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# MILD INDUCED HYPOTHERMIA AND AN URGENT INVASIVE CORONARY STRATEGY – A PROMISING PROTOCOL FOR COMATOSE SURVIVORS OF SUDDEN CARDIAC ARREST

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## INTRODUCTION

Sudden cardiac arrest remains the leading cause of death in developed countries, with an annual incidence ranging from 36 to 81 events per 100.000 inhabitants. Following the initial cardiopulmonary resuscitation, spontaneous circulation can be restored in 40 to 60% of the patients. Because of the usual delays in the “chain of survival”, more than 70% of resuscitated patients typically remain comatose upon hospital admission. This is due to postresuscitation brain injury, which may vary in severity from mild disability to a permanent vegetative state. Because no effective treatment was available in the past, the great majority of comatose survivors of cardiac arrest ultimately died in the hospital or in nursing homes in a permanent vegetative state.

The era of mild induced hypothermia, which began in 2002 following the landmark publication of two independent randomized trials (1, 2), undoubtedly revolutionised the field of postresuscitation treatment. Indeed, with a number required to treat between 7 and 8, hypothermia is a unique intervention in modern cardiovascular medicine. After effective treatment for postresuscitation brain injury became available and comatose patients “started to wake up” during subsequent days of treatment, more efforts have been made to define and treat the cause of cardiac arrest. Because an acute coronary event leading to critical narrowing or complete coronary obstruction is the main trigger of sudden cardiac arrest, urgent coronary angiography followed by percutaneous coronary intervention (PCI) has been increasingly performed upon hospital admission (3,4). We have learned that urgent PCI is feasible, safe, and successful and may improve the survival of patients with resuscitated cardiac arrest. Several hospitals have therefore designed dedicated postresuscitation protocols for comatose survivors of cardiac arrest that incorporate mild induced hypothermia, an urgent invasive coronary strategy and intensive care support related to haemodynamics, respiration, and electrolyte and acid-base balance (5).

## Evolution of postresuscitation management of comatose survivors of cardiac arrest at the University Medical Center in Ljubljana

We used mild induced hypothermia for the first time in September 2003, and since then, it has quickly become the standard of care in comatose survivors of cardiac arrest. Our usual hypothermia protocol is simple and cheap and can be immediately implemented in every hospital (Figure 1). In short, comatose survivors of cardiac arrest are obviously intubated and mechanically ventilated. After achieving the appropriate sedation and muscle relaxation to prevent shivering, hypothermia is induced by the rapid infusion (30 ml/kg in 30 minutes) of cold saline at 4 C (6). Ice packs are simultaneously used to augment cooling. Using this method, we are able to reach a target central temperature between 32 and 34 C in 3 to 4 hours. This target temperature, measured by urinary catheter, is then maintained for 24 hours and followed by spontaneous rewarming, which should not exceed 0.5 C per hour. During and following rewarming, it is very important to prevent temperature rise, shivering and hypovolemia. We initially started the hypothermia protocol only after hospital admission. However, after gaining more experience, we advised our prehospital emergency units and referring hospitals to start hypothermia immediately after re-establishing spontaneous circulation and continue during the transport to our hospital.

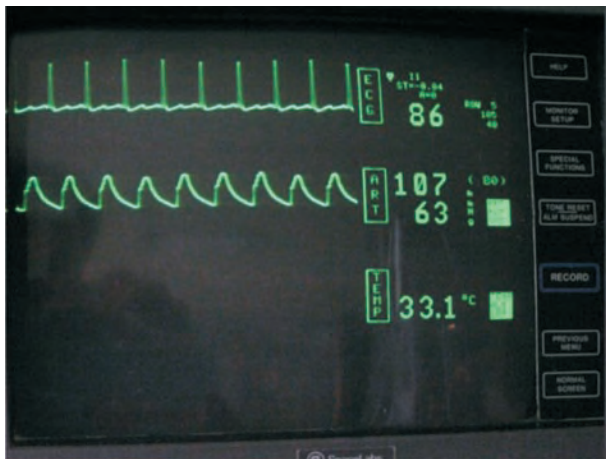
Because we are a primary PCI centre for ST-elevation myocardial infarction (STEMI) with 24-hour service since 2000, we gradually adopted a strategy of urgent coronary angiography and PCI in comatose survivors of cardiac arrest. History of coronary artery disease, chest discomfort before the onset of cardiac arrest, signs of STEMI or other ischemic changes in the postresuscitation electrocardiogram (ECG) argue for an acute coronary cause of arrest and urgent coronary angiography to immediately define the anatomical lesion(s). Indeed, the lesion may be found not only in a high percentage of

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**Figure 1.** Protocol for mild induced hypothermia in comatose survivors of cardiac arrest at the University Medical Centre in Ljubljana (Slovenia)



- **Intubation /mechanical ventilation**
- **Sedation** (midazolam 0.1-0.3mg/kg bolus+infusion)
- **Muscle relaxation** (0.1 mg/kg norcuronium + repeated when shivering)
- **Rapid infusion of 0.9% NaCl at 4 C** (30 ml/kg in 30 minutes)
- **Ice packs** (head, neck, axilla, abdomen)
- **Maintain 32-34 C for 24 hours** (Urinary bladder temperature)

patients with STEMI but also in up to 30% of patients without STEMI in a postresuscitation ECG (3, 4). We also demonstrated that combining urgent invasive coronary strategy with hypothermia is feasible and safe (6). Hypothermia does not compromise the angiographic result of PCI, and there is no excess in arrhythmias and haemodynamic instability requiring more aggressive support with inotropes, vasopressors or an intra-aortic balloon pump (6, 7). Moreover, when the proportion of comatose survivors of out-of-hospital cardiac arrest undergoing hypothermia and urgent invasive coronary strategy increased from 0% between 1995-97 to 90% and 70% between 2006 and 2008, respectively, the survival to hospital discharge concomitantly increased from 24% to 62%. Importantly, survival with good neurological recovery concomitantly increased from 15% to 40%. These impressive but not yet “peer review” published results were independently confirmed by other investigators using the same strategy of aggressive postresuscitation management (5,8). We therefore designed a special “fast track” for comatose survivors of cardiac arrest and complemented the already existing “STEMI-primary PCI” network to offer the benefits of this treatment to patients from remote areas (Figure 2).

**CONCLUSION**

Mild induced hypothermia and an urgent invasive coronary strategy on suspicion of a coronary cause of cardiac arrest should be part of a comprehensive postresuscitation treatment protocol for comatose survivors of cardiac arrest. Such an “organ-oriented” and aggressive treatment strategy may significantly improve the previously dismal survival rates in these patients. It is likely that the best results may be achieved if the treatment of comatose survivors of cardiac arrest is centralised to dedicated interventional cardiology centres that already have a “STEMI-primary PCI” network with a high volume of acute PCI procedures and a competent cardiac intensive care unit.

**Start hypothermia after re-establishment of spontaneous circulation already on the field**



**Urgent transport to dedicated interventional cardiology center with skilled cardiac intensive care unit**



**Urgent coronary angiography-PCI if coronary cause is likely while hypothermia is ongoing**



**Complete 24-hour hypothermia and provide intensive care support**



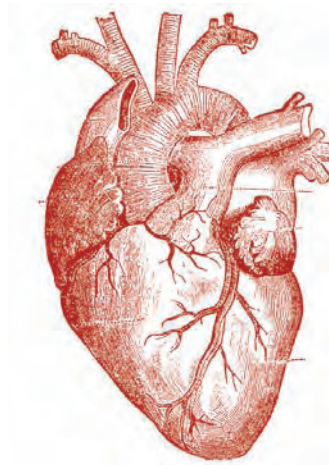
**Figure 2.** “Fast track” for comatose survivors of out-of-hospital cardiac arrest.





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# HIV

*Context:* Montenegro has a low-level HIV epidemic, but the majority of registered HIV cases have been diagnosed in patients between the ages of 20 to 34-years-old. The aim of this study was to assess the level of HIV-related knowledge, attitudes towards people living with HIV (PLHIV) and sexual behaviour among youth aged 15-24 in Montenegro.

*Methods:* A nationally representative cross-sectional survey assessed 1164 people aged 15-24. The data were collected in September 2009 using face-to-face interviews concerning HIV-related awareness and a self-administered questionnaire related to sexual experience.

*Results:* In general, the level of knowledge and accepting attitudes towards people living with HIV were unsatisfactory in the surveyed population, although they reported current sexual activity. According to participant sexual initiation and not as a first episodes of sexual inter-





## HIV-RELATED KNOWLEDGE, ATTITUDES AND SEXUAL RISK BEHAVIOURS AMONG MONTENEGRIN YOUTH

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### ABSTRACT

**Context:** Montenegro has a low-level HIV epidemic, but the majority of registered HIV cases have been diagnosed in patients between the ages of 20- to 34-years-old. The aim of this study was to assess the level of HIV-related knowledge, attitudes towards people living with HIV (PLHIV) and sexual behaviour among youth aged 15-24 in Montenegro.

**Methods:** A nationally representative cross-sectional survey assessed 1164 young people aged 15-24. The data were collected in December 2009 using face-to-face interviewing for topics concerning HIV-related awareness and attitudes, whereas a self-administered questionnaire covered topics related to sexual experiences and behaviour.

**Results:** In general, the level of HIV-related knowledge and accepting attitudes towards people living with HIV were unsatisfactory. Of the surveyed population, slightly more

than half reported current sexual activity. The reported level of sexual risk behaviour differed significantly according to participant sex and region. Early sexual initiation and not using condoms during the first episodes of sexual intercourse were found to be significant predictors of more frequent sexual risk behaviour.

**Conclusions:** Despite the relatively high awareness pertaining to routes of HIV transmission, the surveyed population reported misconceptions related to HIV transmission. A significant proportion of young people have engaged in risky sexual behaviours. These results strongly support the introduction of secondary school curriculum related to HIV/STI prevention as well other risk behaviours observed in this age cohort.

**Keywords** HIV - Young people - Sexual risk behaviour – Montenegro

### INTRODUCTION

Montenegro has a low-level HIV epidemic and an overall HIV prevalence of 0.01%. The HIV infection rate appears to be stable, with 7-12 new cases diagnosed each year. Since 1989 when the index case was detected, there have been 115 registered HIV/AIDS cases and 35 AIDS-related deaths. The most important transmission routes in Montenegro seem to be heterosexual (48%) and homo/bisexual intercourse (36%). Recently, there has been a notable shift from heterosexual to homosexual transmission routes among newly registered cases. More than 60% of registered HIV cases have been diagnosed in patients between the ages of 20 and 34 (1).

The aim of this study was to provide valid data related to HIV knowledge and sexual behaviour. This study was designed as the second in a series regarding HIV/AIDS-related knowledge, attitudes and sexual practices among young people in Montenegro as a part of the national HIV response monitoring and evaluation framework. These

data should be used to establish trends in the behaviour of young people as well as to provide guidance to tailor HIV prevention in Montenegro. The first survey of this kind was conducted in 2007 in a nationally representative sample of young adults aged 18-24 (n=864) (2). Both surveys were financially supported by the Global Fund to Fight AIDS, Tuberculosis and Malaria.

The increasing HIV infection rates in our region and in the countries of Southern and Eastern Europe are mainly driven by risky sexual behaviour among young people (3, 4).

Risky sexual behaviour among adolescents and young people includes early sexual initiation, multiple sexual partners, inconsistent condom use, sexual intercourse under the influence of alcohol and drugs, concurrent relationships, and casual partners. (5).

