainly recommend further pharmacokinetic explorations of this type. More individually adjusted dosing regimens would certainly provide more satisfactory long-term clinical outcomes.

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MATKO MARUŠIĆ - LIFE OF AN EDITOR

Ljiljana Vuckovic Dekic
Institute for Oncology and Radiology of Serbia

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In this charming booklet, Matko Marušić, the founder (1991), Editor-in chief (1991-2009) and Editor Emeritus (since 2009) of the Croatian Medical Journal (CMJ), tells a story of how he made this new medical journal a good and reputable international journal.

The first part of the booklet "The first five years", the author explains, with great deal of humor, his efforts to acquisite new contributions, and how he pre-review the submitted manuscripts. I this part, he lists the most common mistakes, errors and omissions in the submitted manuscript, and explains how to deal with these. The roles of the reviewers, the co-editor-in-chief and entire the editorial staff are also explained, regardless the acceptance or rejection of the manuscript. The final acceptance does not mean that the authors and the editors are now revealed: the author's duties regarding the proofs and editors' duties regarding printing are also listed.

The second part of the booklet "The second five years" is devoted to the "Instructions for authors" – the efforts of the journal to make them clear, understandable and helpful to authors – all in accordance to the CMJ's author-friendly policy.

In the third part entitled "The first ten years" the author explains that the success of the CMJ is due to the constant, persistent, diligent and honest job of not only himself, but also of his coeditor-in chief (Ana Marušić), whose enormous contribution M. Marušić acknowledges with great gratitude.

Finally, in "The end article", the author explains the causes of his decision (the sweet-bitter one, as he emphasizes) to take leave, after 17 years, of his position as editor-in chef of the CMJ. His unsuccessful effort to protect the editorial freedom and independence led to this decision. This conflict originated from the CMJ retractions of several plagiarized articles of Asim Kurjak, whose plagiarism was detected by the editor of the British Medical Journal. Although the international scientific community tried to convince the leadership of the Zagreb Medical School that the CMJ acted according to the highest international standards of the editorship and publication ethics, the pressure was so intense that Matko Marušić left his position of the Editor-in-chief and took position of the dean of the University of Split's School of Medicine. M. M shows no bitterness regarding these sad events; the only trace of his disappointment is that the fairness came not from the colleagues, but from legal authorities.

Matko Marušić - Life of an Editor
ISBN 978-953-176-4780,
English, 51 pages,
60 references,
price Euro 5

The hard work of editing a medical journal is described in detail and in an amusing way in this booklet. I think that is due to the fact that Matko Marušić really loves this job, and spares no effort to help authors to write their articles suitable for reputable international medical journals, to which the CMJ, due to devoted job of its editors, belongs undoubtedly. Therefore, the booklet is equally valuable to all three main actors in the publishing game – authors, reviewers and editors – that certainly would benefit by reading it. And finally, and not less important, this book is so amusing that it is read with great pleasure, in spite of the seriousness of the topic.