This article is focused on HIV-related knowledge, attitudes towards PLHIV and sexual risk behaviour among young people in Montenegro.

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## METHODS

### *Participants*

This cross-sectional household survey was conducted in a nationally representative sample (n=1,200) of young people aged 15-24 in December 2009. The overall participation rate was 97%.

The sampling frame was based on the Census of Populations, Households and Dwellings completed in 2003. A sample size of 1,200 was calculated to provide a precision of 3%, an expected prevalence of 50% for the indicator "Misconception related to HIV transmission", an expected refusal rate of 10% and a confidence interval of 95%. The sample was created as a two-stage stratified probability sample, with the enumeration areas as primary sampling units and households as secondary units. The enumeration areas, as primary sampling units, were stratified according to the type of settlement (urban/rural) and region (northern, central or southern) and were selected in proportion to the number of persons aged 15-24 years. A list of households for the selected enumeration areas (households with at least one member aged 15 to 24 years) was used as a sampling frame for the selection of secondary units. From each previously selected enumeration area, 10 households were selected with equal probability (simple random sample). In households with more than one member aged 15-24, the person with the most recent birthday was sampled.

The questionnaire consisted of two parts. The first part was comprised of questions related to knowledge, attitudes and beliefs and was completed by an interviewer in a face-to-face setting. The second part contained questions related to sexual behaviour and was self-administered.

### *Data collection instruments*

A knowledge, attitudes, practices and beliefs-structured (KAPB) questionnaire, containing 169 variables, was used. The first part of the questionnaire included socio-demographic data and questions related to the following items: personal and family background (education, activity, economic status and parental monitoring), HIV/AIDS-related knowledge, attitudes towards people living with HIV/AIDS, attitudes towards gender sexual roles, peer norms, information sources, sensation seeking, locus of control and self-esteem.

The self-administered portion of the questionnaire included questions regarding types of sexual experiences, age at first intercourse, number of sexual partners during the previous 12 months, number of lifetime partners, condom use at first intercourse, condom use at last intercourse, condom use at last intercourse with non-regular partner, frequency of condom use during the previous 12 months (never, sometimes, always), attitudes towards condom use, self-efficacy in relation to condom use, self-reported symptoms of STIs, HIV testing, concurrent relationships and use of relevant reproductive health services.

The questionnaire was piloted with 20 participants, and those households were omitted from the final sampling frame. The final version of the questionnaire was adjusted in accordance with the pilot survey findings.

### *Measures*

**The HIV/AIDS knowledge scale** contained 7 standardised items (Cronbach's  $\alpha=0.6314$ ) related to knowledge regarding modes of HIV transmission prevention (such as "Is it possible to prevent HIV transmission by having sex exclusively with one healthy and faithful partner?") and including major misconceptions related to HIV transmission (such as the possibility of HIV transmission through mosquito bites, use of public toilettes, or sharing food with an HIV-infected person). All correct answers were coded as 1, while false and "don't know" answers were coded as 0.

**The parental monitoring scale** contained 4 questions – parental awareness of their children's whereabouts in the evening, how their children spent their money and spare time and who their children maintained as friends. The parental monitoring scale was scored as a composite of these four questions (Cronbach's  $\alpha=0.7845$ ) with a theoretical range of 4-12 and a mean score of  $M=11.17$  ( $SD=1.41$ , 95% CI 11.08-11.25), with higher numbers indicating stronger parental control. More than 60% of the respondents had scores of 12, meaning that they had perceptions of high levels of parental monitoring, while more than 90% had scores  $\geq 10$ .

**Attitudes towards PLHIV** were examined through 16 questions measuring acceptance of PLHIV and those populations most at -risk for HIV infection. The acceptance of PLHIV scale was calculated as a score of these 16 recorded items (Cronbach's  $\alpha=0.8189$ ), where negative responses were coded as 0 and positive responses were coded as 1. The possible range of scale values was 0 to 16, with a higher score indicating a more open attitude towards PLHIV.

**The „attitudes towards condom use" scale** was calculated as a 5-point Likert scale measuring concordance with 10 statements about condom use, norms and effectiveness (Cronbach's  $\alpha=0.669$ ). The values recorded ranged from 10 to 50, with higher codes indicating more positive attitudes towards condom use, i.e., stronger motivation. The mean score of this index was 35.12 ( $SD=5.65$ ), with scores ranging from 15 to 50.

**The self-efficacy index** was calculated as a scale consisting of 9 items referring to the assessment of personal ability for condom use (purchasing, negotiation of its use and proper use, Cronbach's  $\alpha=0.825$ ). Values ranged from 9 to 45 and certain items were recorded such that higher scores indicated higher perceptions of self-efficacy. The range of the index was 10-40 with a mean value of 26.92 ( $SD=6.6$ ).

**The Sexual Risk Taking Index** was formed as a scale consisting of 6 behavioural dimensions that were linked with the risk of contracting HIV or another STI (condom use [never/sometimes], sex under the influence of alcohol,



sex under the influence of drugs, more than 2 sexual partners in the previous 12 months, non-condom use at last sexual intercourse with non-regular partner, and concurrent relationships), with a 0-6 score range. For the purpose of logistic regression analysis, the SRT index was dichotomised such that 0 indicated respondents with no risk behaviours experienced and 1 indicated respondents with an index value of 1-5.

### *Statistical Methods*

The frequency distributions for all examined variables were calculated and presented. Univariate associations of potential predictors with sexual risk behaviours were examined. To adjust for multiple predictors of risky sexual behaviours simultaneously, multivariate logistic regression was performed. Separate models were constructed for men and women. All variables that were significant in the univariate analysis as well as those shown to be significant in the literature were included in the logistic regression model. The backward stepwise procedure was used to determine the final model. The fitness of the final model was assessed using the Hosmer-Lemeshow test (6).

## **RESULTS**

### *Demographic characteristics*

The mean age of the participants was 19.28 years (SD=2.63). Almost half of the participants, 555, were from the central region (47.7%), 346 (29.7%) were from the northern part of Montenegro, and 263 (22.6%) were from the southern coastal region. Of all participants, 848 (72.9%) were from urban areas, and 316 (27.1%) were from rural settlements. The basic socio-demographic characteristics of the sample are presented in Table 1.

### *HIV/AIDS Knowledge*

The proportion of respondents who provided correct answers to individual HIV knowledge questions varied from 56.5% („Could HIV be contracted if using a glass used by an HIV-infected person?“) to 86% for showing awareness of condom use as the best prevention method concerning the sexual transmission of HIV. Misconceptions regarding HIV transmission were still present to a significant extent. More than one-third of respondents believed that HIV could be transmitted through sharing a meal with an HIV-infected person or using a public toilette. One-fifth (20.3%) were not aware that HIV could be transmitted by having sexual intercourse with a healthy looking person.

The mean score of the HIV/AIDS knowledge scale was 4.88 (SD=1.75, 95% CI 4.78-4.98). Slightly less than half of the respondents had scores of 6 or 7, while almost one-fourth had scores  $\leq 3$ . Only 21.2% of the respondents gave correct answers to all 7 questions. There was no difference between genders ( $t=-1.122$ ,  $p=0.262$ ).

There was no difference in the knowledge level shown between male and female participants ( $t=-1.122$ ,  $p=0.262$ ). A regional comparison of the HIV/AIDS knowledge scale revealed significant differences ( $F=16.459$ ,  $df=2$ ,  $p<0.001$ ). The best knowledge scores were derived from respondents of the coastal region, and the worst were from respondents of the northern region. Respondents from urban settlements scored significantly higher in comparison to their peers from rural settlements ( $M=5.0$  compared to  $M=4.5$ ,  $t=4.51$ ,  $p<0.001$ ). Respondents under the age of 18 scored lower than those over 18 (39.9% of respondents aged 15-17 compared to 47.3% of respondents 18-24 had high scores [6 or 7],  $\chi^2=5.71$ ;  $df=1$ ,  $p<0.05$ ). Exposure to HIV-related information at school (from a basic level up to participation in peer education programmes) was correlated with significantly higher HIV knowledge index scores ( $F=11.395$ ,  $p<0.001$ ).

### *Attitudes towards PLHIV*

The results revealed a significant stigma towards PLHIV as expressed by the fact that 45% of the surveyed young people would not share a meal, 35% would not share a desk and 32% would not hang out with an HIV-infected person, while 67% of young people would not buy food from an HIV-infected vendor. Thirty-three percent declared that an HIV-infected teacher should not be allowed to continue teaching, while 22% thought that HIV-infected pupils should not be allowed to continue attending regular school.

The mean score of this scale was 8.36, with 8.13 (95% CI 7.83-8.42) among males, and a slightly higher score of 8.63 among females (95% CI 8.32-8.95,  $t=-2,310$ ,  $p=0,021$ ). Only 40% of the surveyed young people had scores above the median value (Med=9), while only one-sixth of the respondents scored  $\geq 13$ , which indicated a high level of acceptance towards PLHIV. The level of acceptance of PLHIV was positively correlated with education level ( $\rho=0.124$ ,  $p<0.001$ ) and scores on the HIV/AIDS knowledge scale ( $\rho=0.443$ ,  $p<0.001$ ).

Univariate analysis revealed that scores of this scale varied insignificantly depending on the type of settlement (19.3% of urban and 8.6% of rural youth surveyed expressed high levels of acceptance,  $\chi^2=19.197$ ;  $df=2$ ,  $p<0.001$ ), the size of the place in which participants had lived the majority of their lives (higher acceptance in places with more than 10,000 inhabitants,  $\chi^2=24.99$ ;  $df=6$ ,  $p<0.001$ ), age (younger respondents revealed lower levels of acceptance,  $\chi^2=23.83$ ;  $df=2$ ,  $p<0.001$ ), and the education levels of both parents and the participants themselves (12.2% of young people with primary education compared to 19% of young people with secondary and 26% with college/university education revealed high levels of acceptance toward people living with HIV,  $\chi^2=21.08$ ;  $df=4$ ,  $p<0,001$ ). There were no differences between participants of various regions ( $F=1.59$ ,  $p>0,05$ ). A higher frequency of discussions with participants' mothers





**Table 1.** Basic socio-demographic characteristics of the sample by gender

Age	Males (n=620)	Females (n=544)	Total (n=1164)
15	58 (9.4)	62 (11.4)	120 (10.3)
16	67 (10.8)	61 (11.2)	128 (11.0)
17	73 (11.8)	62 (11.4)	135 (11.6)
18	64 (10.3)	61 (11.2)	125 (10.7)
19	52 (8.4)	45 (8.3)	97 (8.3)
20	76 (12.3)	59 (10.8)	135 (11.6)
21	51 (8.2)	49 (9.0)	100 (8.6)
22	74 (11.9)	52 (9.6)	126 (10.8)
23	55 (8.9)	50 (9.2)	105 (9.0)
24	50 (8.1)	43 (7.9)	93 (8.0)
<b>Type of settlement *</b>			
Urban	436 (70.3)	412 (75.7)	848 (72.9)
Rural	184 (29.7)	132 (24.3)	316 (27.1)
Lived with both parents by the age of 18	559 (90.7)	491 (90.9)	1050 (90.8)
Currently live with parents	583 (94.3)	505 (93.5)	1088 (94.0)
<b>Mother's education</b>			
Up to primary school completed	90 (14.6)	91 (16.9)	181 (15.7)
Secondary school Completed	430 (69.8)	354 (65.7)	784 (67.9)
College/university	96 (15.6)	94 (17.4)	190 (16.5)
<b>Father's education</b>			
Up to primary school completed	41 (6.8)	50 (9.4)	91 (8.0)
Secondary school Completed	416 (68.5)	354 (66.3)	770 (67.5)
College/university	150 (24.7)	130 (24.3)	280 (24.5)
<b>Participant's occupation**</b>			
Secondary school student	249 (40.2)	240 (44.1)	489 (42.0)
College/University student	167 (26.9)	164 (30.1)	331 (28.4)
Employed	100 (16.1)	64 (11.8)	164 (14.1)
Unemployed/housekeeper	104 (16.8)	76 (13.9)	180 (15.4)
<b>Family socio-economic status</b>			
Worse than average	32 (5.2)	23 (4.2)	55 (4.7)
Average	466 (75.3)	401 (73.7)	867 (74.5)
Better than average	121 (19.5)	120 (22.1)	241 (20.7)
<b>Marital status</b>			
Single	602 (97.1)	511 (94.5)	1113 (95.9)
Married	14 (2.3)	26 (4.8)	40 (3.4)
Cohabiting	3 (0.5)	2 (0.4)	5 (0.4)
Divorced	1 (0.2)	2 (0.4)	3 (0.3)
<b>Size of the settlement where participants had lived the majority of their lives</b>			
Village	126 (20.4)	87 (16.1)	213 (18.3)
Place with <10,000 inhabit.	87 (14.1)	80 (14.8)	167 (14.4)
More than 10,000 and less than 50,000 inhabitants	172 (27.4)	175 (32.5)	347 (29.9)
>50,000 inhabitants	234 (37.8)	200 (36.9)	434 (37.4)
<b>Gender differences: * p&lt;0.05, ** p&lt;0.01, *** p&lt;0.001</b>			



**Table 2.** Sexual behaviour by gender

	Males (n=620) N (%)	Females (n=544) N(%)	Total (n=1164) N (%)
Have had vaginal intercourse***	397 (64.0)	197 (36.2)	594 (51.7)
Have had anal intercourse***	263 (43.1)	88 (16.4)	351 (30.6)
Have had oral intercourse***	131 (21.5)	33 (6.1)	164 (14.3)
<b>Age at first sexual intercourse</b>			
14 and younger	44(10.6)	1 (0.5)	45 (7.5)
15	49 (11.9)	3 (1.5)	53 (8.7)
16	111 (27.0)	14 (7.2)	125 (20.6)
17	87 (21.2)	33 (16.9)	120 (19.8)
18	70 (17.0)	57 (29.2)	127 (21.0)
19	31 (7.5)	43(22.1)	74 (12.2)
20 and older	19 (4.6)	43 (22.1)	61 (10.1)
<b>Number of partners during previous 12 months***</b>			
0	25 (6.3)	13 (6.7)	38 (6.4)
1	142 (35.7)	146 (75.3)	288 (46.6)
2	95 (23.9)	25 (12.9)	120 (20.3)
3	45 (11.3)	8 (4.1)	53 (9.0)
4+	91 (22.8)	2 (1.0)	93 (15.7)
<b>Number of lifetime partners***</b>			
1	56 (14.5)	105 (54.4)	161 (27.9)
2	50 (13.0)	33 (17.1)	83 (14.4)
3	46 (11.9)	28 (14.5)	74 (12.8)
4+	233 (60.6)	27 (14.0)	260 (44.9)
Condom use at first intercourse	262 (63.9)	119 (60.7)	381 (62.9)
Condom use at last intercourse*	268 (65.8)	107 (55.2)	375 (62.4)
Had a non-regular partner during the previous 12 months***	182 (44.5)	21 (10.7)	203 (33.6)
Condom use at last intercourse with non-regular partner	207 (70.9)	32 (55.2)	239 (67.9)
Sex under the influence of alcohol in the previous 12 months	156 (38.1)	39 (19.9)	195 (32.2)
Sex under the influence of drugs in the previous 12 months	11 (2.7)	1 (0.5)	12 (2.0)
Concurrent relationships (ever)	204 (49.9)	11 (5.6)	15 (35.3)
<b>Condom use during the previous 12 months **</b>			
Never	46 (11.7)	38 (21.0)	84 (14.6)
Sometimes	160 (40.7)	77 (42.5)	237 (41.3)
Always	187 (47.6)	66 (36.5)	253 (44.1)
<b>Condom use during oral sex during the previous 12 months **</b>			
Never	141 (49.0)	73 (73.0)	214 (55.2)
Sometimes	68 (23.6)	13 (13.0)	81 (20.9)
Always	79 (27.4)	14 (14.0)	93 (24.0)
<b>Gender differences: * p&lt;0.05, ** p&lt;0.01, *** p&lt;0.001</b>			





regarding sexual topics was shown to be a significant predictor of an accepting attitude (20.8% of those who talked with their mothers about topics related to sex often/always had positive attitudes towards PLHIV compared to 14.8% of those who talked with their mothers rarely or never,  $\chi^2=17.958$ ;  $df=2$ ,  $p<0.001$ ). A positive association between exposure to HIV-related information and a higher level of acceptance of PLHIV was revealed ( $\rho=0.143$ ,  $p=0.000$ ).

### Sexual behaviour

Approximately one -half of the participants declared having had certain sexual experiences. The major characteristics of the sexual behaviour of the surveyed population are summarised in Table 2. Respondents from the coastal region reported sexual activity to a greater extent than their peers from the central and northern regions.

On average, the young people surveyed first experienced sexual intercourse at the age of 17.2 ( $SD=1.8$ ), with males (16.6,  $SD=1.7$ ) reporting significantly earlier sexual experiences ( $t=-12.91$ ,  $p<0.001$ ) than females (18.4,  $SD=1.6$ ). The majority of sexually active respondents (61.4%) first had sexual intercourse between the ages of 16 and 18 (65.2% males and 53.3% females), with 22.4% older than 18 and 16.2% (22.6% males and 2.6% fe-

males) younger than 16 at their first sexual intercourse. Only 8.2% declared that they had entered into sexual activities too early, with no difference between genders.

Sixty-three percent of young people used condoms during their first intercourse. A positive association between condom use at first intercourse and exposure to HIV-related information at school was observed ( $\rho=0.18$ ,  $p=0.000$ ).

From those who reported having had sexual intercourse, 93.3% were sexually active during the previous 12 months. Of all participants, 48.6% had only one partner during the previous 12 months, while 15.7% had 4 or more partners, with males having had significantly more partners than females ( $Med_m=2$ ,  $M_m=2.63$ ,  $SD_m=2.76$ ;  $Med_f=1$ ,  $M_f=1.31$ ,  $SD_f=2.16$ ,  $t=9.197$ ,  $p<0.001$ ).

A higher extent of parental monitoring was correlated with reduced numbers of partners (Figure 2).

More than one -third of the respondents who were sexually active before the age of 16 had 4 or more partners during the previous 12 months ( $\chi^2=60.72$ ;  $df=5$ ,  $p<0.001$ ). Young people with higher scores on the sensation-seeking scale were prone to higher numbers of sexual partners ( $\chi^2=9,969$ ;  $df=1$ ,  $p<0,01$ ). Considering the number of lifetime partners, 54.4% of females had just one partner, while more than three -fifths of males had 4 or more partners.

One -fourth of men and 3.1% of women reported that their last sexual intercourse was casual ( $\chi^2=43.25$ ;  $df=1$ ,  $p<0,001$ ).

Early sexual initiation was a significant predictor of more frequent sex with non-regular partners (57.8% of those who had sexual intercourse  $\leq 16$  years of age had experienced sex with a non-regular partner compared to 19.2% of those respondents who first had sexual intercourse at age 17 or older,  $\chi^2=94.17$ ;  $df=1$ ,  $p<0,001$ ). A positive correlation was revealed between higher scores on the sensation-seeking scale and sex with non-regular partners ( $\rho=0,269$ ,  $p<0,001$ ).

Inconsistent condom use was associated with the perception that condoms destroy the spontaneity of the sexual act ( $\rho=-0,259^{***}$ ) or create erectile dysfunction ( $\rho=-0,212^{***}$ ) and beliefs that the person suggesting condom use does not have confidence in the partner ( $\rho=-0,102^*$ ). A higher level of positive attitudes towards condom use ( $\rho=0,298^{***}$ ) and a higher perception of self-efficacy related to the negotiation and proper use of condoms ( $\rho=0,197^{***}$ ) were shown to be positively associated with more frequent condom use.

### Sexual Risk Behaviours

The survey results revealed that more than three -fourths of respondents, to different extents, have engaged in sexual risk behaviour. Males engaged in sexual risk behaviour to a much greater extent than females ( $\chi^2=86.03$ ;  $df=6$ ,  $p<0,001$ ). The mean score of this index was 1.81 (2.20 for males, 1.02 for females), with 41.4% of males and just 7.8% of females having scores  $\geq 3$ .

Figure 2. Number of partners in the previous 12 months with regard to the level of parental monitoring

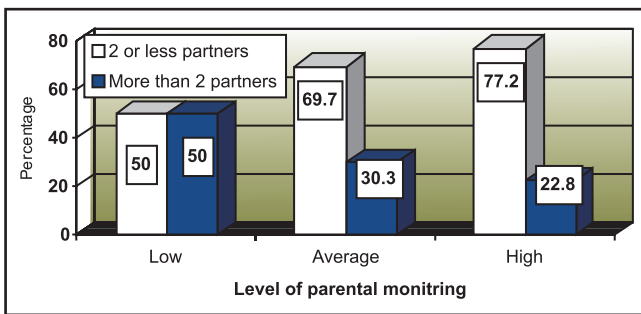
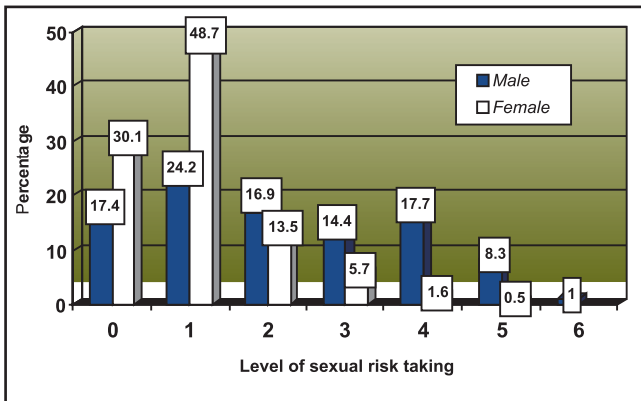


Figure 1. Sexual risk -taking by gender





	Total (N=486)	Women (N=337)	Men (N=149)
<b>Respondent's education level</b>			
Completed primary school or less ®			1
Secondary school			0.0006
College/university			0.0017
<b>Father's education level</b>			
Completed primary school or less ®	1	1	
Secondary school	0.51 (0.15-1.73)	0.79 (0.09-6.59)	
College/university	0.37 (0.18-0.78)	0.17 (0.05-0.62)	
<b>Mother's education level</b>			
Completed primary school or less ®	1	1	
Secondary school	0.46 (0.17-1.23)	0.32 (0.06-1.57)	
College/university	1.23 (0.57- 2.64)	1.53 (0.47-4.95)	
"Most of my friends think it's normal to have sex on the first date."	1.79 (1.04-3.08)		2.76 (1.45-5.27)
"Most of my friends have negative attitudes towards condom use."	2.24 (1.08-4.66)		
Condom use at first sexual intercourse	0.16 (0.08-0.30)	0.08 (0.03-0.25)	0.22 (0.09-0.51)
Sexual initiation at the age ≤15	2.34 (1.23 – 4.45)		2.39 (1.20-4.81)
Talking about sexual life with the partner (often/always)	1.66 (0.93-2.95)	2.95 (1.01-8.56)	
Talking about sexual life with best friend (often/always)	1.85 (1.01-3.41)		
Attitudes towards condom use	0.95 (0.91-0.98)		0.93 (0.87-0.98)
High level of parental monitoring	0.57 (0.32-1.02)		0.41 (0.20-0.87)
<b>Gender</b>			
Male®	1		
Female	0.52 (0.30- 0.42)		
<b>Age</b>	1.23 (1.09- 1.38)		

**Table 3.** Logistic regression models regarding sexual risk -taking by gender (odds ratios and 95% confidence intervals)

The overall logistic regression model was constructed including 486 participants. Separate models were constructed for male and female participants to understand the gender-induced differences in the predictors of sexual risk behaviours. All three models are presented in Table 3.