Ljiljana Vučković-Dekić

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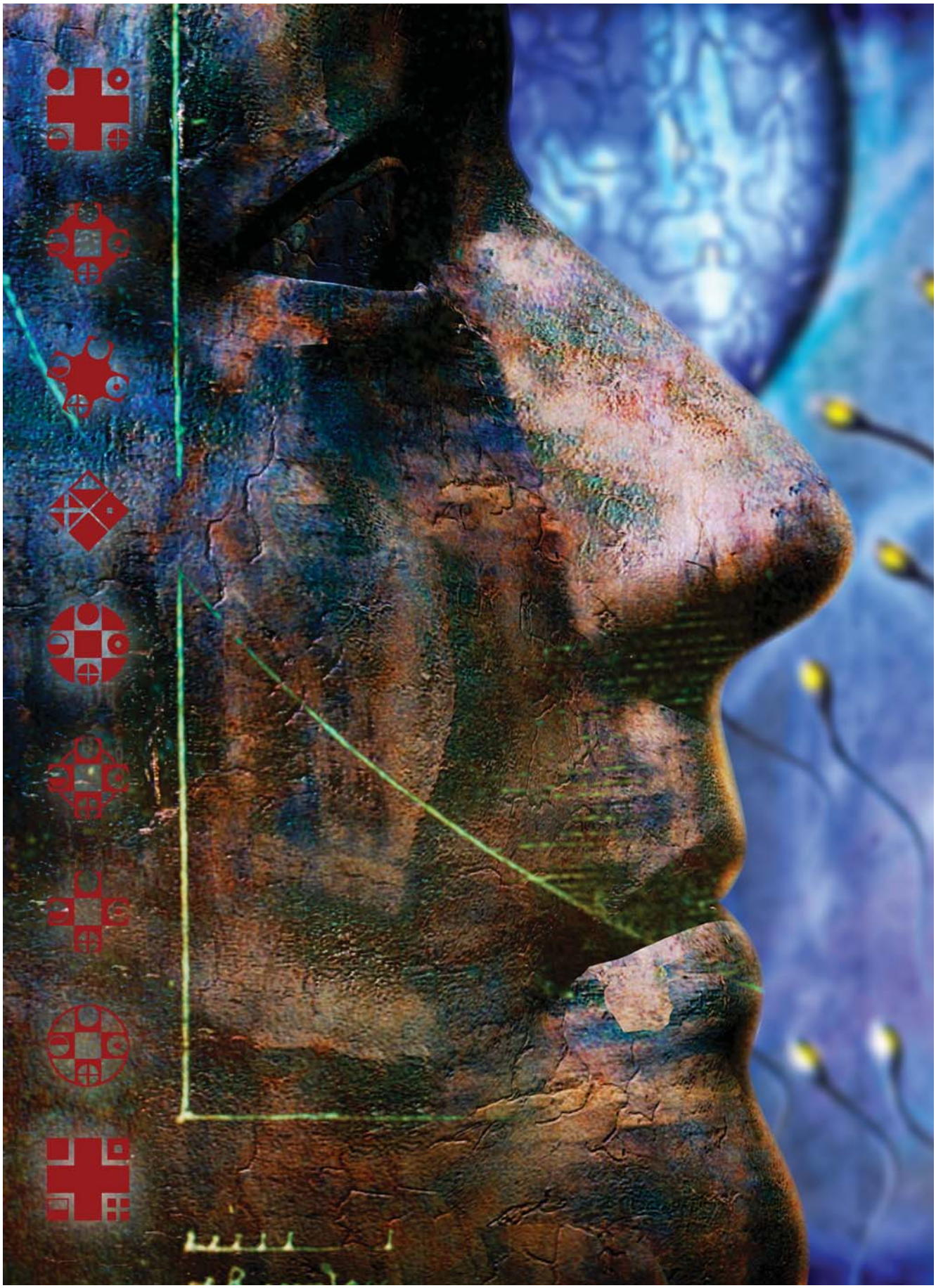
Correspondence to: Ljiljana Vuckovic Dekic,
Institute for Oncology and Radiology of Serbia, Pasterova 14, 11 000 Belgrade, e-mail: ljiljanavd@gmail.com



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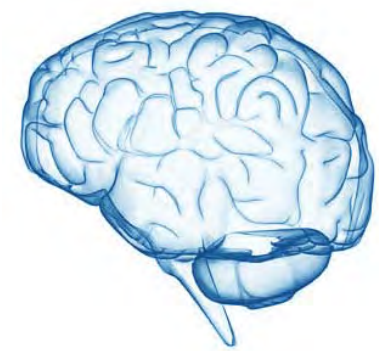

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THE MEDICAL FACULTY KRAGUJEVAC
Svetozara Markovica 69, 34000 Kragujevac, SERBIA
P.O. Box 124
Tel. +381 (0)34 30 68 00 • Tfx. +381 (0)34 30 68 00 ext. 112
e-mail: sjecr@medf.kg.ac.rs

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