Females were less prone to sexual risk behaviour (OR=0.52) than their male peers. The logistic regression model for all respondents revealed that higher education levels of the father or mother as well as higher levels of parental monitoring were significant prevention factors for sexual risk behaviours (OR=0.57). Condom use at first intercourse was shown to have the strongest protective effect concerning all respondents (OR=0.16) as well as males (OR= 0.08) and females (OR=0.22) alone. More positive attitudes towards condom use were significant in the model for all respondents as well as that for male respondents, while more frequent discussions about sexual life with the partner was shown to be a risk factor in the model for all respondents (OR=1.66) and for women (OR=2.95). Early sexual initiation was a significant risk factor in the overall model (OR=2.34) as well as in that for the male participants (OR=2.39).

## DISCUSSION

The present study evaluated the level of HIV-related knowledge and accepting attitudes towards PLHIV as well as the level of risk behaviours related to the transmission of HIV and other STIs.

Overall, the level of knowledge was satisfactory in terms of the prevention of sexual transmission of HIV. However, misconceptions related to HIV transmission were still present in more than one -third of the surveyed population. The knowledge level in this study was slightly higher than that in a previous study from 2007, which surveyed a population aged 18-24 (2). A regional comparison revealed significant differences in the knowledge levels between participants from the north region and other parts of Montenegro (central and coastal regions). Peer education programmes as well as other prevention programmes were significantly correlated with increased levels of knowledge. The knowledge level of those surveyed was similar to that of young people in Croatia, but unlike in the Croatian participants, no gender differences were observed in this study (7, 8).





The level of acceptance of PLHIV was unsatisfactory, as only one -sixth of the surveyed population scored more than 13 on a 0-16 scale, with slightly more positive attitudes among the female respondents. Older respondents, respondents from urban settlements and respondents with higher levels of HIV-related knowledge were more open towards possible contact with HIV-infected people. Having received HIV-related information at school was also associated with higher levels of acceptance of PLHIV.

Slightly more than half of the surveyed population were reported being sexually active, with significantly more prevalent sexual activity among males. The results showed significant gender differences in sexual behaviour, with women taking fewer risks than men, which is consistent with the findings in a large number of questionnaire and experimental studies (9,10).

The level of sexual risk behaviours reported varied from 2% for sex under the influence of drugs to 56% for inconsistent condom use. Sixteen percent of participants engaged in sexual activity under the age of 16, which is consistent with the results reported in studies conducted in other central and south-eastern European countries (11). Additionally, 72% of males and 28.5% of females had 3 or more partners thus far, with 33% of males and 5% of females reporting multiple partners within the previous 12 months. Respondents reporting early sexual initiation were significantly more likely to engage in other sexual risk behaviours such as multiple partners, inconsistent condom use and sex under the influence of alcohol (12).

Consistent condom use is considered one of the most effective prevention strategies for reducing the risk of transmission of HIV and other STIs (13, 14). Individuals who used condoms during their first intercourse were significantly more likely to subsequently use condoms regardless of the partner. Contraception used during first intercourse was associated with subsequent consistent contraception use (15). Of those who consistently used condoms during the previous 12 months, 92% had used condoms during first intercourse, while 65% of those using condoms at first intercourse remained faithful to that habit. During participants' last sexual intercourse, condoms were used by 66% of respondents, with significantly higher percentages among males than females, while almost the same proportion (68%) used condoms during the last intercourse with non-regular partners, showing no difference in this study population compared to young people surveyed in Montenegro in 2007 (2). Despite the relatively high level of knowledge with regard to the sexual transmission of HIV, consistent condom use remains a habit in less than half of the surveyed population.

Older respondents were more prone to risk-taking behaviours than their younger peers. Self-esteem and self-efficacy were not found to be predictive of sexual risk behaviours, which is consistent with a survey of Slovak students but not with the findings of several other studies (16, 17, 18), where they were found to be predictive for protective as well as for risk-taking behaviours such as multiple partners (11).

Condom use at first intercourse proved to be the strongest protective factor in both genders for not engaging in sexual risk behaviours, which is consistent with the findings in several studies related to condom use predictors (19, 20, 21). Respondents who used condoms during first intercourse were significantly more frequent condom users at last intercourse, regardless of partner, and were consistent condom users in general. Positive attitudes towards condom use and stronger self-efficacy were predictors of condom use, which is consistent with other studies that investigated predictors of condom use among adolescent and young adult populations (22).

A higher level of parental monitoring was a significant protective factor in the overall logistic regression model for the level of sexual risk-taking and in the model for male respondents, which is consistent with the findings in other surveys showing that supervision is related to boys' risk behaviours (23,24,25).

Among the limitations of cross-sectional studies is that the causality of the associations revealed cannot be established. Further limitations result from the possible participation and recall bias due to the retrospective nature of the study (frequency of condom use, number of partners (26), age at first sexual intercourse (27,28)) and the provision of socially desirable answers (29). The methodological advantages of this study result from the reliable sampling framework and pre-tested data collection methods. The validity of the self-reported data was further increased by using anonymous questionnaires, which were partially self-administered, and pre-testing the questionnaire for understanding and precise recall of certain required data. To increase the response rate, special attention was paid to selection and training of the interviewers considering the sensitive nature of the behaviours to be studied. A response rate of 97% is relatively high and contributes to the validity of the data (30).

## IMPLICATIONS

In light of raising awareness related to HIV/AIDS through the GFATM,- the subject „Healthy Life Styles“ was included in the primary school curriculum as optional subject for 8<sup>th</sup> or 9<sup>th</sup> graders. It was designed to cover not only sexual and reproductive health in relation to HIV, but also healthy nutrition, mental health, communication, prevention of injuries, physical activity, substance abuse, and communal hygiene,.

Our results indicate that condom use at first intercourse was a strong predictor, for both boys and for girls, for subsequent more frequent condom use. Other studies have shown that school-based HIV prevention programmes have significant effects on students' self-efficacy for condom use and intentions to adopt prevention practices (31). This is a strong argument for condom promotion at early adolescence prior to entering into sexual activities, at higher grades of primary school and lower grades of secondary school.



However, further research in the adolescent population is needed to obtain a more precise picture of the sexual behaviour of this age group and clarify which other factors contribute to different types of risk behaviours.

### Conclusions

These findings confirm the hypothesis of significant gender and regional differences in the level of knowledge and accepting attitudes towards PLHIV and in the prevalence of risk sexual behaviour. Provision of HIV-related educational activities at school was positively associated with a higher level of HIV-related knowledge and acceptance of PLHIV (31) as well as a higher rate of condom use at first intercourse and consistent condom use thereafter. These results strongly support the idea of in-school HIV-related educational activities as well as the introduction of secondary school curriculum related to HIV/STI prevention and other aspects of risk behaviour characterising this age population.

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# FLUORIDE RELEASE FROM GLASS IONOMER CEMENTS CORRELATES WITH THE NECROTIC DEATH OF HUMAN DENTAL PULP STEM CELLS

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## ABSTRACT

Glass ionomer cements (GICs) are commonly used as restorative materials. The effect of GICs on different cell types varies. Stem cells from Human Exfoliated Deciduous teeth, SHED are a source for dental tissue regeneration. The necrosis and inflammation that eventually follows necrosis can disturb this regenerative process.

We tested seven GICs including Fuji I, Fuji II, Fuji VIII, Fuji IX, Fuji plus, Fuji triage and Vitrebond for their necrotic induction potential in human SHEDs. We also correlated these effects with eluate fluoride release. The toxicity of GICs was tested via a lactate dehydrogenase assay and flow cyto-

metric analysis of propidium iodide and Annexin V stained cells. The concentration of fluoride was measured by HPLC. The Fuji I and Fuji II GICs had a significantly lower cytotoxic effect on SHEDs compared to other tested GICs, as evaluated by the LDH assay. The results obtained from the flow cytometric analyses were similar. The Fuji I and Fuji II eluates released the lowest concentrations of fluoride and induced the lowest percentages of SHED death. Fluoride release correlated with GIC cytotoxicity.

**Keywords:** glass ionomer cements, cytotoxicity, fluoride, Stem cells from Human Exfoliated Deciduous teeth

## INTRODUCTION

Pulp has an important role in the formation of dentin. Dentin formation begins when dental pulp mesenchymal stem cells differentiate into odontoblasts and start the deposition of collagen matrix and subsequent mineralisation [1]. Dentin formation continues through life due to tooth aging, as well as in response to physical and/or chemical injuries [2]. The main goal of restorative dentistry is to restore teeth using adequate treatments that will protect pulp function. To avoid any additional damage to pulp tissue during operative procedures caused by the toxicity of restorative materials or the penetration of bacteria, several layers of a specific material between the restorative material and the dental tissue must be applied [3, 4]. Calcium hydroxide-based products, adhesive systems and glass ionomer cements (GICs) are typically used for this purpose. GICs were introduced by Wilson and Kent in 1971 as a mixture of a calcium or strontium alumino-fluoro-silicate glass powder (base) and a water-soluble polymer (acid) [5]. Several variations of glass-ionomer materials were subsequently developed. Later

variants of GICs demonstrated enhanced flexural strength, diametral tensile strength, elastic modulus and wear resistance, but their main disadvantage is higher cytotoxicity in comparison with conventional GICs. The responses to GICs differ by cell type. Thus, it is important to evaluate the cytotoxicity of GICs to SHEDs [6-9].

It has been suggested that the pattern of cell death pattern could be an important method of evaluating the irritation potential of dental materials. Apoptotic cells are removed by phagocytosis and with little inflammatory response, in contrast to the inflammation and injury to the surrounding tissues induced by the necrotic process [10]. As dental pulp stem cells are the main source for dental tissue regeneration, it is important to evaluate the potential of GICs to induce necrosis of SHEDs and subsequent inflammation in the surrounding tissue. We evaluated the potential of seven commonly used biomaterials, Fuji I, Fuji II, Fuji VIII, Fuji IX, Fuji Plus, Fuji Triage and Vitrebond, to induce necrosis of human SHEDs.

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## MATERIALS AND METHODS

### Cell culture

Human SHEDs were purchased from AllCells (Emeryville, California USA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 100 IU/mL penicillin G and 100 µg/mL streptomycin (Sigma-Aldrich Chemical, Munich, Germany). SHEDs passaged 6 times were used throughout these experiments.

### Tested materials

The cytotoxic effects of seven glass ionomer cements, Fuji I, Fuji II, Fuji VIII, Fuji IX, Fuji plus, Fuji Triage (GC America, Alsip, IL, USA) and Vitrebond (3M ESPE, London, UK), were tested in this study. Separate GIC samples were prepared according to the manufacturers' directions at room temperature and then placed into open plastic rings 5 mm in diameter by 2 mm deep. After consolidation, the samples were removed from rings and dry heat sterilised for 1 hour at 170 °C. The samples were then incubated in complete culture medium (150 µL per sample) for 72 hours at the 37°C and in a 5% CO<sub>2</sub> atmosphere. The sample dimensions and immersion conditions were chosen to approximate the GIC mass and the dentin-exposed surface area typically used in restorative dentistry patient procedures. The medium exposed to each GIC sample was used for testing in a cell-culture system.

### Evaluation of toxicity using the LDH assay

The cytotoxicity of GICs was examined via a *Cytotoxicity Detection Kit* (LDH) (Roche Applied Science). SHEDs were diluted with DMEM medium to 1 x 10<sup>5</sup> cells/mL, and aliquots (1 x 10<sup>4</sup> cells/100 µL) were placed in individual wells in 96-well plates. The next day, the media were exchanged with media exposed to a GIC mixed with fresh medium in a 1:1 ratio to a final volume of 100 µL. Each eluate was tested in triplicate. Two groups of control wells were prepared: low control (medium was exchanged with fresh medium) and high control (medium was exchanged with medium containing 1% Triton X). The cells were incubated at 37°C in a 5% CO<sub>2</sub> incubator for 24 h. After treatment, the supernatant (100 µL) was transferred to a new plate and incubated with an equivalent volume of substrate solution. After incubating the plates for 30 minutes at RT, 50 µl/well of stop solution was added, and the plates were spectrophotometrically examined at 450 nm. The percentage of dead cells was calculated using the formula:

$$\% \text{ of dead cells} = \frac{\text{exp. value} - \text{low control}}{\text{high control} - \text{low control}} \times 100$$

### Apoptosis assay

SHEDs exposed to GIC eluates were examined by flow cytometry using Annexin V FITC (BD Pharmingen, San Jose, CA, USA) Propidium Iodide (Sigma-Aldrich Chemical Company, Munich, Germany) staining. After the SHEDs reached subconfluency, the flask medium was replaced with mixture of medium exposed to GICs

and fresh, complete DMEM (ratio 1:1) (volume, 4 mL). The SHEDs exposed to the GICs eluate were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 24 h. The cultured cells were washed twice with cold phosphate-buffered saline (PBS, Sigma Aldrich) and resuspended in 1x binding buffer (10x binding buffer: 0.1 M HEPES/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl<sub>2</sub>) at a concentration of 1 x 10<sup>6</sup>/mL. Annexin FITC (5 µL) and propidium iodide (PI) (5 µL, 50 µg/ml in PBS) were added to 100 µL of the cell suspension and incubated for 15 min at room temperature (25°C) in the dark. After incubation, 400 µL of 1 x binding buffer was added to each tube. The stained cells were analysed within 1 hour using FACS Calibur (BD, San Jose, USA) and CellQuest software. Because Annexin V FITC staining precedes the loss of membrane integrity that accompanies the later stage identified by PI, Annexin V FITC positive and PI negative staining indicates early apoptosis, whereas Annexin V FITC negative and PI negative staining indicates cell viability. Cells that are in late apoptosis or already dead are both Annexin V FITC and PI positive, and dead cells are PI positive only [11].

### Quantification of fluoride in medium exposed to GIC

The fluoride (F) concentrations of each eluate were assayed by high performance liquid chromatography using a Chromeleon® Chromatography Workstation (Dionex, Wien, Austria) equipped with a GP50 gradient pump, conductivity detector, ASRS ultra 4 mm. An Ionpac AS15 column and an AG15 guard column were used. Potassium hydroxide was used as the eluent. The flow rate was 1.0 mL/min. All results were analysed on Chromeleon 6.7 Chromatography Management Software.

### Statistical Analysis

The cytotoxicity was expressed as mean + standard deviation. One-way ANOVA tests and linear regression were used to analyse the data. A p < 0.05 was considered statistically significant.

## RESULTS:

### LDH assay

Stem cell death, as evaluated by an LDH test 24 hours after incubation in GIC eluates, indicated very similar cytotoxic effects for the Fuji VIII, Fuji IX, Fuji plus, Fuji triage and Vitrebond GICs. These eluates induced necrosis in approximately 50% of the SHEDs. The Fuji I (25.14% dead cells) and Fuji II (28.56% dead cells) GICs demonstrated significantly less cytotoxicity (Figure 1).

### Fluoride release

We wanted to know if the leaching of ionic components into the biomaterial eluates could account for the cytotoxic effect of the GICs. For that purpose, one of the major ions present in all tested GICs, F<sup>-</sup>, was quantified in the eluates (Figure 2). There was a strong correlation between the cytotoxic effects of GICs and fluoride release. Pear-



son's correlation coefficient ( $r^2$ ) value demonstrated a high correlation between F- release and cytotoxicity ( $r^2=0.848$ ,  $p=0.003$ ). The more cytotoxic GICs (Fuji Plus, Vitrebond, Fuji IX, Fuji triage and Fuji VIII) released more F- than the other tested GICs. The less cytotoxic materials (Fuji I and Fuji II) released less F-.

### Apoptosis assay

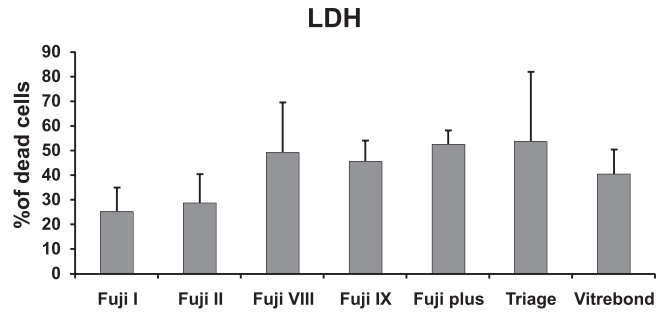
To more closely investigate the effects of GICs on human SHEDs, we performed apoptosis assays. These assays confirmed the results obtained by the LDH test (Figure 3). All GICs induced permeabilisation of the SHED membranes (propidium iodide-positive cells). The highest percentages of dead cells were recorded after treatment with Fuji VIII, Fuji IX or Vitrebond (Figure 3). In addition, there was a good correlation between the percentage of dead cells as measured by the apoptosis assay and fluoride release ( $r^2 = 0.717$ ,  $p=0.016$ ).

## DISCUSSION

The biological compatibility of dental materials is essential for avoiding or limiting pulp tissue irritation or degeneration. GIC formulations contain organic monomers and different ions that may diffuse through the dentin tubules and reach the pulp tissue. These ions can affect the vitality of the odontoblast layer and interfere with pulp homeostasis and healing [12,13]. SHEDs are adult stem cells that are able to regenerate a dentin-pulp-like complex, composed of mineralised matrix with tubules lined with odontoblasts and fibrous tissue containing blood vessels in an arrangement similar to the dentin-pulp complex found in normal human teeth [14]. Dentin formation continues through life in response to physical and/or chemical injuries and tooth aging. As SHEDs are involved in the damaged pulp repair processes, we selected them as an adequate cell culture system for testing the effects of GICs.

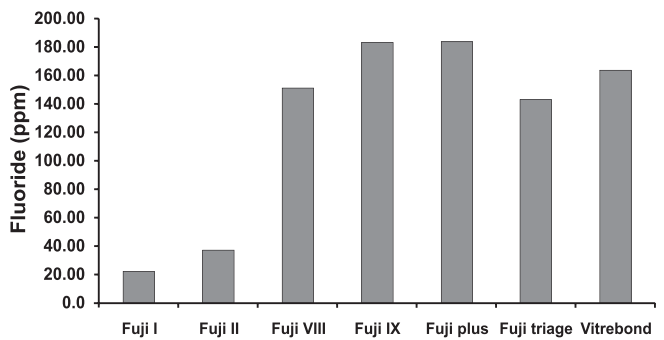
To explore the cell damage potential of GICs, we used an LDH assay. Lactate dehydrogenase is a cytoplasmatic enzyme released after cell membrane disruption. The LDH assay measures the activity of LDH in cell supernatants, thus, indirectly measuring cell death associated with a loss of membrane integrity [15]. The results of the LDH assay indicated that eluates from the Fuji VIII, Fuji IX, Fuji plus, Fuji Triage and Vitrebond samples were highly cytotoxic to human SHEDs (Figure 1), and there was no significant difference between them. The Fuji I and Fuji II eluates were slightly less cytotoxic, suggesting better biocompatibility. Our results are in agreement with previous reports showing that GICs are toxic to dental pulp [16] and pluripotent mesenchymal precursor cells [17].

GICs eluates induced similar toxicity as evaluated by flow cytometric analysis of PI-stained cells. These data indicated that the highest percentage of damaged cells was attained after treatment with Fuji plus, Fuji IX or Vitrebond GICs, a moderate percentage was attained after treatment with Fuji

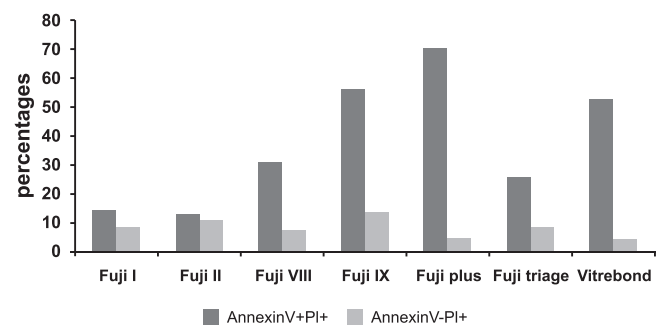


**Figure 1.** Stem cell necrosis as evaluated by the LDH assay. Each column represents the percentage of dead cells (mean value and standard deviation of 3 experiments with 3 replicates).

The least cytotoxic GICs were Fuji I (25.14%) and Fuji II (28.56%), while Fuji Plus (52.48%), Vitrebond (54.18%), Fuji VIII (49.13%), Fuji IX (45.20%) and Fuji Triage (53.68%) demonstrated higher cytotoxic effects on SHEDs. There was a statistically significant difference between Fuji I and Vitrebond ( $p=0.04$ ).



**Figure 2.** Quantification of Fluoride in the eluates of tested GICs.



**Figure 3.** The percentage of dead cells as evaluated by the apoptosis assay. The highest percentage of dead cells was attained after treatment with Fuji IX (69.6%), Vitrebond (57.20%) or Fuji Plus (74.9%). A moderate percentage of dead cells was attained after treatment with Fuji VIII (38.40%) or Fuji Triage (34.10%). The lowest percentage of necrosis was attained after treatment with Fuji I (22.8%) or Fuji II (24.3%). The results of a representative experiment are presented.





trriage or Fuji VIII GICs and the lowest percentage was attained after treatment with Fuji I or Fuji II GICs (Figure 2).

These differences in cytotoxic effects between different GICs on human SHEDs appear to be related to the amount of fluoride released. The most toxic GICs, Fuji Plus, Vitrebond and Fuji VIII, released a higher amount of F anions than the other tested materials (Figure 3). In addition, low levels of released fluoride were noticed in the Fuji I and Fuji II eluates, which were the least cytotoxic GICs (Figure 2). Although it is known that the main advantage of using GICs as adhesive restorative materials is the long-term antibacterial effect due to fluoride release [3, 9, 18], we are the first group to demonstrate that the fluoride release of GICs has a direct correlation with cytotoxic effects on human SHEDs. GICs achieve maximum fluoride release 24 h after the initial setting [19], and that fluoride release has a significant potential for inducing pulpal toxicity [20]. The potential of fluoride anions to induce necrosis of Swiss-strain mouse hepatocytes [21] and primary culture of rat thymocytes [22] has been reported previously but in this study, we are first to show that fluoride release directly correlates with GIC cytotoxic effects in human SHEDs.

#### Acknowledgements

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# HEPATOTOXICITY OF TEMSIROLIMUS AND INTERFERON ALPHA IN PATIENTS WITH METASTASISED RENAL CANCER: A CASE STUDY

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## HEPATOKSIČNOST TEMSIROLIMUSA I INTERFERONA ALFA KOD PACIJENATA SA METASTATSKIM KARCINOMOM BUBREGA: SERIJA SLUČAJEVA

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### ABSTRACT

*Temsirolimus is a drug used for the treatment of renal cell carcinoma. The target of action of temsirolimus is mTOR (mammalian target of rapamycin) kinase, a cellular protein that regulates the growth of tumour cells and blood vessels. The aim of the present study was to determine whether temsirolimus has greater hepatotoxic potential than standard therapies for renal cancer, including interferon alpha and vinblastine.*

*The current study was conducted on patients treated at the Institute for Radiology and Oncology of Serbia, Belgrade for metastasised renal cell carcinoma. In total, nine patients were administered 25 mg of temsirolimus per week for four weeks. Another fourteen patients were treated with standard therapy, including interferon alpha (6 MJ, three times a week) and vinblastine (10 mg, two days per cycle, for four cycles). Biochemical parameters of liver function (aspartate amino-transferase, alanine amino-transferase, alkaline phosphatase, lactate dehydrogenase,  $\gamma$  glutamine trans-peptidase, bilirubine [direct and total] and serum proteins) were analysed prior to administration and four weeks after treatment.*

*In total, six patients developed hepatotoxicity, which was defined as a 3-fold increase in aspartate amino-transferase and alanine amino-transferase levels after the administration of therapy. Three patients showing signs of hepatotoxicity received temsirolimus, and three were treated with interferon alpha and vinblastine. Except for the level of aspartate amino-transferase, the studied factors including age, sex, drug, diabetes, heart failure, hypertension, nephrectomy, stage of cancer and serum urea and creatinine levels were not associated with hepatotoxicity. Namely, in patients who experienced hepatotoxicity, the aspartate amino-transferase content was significantly lower prior to the administration of drugs ( $13.3 \pm 6.1$  vs.  $20.1 \pm 7.4$ ;  $T = -2.400$ ,  $df = 12$ ,  $p = 0.033$ ).*

*The results of the present case study suggest that temsirolimus is not more hepatotoxic in patients with metastasised renal cancer than standard therapies such as interferon alpha and vinblastine.*

**Key words.** Temsirolimus; liver; toxicity; metastasised renal cancer.

### SAŽETAK

*Temsirolimus se koristi u lečenju karcinoma bubrega, jer onemogućava stvaranje novih krvnih sudova neophodnih za širenje tumora. Ove efekte temsirolimus postiže inhibicijom posebne tirozin – kinaze (kinaze za koju se vezuje rapamicin kod sisara). Cilj naše studije je bio da ispita da li temsirolimus ima veći hepatotoksični potencijal od standardne terapije metastatskog karcinoma bubrega, kombinacije interferona alfa i vinblastina.*

*Studija je sprovedena kod pacijenata sa metastatskim karcinomom bubrega lečenih na Institutu za onkologiju i radiologiju Srbije u Beogradu. Bilo je 9 pacijenata koji su uzimali temsirolimus 25 mg nedeljno, tokom 4 nedelje. Drugih 14 pacijenata je bilo na standardnoj terapiji (interferon alfa 6 M. tri puta nedeljno) i vinblastin 10 mg dva dana u ciklusu, tokom 4 ciklusa. Kod pacijenata su analizirani biohemijski parametri funkcije jetre na dolasku i četiri nedelje nakon uvođenja terapije: transaminaze (aspartat aminotransferaza i alanin aminotransferaza), alkalna fosfataza, laktat dehidrogenaza, gama glutamin transpeptidaza, bilirubin (direktni i ukupni), i proteini u serumu.*

*Bilo je šest pacijenata kod kojih je došlo do oštećenja jetre, definisanog kao najmanje trostruki porast serumskog nivoa aspartat aminotransferaze i alanin aminotransferaze posle primene terapije: troje od njih je primilo temsirolimus, a troje interferon alfa i vinblastin. Nijedan od ispitivanih faktora (starost, pol, lek, dijabetes, insuficijencija srca, hipertenzija, nefrektomija, stadijum carcinoma, nivo uree i kreatinina u serumu) nije bio udružen da hepatotoksičnošću, izuzev nivoa aspartat aminotransferaze, koji je pre primene lekova bio značajno niži kod pacijenata koji su razvili oštećenje jetre ( $13.3 \pm 6.1$  vs.  $20.1 \pm 7.4$ ;  $T = -2.400$ ,  $df = 12$ ,  $p = 0.033$ ).*

*Rezultati naše serije slučajeva sugerišu da temsirolimus nije više hepatotoksičan kod pacijenata sa metastatskim karcinomom bubrega od standardne terapije kombinacijom interferona alfa i vinblastina.*

**Ključne reči.** Temsirolimus; jetra; toksičnost; metastatski karcinom bubrega.

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## INTRODUCTION

Temsirolimus binds mTOR (mammalian target of rapamycin) kinase, a cellular protein that regulates the growth of tumour cells and blood vessels. An intravenous preparation of temsirolimus was developed by Wyeth and received marketing authorisation from the Food and Drug Administration in May 2007 and from the European Medicine Agency in November 2007 for the treatment of metastasised renal cell cancer (RCC).<sup>1,2</sup>

Kinase mTOR (mammalian target of rapamycin) is a component of intracellular signalling pathways involved in the growth and division of cells and in the cellular response to hypoxia. Temsirolimus binds FKBP-12, an intracellular protein, forming a complex that inhibits signals initiated by mTOR. The blockage of mTOR signals prevents the production of proteins that regulate cell cycle progression and angiogenesis.<sup>3,4,5,6</sup>

In general, temsirolimus is efficacious in patients with metastasised renal cell cancer, and it causes adverse effects of moderate severity. The most frequent side effects of temsirolimus include rash, nausea, weakness, inflammation of mucous membranes, anorexia and anemia.<sup>7,8,9</sup>

The aim of the present study was to determine whether temsirolimus is more hepatotoxic than standard therapies for metastasised renal cancer, including interferon alpha and vinblastine.

## MATERIALS AND METHODS

### The patients

The present observational study was conducted on patients treated at the Institute for Oncology and Radiology of Serbia, Belgrade for metastasised renal cell cancer from January 1<sup>st</sup>, 2000 to April 1<sup>st</sup>, 2007. In total, 23 patients were included in the study. Nine patients (average age  $59.2 \pm 7.2$  years) received 25 mg of temsirolimus per week (for four weeks) and 14 patients (average age  $54.6 \pm 9.8$  years) were treated with interferon alpha (6 MJ, three times a week) and vinblastine (10 mg, two days per cycle, for four cycles). The study was approved by the research committee of the Institute for Oncology and Radiology of Serbia.

### The variables

Biochemical parameters of liver function (aspartate amino-transferase, alanine amino-transferase, alkaline phosphatase, lactate dehydrogenase,  $\gamma$  glutamine trans-peptidase, bilirubine [direct and total] and serum proteins) and the concentration of serum urea, creatinine, sodium, potassium and calcium were analysed prior to drug administration and four weeks after treatment. The patient's age, sex, chronic diseases, therapy and stage of cancer were obtained from their files.

### Statistics

The prevalence of each risk factor was determined for patients with liver injury (cases) and patients with normal

serum levels of liver enzymes (controls). Differences between patients with liver injury and those in the control group were assessed with a Student T-test for continuous variables and with a Fisher's exact test for frequencies. Differences were considered significant when the probability of the null hypothesis was less than 0.05. To estimate the association between potential risk factors and hepatotoxicity, crude and adjusted odds ratios (OR) with 95% confidence intervals (95% CI) were calculated using logistic regression<sup>10,11</sup>.

## RESULTS

The study population included 23 patients with metastasised renal cell cancer. The characteristics of patients with and without liver injury are shown in Table 1. Significant differences in the age, sex, nephrectomy, hypertension, chronic heart failure, diabetes, drugs, grade of tumour, skin rash, serum urea level and creatinine and alanine amino-transferase levels content of patients were not observed among groups. However, prior to treatment, significant differences in the serum level of aspartate amino-transferase were detected (see Table 1).

The results of logistic regression analysis (Cox & Snell  $R^2 = 0.486$ , Nagelkerke  $R^2 = 0.713$ , Hosmer and Lemeshow  $\chi^2 = 3.187$ ,  $df = 8$ ,  $p = 0.922$ ) with adjustments for potential confounders are shown in Table 2. As shown in the table, significant associations between liver injury and the studied factors were not observed. Although the adjusted odds ratio for drugs and tumour grade were 16.64 and 4.88, respectively, the confidence limit included the value of one, indicating that the association was not significant.

## DISCUSSION

In several phase II and III clinical trials on temsirolimus, the following adverse effects were observed: skin rash (47%), weakness (51%), inflammation of mucous membranes (41%), nausea (37%), edema (35%) and loss of appetite (32%).<sup>12,13</sup>

Serious adverse reactions to temsirolimus have been reported, including hypersensitivity reactions (skin redness, chest pain and/or breathing difficulties), extreme hyperglycaemia, interstitial lung disease, intestinal perforation and acute renal insufficiency. The most frequent laboratory abnormalities in patients receiving temsirolimus were anaemia (94%), hyperglycaemia (89%), hyperlipidaemia (87%), hypertriglyceridemia (83%), increased serum levels of alkaline phosphatase (68%), aspartate amino-transferase (38%) and creatinine (57%), lymphopenia (53%), hypophosphatemia (49%), decreased platelet count (40%), and leukopenia (32%).<sup>14,15,16</sup>

In the present study, surrogate markers for liver injury (a 3-fold increase in the serum level of aspartate amino-transferase and alanine amino-transferase compared to





Variable	Patients with elevated serum levels of liver enzymes (3-fold higher than the baseline (n=6))	Patients without elevated serum levels of liver enzymes (n=17)	Test value and significance of null hypothesis	Crude odds ratios and confidence intervals (1.96*SE)
Sex (M/F)	5/1 (83%/17%)	12/5 (71%/29%)	Fisher's p = 1.000	2.08 (0.19, 22.66)
Age (years, mean ± SD)	60.1 ± 7.3	54.5 ± 9.1	T = 1.380, p = 0.182	1.09 (0.96, 1.25)
Nephrectomy (yes/no)	6/0 (100%/0%)	16/1 (94%/6%)	Fisher's p = 1.000	504.14 (0.00, >1000)
Diabetes mellitus (yes/no)	0/6 (0%/100%)	4/13 (24%/76%)	Fisher's p = 0.539	0.00 (0.00, >1000)
Hypertension (yes/no)	2/4 (34%/66%)	6/11 (36%/64%)	Fisher's p = 1.000	0.92 (0.12, 6.56)
Tumour grade (C64/C65/C61/C67/C25)	4/1/1/0/0 (66%/17%/17%/0%/0%)	13/2/0/1/1 (76%/12%/0%/6%/6%)	$\chi^2 = 3.679$ , p = 0.451	0.97 (0.40, 2.38)
Chronic heart failure (yes/no)	2/4 (34%/66%)	6/11 (36%/64%)	Fisher's p = 1.000	0.92 (0.13, 6.56)
Skin rash (yes/no)	3/3 (50%/50%)	6/11 (36%/64%)	Fisher's p = 1.000	1.83 (0.28, 12.07)
Drug (temsirrolimus/interferon+vinblastine)	3/3 (50%/50%)	6/11 (36%/64%)	Fisher's p = 0.643	1.83 (0.28, 12.07)
Serum urea content before treatment (mM/l)	6.4 ± 1.1	6.7 ± 3.1	T = -0.200, p = 0.843	0.96 (0.67, 1.39)
Serum creatinine content before treatment (µM/l)	117.5 ± 28.4	120.7 ± 47.0	T = -0.156, p = 0.877	0.99 (0.98, 1.02)
Serum aspartate amino-transferase content before treatment (IU/l)	13.4 ± 6.1	21.1 ± 8.4	T = -2.400, p = 0.033*	0.83 (0.67, 1.01)
Serum alanine amino-transferase content before treatment (IU/l)	15.5 ± 12.4	20.1 ± 7.4	T = -1.090, p = 0.288	0.94 (0.83, 1.06)

\*significant difference

**Table 1.** Characteristics of the Patients.

baseline values) did not display a stronger association with temsirolimus than standard medications for metastasised renal cancer. However, in three out of nine patients (33%) temsirolimus was associated with liver injury. The causal relationship between temsirolimus and liver injury was rated as probable because liver injury was temporally related to the administration of temsirolimus, and liver enzyme serum levels normalised after temsirolimus treatments were ceased/challenging. The standard therapy (interferon

alpha plus vinblastine) caused liver injury in 3 out of 11 patients (27%); however, the observed difference in the rate of liver injury among groups cannot be considered significant due to the small number of patients. Hepatotoxicity was observed in both therapeutic regimens and should be taken into account during the treatment of patients.

Temsirolimus is administered to patients with metastasised renal cell cancer due to its effectiveness.<sup>11</sup> The results of the present study suggest that mild liver injury can be

**Table 2.** Crude and adjusted odds ratios of the risk factors for liver injury in patients with metastasised renal cell cancer receiving temsirolimus or interferon alpha and vinblastine.

Risk factors	Crude OR (95% CI)	Adjusted* OR (95% CI)
Drug (temsirrolimus/interferon+vinblastine)	1.83 (0.28, 12.07)	16.64 (0.07, 4142.91)
Tumour grade	0.97 (0.40, 2.38)	4.88 (0.13, 180.87)
Hypertension	0.92 (0.12, 6.56)	0.75 (0.01, 99.18)
Serum urea content before treatment	0.96 (0.67, 1.39)	0.80 (0.18, 3.45)
Serum aspartate amino-transferase content before treatment	0.83 (0.67, 1.01)	0.77 (0.58, 1.02)

\* Adjusted for age†, sex†, nephrectomy†, hypertension, chronic heart failure†, diabetes†, drug, tumour grade, skin rash†, serum level of urea, creatinine†, aspartate amino-transferase and alanine amino-transferase†.

†Crude and adjusted odds ratios are not shown in the table for the sake of clarity.

OR = odds ratio



expected with the use of temsirolimus. To prevent severe forms of liver injury, serum levels of liver enzymes should be measured weekly during the first month of therapy and monthly thereafter.

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## NITRIC OXIDE IN HEMODIALYSIS PATIENTS

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## AZOT OKSID KOD PACIJENATA NA HEMODIJALIZI

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### ABSTRACT

Nitric oxide (NO) is a molecule that has an important role in many physiological and pathological conditions, necessitating more studies to elucidate its function in various diseases. Patients with renal failure present impaired NO activity that may influence disease development and prognosis. The aim of the study was to measure NO in hemodialysis (HD) patients as related to HD duration and erythropoietin (rhEpo) therapy. The NO concentration was measured in 76 HD patients and 30 healthy control subjects. The patients were divided into three groups by the duration of HD: group I, <5 years (n=26); group II, 5-10 years (n=22); and group III >10 years (n=28). The HD patients were divided into two groups based on the receipt of rhEpo therapy: group I without rhEpo therapy (n=37) and group II with rhEpo therapy (n=39). In an evaluation study of NO level after the 6<sup>th</sup> month of rhEpo therapy, the HD patients were divided into 2 groups: group I without rhEpo therapy (n=20) and group II with rhEpo therapy (n=27). The NO level in HD patients was measured at 0, 3 and 6 months. Routine haematological parameters were assayed along with NO. HD patients presented an increased NO level compared to controls ( $p < 0.001$ ). There were no statistical differences in NO levels observed among the HD duration groups. Patients receiving rhEPO supplementation therapy with rhEpo presented decreased NO levels ( $p < 0.01$ ). In the evaluation study, HD patients receiving rhEpo therapy again presented lower NO levels ( $p < 0.05$ ).

These results indicate that the increased NO values observed in HD patients is probably due to the induction of HD membrane and/or a lack of renal excretion. Erythropoietin therapy decreased NO levels, which may improve patient condition and contribute to better disease outcome.

**Key words:** nitric oxide, hemodialysis, erythropoietin

### SAŽETAK

Molekul azotnog monoksida (NO) ima važnu ulogu u mnogim fiziološkim i patološkim stanjima na šta ukazuju mnoge studije koje objašnjavaju njegovu funkciju u različitim bolestima. Bubrežne bolesti pokazuju poremećaj aktivnosti NO koja utiče na razvoj bolesti i njenu prognozu. Cilj ove studije je da se kod pacijenata na hemodijalizi (HD) ispita povezanost NO-a sa dužinom trajanja hemodijalize i sa eritropoetskom terapijom (rhEpo). Koncentracija NO-a je određena kod 76 pacijenata na HD i kod 30 zdravih ispitanika kao kontrolne grupe. U odnosu na dužinu trajanja HD, pacijenti su podeljeni u tri grupe: I grupa - < 5 godina (n= 26); II grupa 5-10 godina (n=22); i III grupa > 10 godina (n=28). U odnosu na rhEpo terapiju pacijenti na HD su podeljeni u dve grupe: I grupa - bez terapije (n=37); II grupa - sa rhEpo terapijom (n=39). U evaluacijskoj studiji NO-a, u toku šestomesečne terapije rhEpo-om, pacijenti na hemodijalizi su podeljeni u dve grupe: I grupa -bez rhEpo terapije (n=20); II grupa - sa rhEpo terapijom (n=27). Nivo NO-a kod pacijenata na HD je određivan posle 0, 3 i 6 meseci. Ispitani su rutinski hematološki parametri i NO. U odnosu na kontrolnu grupu, NO pokazuje porast kod pacijenata na HD ( $p < 0.001$ ). U odnosu na dužinu trajanja HD, NO ne pokazuje statističku razliku. Kod pacijenata na HD koji primaju suplementnu rhEpo terapiju, nivo NO-a opada ( $p < 0.01$ ). U evaluacijskoj studiji kod pacijenata na HD nivo NO-a opada u odnosu na rhEpo terapiju ( $p < 0.05$ ).

Na osnovu rezultata možemo zaključiti da je porast NO-a kod pacijenata na HD uzrokovan hemodijaliznom membranom i / ili nedostatkom renalne ekskrecije. Terapija rhEpo-om pokazuje pozitivne efekte preko smanjenja nivoa NO-a što može poboljšati stanje pacijenta i doprinosi boljem ishodu bolesti.

**Ključne reči:** azot monoksid, hemodijaliza, eritropoetin.

### Abbreviations:

ESRD - end-stage renal disease  
HD - haemodialysis  
NO - nitric oxide

NOS - nitric oxide synthase  
ONOO- - peroxyntirite  
rhEpo - erythropoietin

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## INTRODUCTION

Underlying concentration changes in biological molecules in hemodialysis (HD) patients are common and may be useful indicators of disease state. In recent decades, there has been much interest focused on nitric oxide (NO) and its link to renal disease.

Nitric oxide (NO), known as "endothelium-derived relaxing factor", is produced from L-arginine, oxygen and NADPH by nitric oxide synthase (NOS) enzymes. It is an important messenger molecule involved in many pathological and physiological processes (1). Its physiological role is related to vessel homeostasis via the inhibition of vascular smooth muscle contraction and growth, inhibition of platelet aggregation, and inhibition of white blood cell adhesion to the endothelium (2). NO also plays a role in inflammation and immune responses when generated by the phagocytes during the process of killing bacteria (via DNA damage) (3). Therefore, NO is a crucial physiological messenger molecule that contributes to blood pressure regulation, blood clotting control, immune defence, sight, smell and likely the processes of learning and memory. However, NO also contributes to pathologic states such as hypertension, stroke, diabetes mellitus, renal disease, impotence and long-term depression. NO, a small molecule, is a free radical, which makes it very reactive with the metal centres of cell proteins and other reactive groups. NO overproduction may result in an oxidant effect with the superoxide anion  $O_2^-$  producing a toxic molecule of peroxynitrite (ONOO<sup>-</sup>). Peroxynitrite contributes to vascular cell impairment, stroke and other neurological problems. Furthermore, a high NO level during severe bacterial infection can cause a blood pressure decrease resulting in septic shock and tissue damage. Prolonged exposure to a high NO level may lead to inflammation and malignant states including juvenile diabetes, multiple sclerosis, arthritis, colitis, chronic renal disease, carcinomas and other chronic diseases. NO also promotes tumour progression and metastasis due to angiogenesis, vessel maturation and dilatation (4, 5, 6). As a reactive agent, NO has been reported to be involved in renal failure pathogenesis. Some authors have confirmed that chronic renal failure in rats might be caused by low NO levels. In contrast, increased NO concentration might cause platelet dysfunction followed by bleeding, which is common in uremic patients. NO production may correlate with the degree of chronic renal failure, and therefore, it may serve as an indicator of the disease state, including haematological parameters and creatinine clearance. According to Brunini et al., conflicting data are available on the systemic production of NO in chronic renal failure patients (7). As the haematological parameters in HD patients have been impaired, erythropoietin (rhEpo) therapy is very effective. In addition to its main role in controlling erythropoiesis, NO has other beneficial effects such as an anti-inflammatory effect, an anti-apoptotic effect, an anti-free radical formation effect and the ability to enhance the proliferation of smooth muscle cells (angiogenesis) (8, 9).

The aim of the study was to evaluate nitric oxide (NO) levels in patients with end-stage renal disease (ESRD), taking into account their HD duration period and rhEpo supplementation therapy.

## METHODS

A group of 76 HD patients was enrolled. Their haematological parameters (haemoglobin, haematocrit, red blood cell count and white blood cell count) and NO level were measured and compared with the control group values. The control group consisted of 30 sex- and age-matched healthy subjects. The HD patients were divided into 3 groups based on HD duration: group I, <5 years (n=26); group II, 5-10 years (n=22); and group III, >10 years (n=28). HD patients were also divided into two groups based on the receipt of rhEpo therapy: group I did not receive rhEpo therapy (n=37), and group II received rhEpo therapy (n=39). In an evaluation study of NO levels during the 6th month of rhEpo, HD patients were divided again into 2 groups: group I did not receive rhEpo therapy (n=20), and group II received rhEpo therapy (n=27). The NO level in HD patients was examined at 0, 3 and 6 months. rhEpo doses (Eprex-Cilag-Janssen) were given subcutaneously following an HD session (20-25 U/kg weekly) to achieve 10-11 g/dl haemoglobin and 35% haematocrit. All patients were treated with HD for 4 hours, 3 times per week, using bicarbonate HD and polysulphone HD membranes. Blood samples were taken from the cubital vein before each HD session.

Standard laboratory techniques were used to measure the haematological parameters of haemoglobin, haematocrit, red blood cell count and white blood cell count. A microplate assay kit was used (OXIS, Oregon, USA) was used to assay NO levels. This method is based on NO degradation converting nitrates to nitrites by the enzymatic reduction with nitrate reductase using the Griess reagent.

Student's t test was used for statistical analysis with a significance level of 0.05.

## RESULTS

The haematology test values in the HD patients were significantly different from the values in the control group, with a lower haemoglobin ( $p < 0.05$ ), lower haematocrit ( $p < 0.01$ ) and lower red blood cell count ( $p < 0.05$ ). There was no significance difference found in white blood cell count. An increased NO value was observed in HD patients compared to controls ( $p < 0.001$ ) (Table 1).

	Control group n=30	HD patients n=45	P
Haemoglobin (g/L)	121 ± 19	104 ± 16	0.05
Haematocrit	0.42 ± 0.14	0.30 ± 0.11	0.01
Red blood cells (X10 <sup>12</sup> /L)	4.41 ± 1.0	3.80 ± 0.9	0.05
White blood cells (X10 <sup>9</sup> /L)	6.90 ± 1.8	6.50 ± 1.5	N.S.
NO (µmol/L)	57.419	150.238	0.001

N.S., not statistical

**Table 1.** Haematological parameters and nitric oxide levels in HD patients



No significant differences in NO levels were noted by HD duration: <5 years ( $155.9 \pm 17 \mu\text{mol/L}$ ); 5 - 10 years ( $168.6 \pm 31 \mu\text{mol/L}$ ); and >10 years ( $166.7 \pm 47 \mu\text{mol/L}$ ), (Figure 1).

In HD patients receiving rhEpo supplementation therapy, a lower NO level was observed ( $151.9 \pm 36 \mu\text{mol/L}$ ) when compared with the HD patients not receiving rhEpo therapy ( $174.6 \pm 40 \mu\text{mol/L}$ ) ( $p < 0.01$ ) (Figure 2).

In a 6-month evaluation study, NO level decreased if a HD patient was receiving rhEpo supplementation therapy. There was a decrease from  $146 \pm 35 \mu\text{mol/L}$  to  $130 \pm 37 \mu\text{mol/L}$  at the 3<sup>rd</sup> month and from  $122 \pm 34 \mu\text{mol/L}$  by the 6<sup>th</sup> month ( $p < 0.05$ ). No significant difference in NO level was noted in the control group (Figure 3).

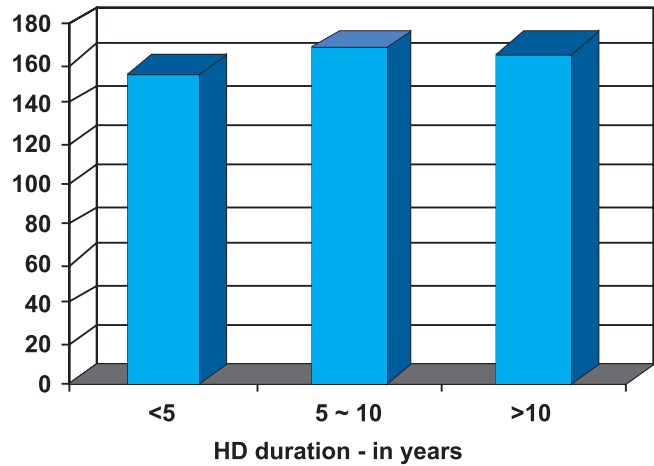


Figure 1. NO levels in the patients based on differing HD duration

## DISCUSSION

In renal physiology, NO plays important roles in processes such as glomerular haemodynamics, the tubuloglomerular negative feedback mechanism and renin release. Furthermore, physiological interactions between NO, renin-angiotensin II and superoxide anions provide a coordinated regulation of kidney function. The imbalance of these interactions may be linked to renal pathophysiology (10, 11). Uremic plasma induces endothelial NO synthesis, which leads to platelet dysfunction and other uremic manifestations. Several studies in experimental animals and humans have demonstrated an accumulation of NO metabolites during uraemia. Interestingly, uremic plasma can provoke NO production in endothelial cells. This enhancement in NO production may be related to the type of HD membrane used and seems to be more pronounced in patients who present with hypotension during HD, which is also common for extracorporeal circulation (7, 12). Therefore, HD *per se* may stimulate NO production via inducible NOS (nitric oxide synthase) as a part of the inflammatory response. However, there are differences between the biocompatibility of HD membranes regarding NO induction. The activity of endothelial NOS can be enhanced during HD sessions via the interaction of lymphomonocytes with the membranes, possibly mediated by TNF-alpha and IL-1 beta production (13). According to our results, NO levels were higher in HD patients than controls. This finding correlates with the results of other studies where the observed baseline NO generation was higher. The chronic hypotensive state in HD patients is associated with longer HD times and higher nitrate/nitrite plasma levels, suggesting enhanced NO production (14). In contrast, in 2000, Schmidt et al. suggested that the cause of higher NO levels in HD patients is a lack of renal excretion. In this study, no increased NO production in HD patients was recorded. Therefore, they suggest that NO production is low in these patients and may contribute to hypertension and disease progression. They support their opinion by presenting NO decreases after HD sessions (15, 16). These previous results may explain why, in the present study, the HD patient NO levels did not show any significant

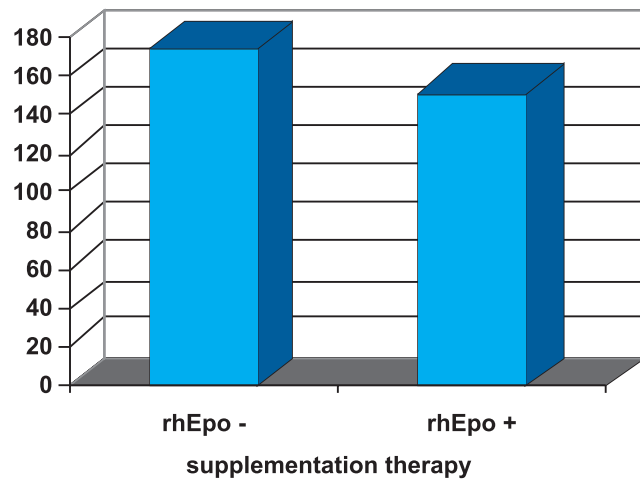


Figure 2. NO levels in HD patients undergoing rhEpo supplementation therapy

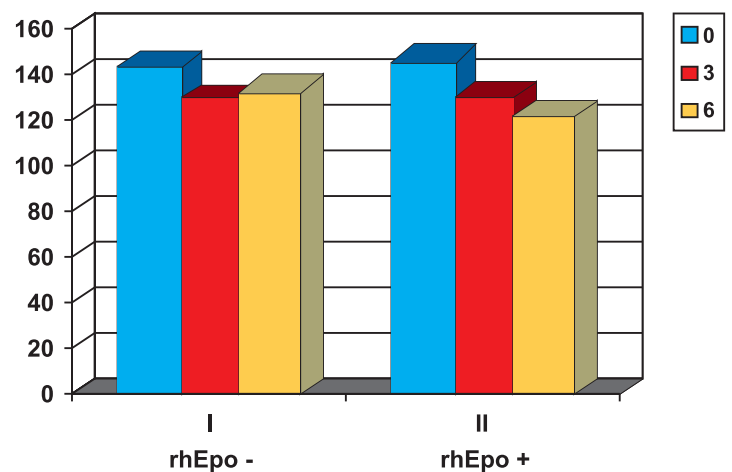


Figure 3. Evaluation of NO levels in HD patients after 3 and 6 months of rhEpo supplementation therapy



difference related to HD duration time. In HD patients receiving rhEpo therapy, a decrease in NO level was observed, in concordance with the results of other studies. The role of rhEpo in decreasing NO level in HD patients may diminish the prooxidative effects of NO and the accompanying toxic influence. Therefore, rhEpo augments urinary nitrate/nitrite concentrations (17). According to a 2006 study by Desai et al., a 24 hour incubation of human aortic endothelial cells with rhEpo results in a downregulation of endothelial NOS protein expression. Thus, rhEpo significantly reduces NO production by both quiescent and proliferating endothelial cells (18). However, some studies indicate that prolonged rhEpo supplementation therapy may cause adverse effects due to its effect on NO. Nevertheless, Krapf et al. suggest that rhEpo may provoke arterial hypertension in HD patients. The vasoconstrictive effects of NO might be caused by both decreased systemic NO production and resistance to NO vasodilatation (19).

## CONCLUSION

Based on the results of the present study, we conclude that NO values are increased in HD patients due to HD membrane induction as a part of the inflammatory response and/or a lack of renal excretion. Erythropoietin therapy, though it demonstrated the adverse effect of causing vasoconstriction, also demonstrated a beneficial effect by decreasing NO levels and preventing its prooxidative effect in combination with the superoxide anion. Such prevention may improve disease conditions and contribute to better disease outcomes.

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When reporting experiments on human subjects, it should be indicated whether the procedures followed were in accordance with ethical standards of the Committee on human experimentation (or Ethics Committee) of the institution in which they were done and in accordance with the Helsinki Declaration. Hazardous procedures or chemicals, if used, should be described in details, including the safety precautions observed. When appropriate, a statement should be included verifying that the care of laboratory animals followed accepted standards.

Statistical methods used should be outlined.

## RESULTS

Results should be clear and concise, and include a minimum number of tables and figures necessary for proper presentation.

## DISCUSSION

An exhaustive review of literature is not necessary. The major findings should be discussed in relation to other published work. Attempts should be made to explain differences between the results of the present study and those of the others. The hypothesis and speculative statements should be clearly identified. The Discussion section should not be a restatement of results, and new results should not be introduced in the discussion.

## ACKNOWLEDGMENTS

This section gives possibility to list all persons who contributed to the work or prepared the manuscript, but did not meet the criteria for authorship. Financial and material support, if existed, could be also emphasized in this section.

## REFERENCES

References should be identified in the text by Arabic numerals in parentheses. They should be numbered consecutively, as they appeared in the text. Personal communications and unpublished observations should not be cited in the reference list, but may be mentioned in the text in parentheses. Abbreviations of journals should conform to those in Index Serbian Journal of Experimental and Clinical Research. The style and punctuation should conform to the Serbian Journal of Experimental and Clinical Research style requirements. The following are examples:



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Book: 17. Sherlock S. Diseases of the liver and biliary system. 8th ed. Oxford: Blackwell Sc Publ, 1989.

Chapter or article in a book: 24. Trier JJ. Celiac sprue. In: Sleisenger MH, Fordtran JS, eds. *Gastro-intestinal disease*. 4th ed. Philadelphia: WB Saunders Co, 1989: 1134-52.

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Tables should be typed on separate sheets with table numbers (Arabic) and title above the table and explanatory notes, if any, below the table.

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## LETTERS TO THE EDITOR

Both letters concerning and those not concerning the articles that have been published in *Serbian Journal of Experimental and Clinical Research* will be considered for publication. They may contain one table or figure and up to five references.

## PROOFS

All manuscripts will be carefully revised by the publisher desk editor. Only in case of extensive corrections will the manuscript be returned to the authors for final approval. In order to speed up publication no proof will be sent to the authors, but will be read by the editor and the desk editor.





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