In this study we investigated the influence of low dosage X - ray irradiation on the incidence of chromosomal damage and changes in mitotic index (MI) in cultured peripheral lymphocytes of the Bosnian and Herzegovinian mountain horse following in vitro irradiation. X-ray irradiation induced a dose-dependent decrease in MI but only the dose of 0.5 Gy induced a significant decrease (p<0.05) in comparison with the control and other dose groups. The analysis of chromosomal damage revealed a clear dose-dependent increase in the incidence of chromosomal damage per metaphase. Significant differences (p<0.05) were detected by analysis of variance and the LSD test confirmed significant differences between cells that received 0.2 Gy and 0.5 Gy when compared to the control cells and cells that received 0.1 Gy. However, Scheffe's test assigned significance only to the differences established between the cells that received 0.5 Gy and the other groups of lymphocytes.

Key words: chromosomal damage, cytogenetic dosimetry, horse, ionizing irradiation
Numerous factors may induce chromosomal damages and X-rays are a potent mutagen and carcinogen, which was observed shortly after their discovery. Numerous studies on individuals that were occupationally, therapeutically or accidentally exposed to X-rays, as well as in vitro experiments, confirmed genomic instability as a possible outcome of this type of irradiation (Krishnaja and Sharma, 2004; Balcer and Harrison, 1988).

The sensitivity of the DNA molecule to genotoxic agents makes each biological individual a potential dosimeter of an agent's biological effects. DNA molecules are the target of ionizing irradiation and their damages are indubitably involved in the process of malignant transformation induced by irradiation. Considering that the DNA molecule is a vital chromosome constituent, the analysis of chromosomal aberrations in peripheral blood lymphocytes (or bone marrow cells) is an acknowledged basis of cytogenetic dosimetry. Chromosomal mutagen effects are of particular interest as they can be observed at the level of the cell. Proportional relation among the number of mutations, the applied dosage and the ability of the smallest dosages to induce mutations are well established (Emery, 1986).

Lymphocytes that continuously migrate between the bloodstream and extra cellular matrix may represent "circulating dosimeters" (IAEA, 1986). Following in vitro irradiation of lymphocytes a pronounced decrease in response to mitogenic stimulus of phytohaemaglutinine occurs (Barell and Blomgren, 1976; Barell et al., 1977). The higher the dosage – the lower the ability of lymphocytes to undergo blast transformation and commence mitosis (Gantenberg et al., 1991).

The capacity of ionizing irradiation to induce chromosomal damage has been confirmed in numerous studies (daCruz et al., 1994; Kaplan and Morgan, 1998; Lambert et al., 1998; He et al., 2000; Thierens et al., 2000; Boyle et al., 2002). Genetic changes caused by ionizing irradiation may be passed along next cell generations. The main issue in studies of damages induced by irradiation is determination of the dosage of the absorbed energy, which may enable the assessment and prognosis of the outcome of potential irradiation disease.

Our study was aimed at examining the correlation between the irradiation dosage and the number of chromosomal aberrations as well as alteration of mitotic index in Bosnian and Herzegovinian mountain horse lymphocytes following in vitro irradiation with a low dosage of X-rays.

Materials and methods / Materijal i metode rada

A total of eight Bosnian and Herzegovinian mountain horses of both sexes (5 males and three females) and different age, were used in this study. Horse blood was sampled by vein-puncture from the jugular vein into sterile vacuum tubes containing heparin. Tubes were irradiated using therapeutical Tele-
A cobalt apparatus, Tehraton 780. The blood was irradiated in vacuum tubes wrapped in a 0.5 cm thick layer of cotton. Irradiation field size was 15 x 15 CM at a distance of 80 cm. Blood samples were treated with individual dosages of 0.1, 0.2 and 0.5 Gy. The applied method of peripheral blood lymphocytes cultivation, recommended by IAEA (2001) was slightly modified to suit the conditions in our laboratory.

Sterile vials were filled with 7 ml of nutrient medium (RPMI 1640 or MEM), 2 ml of foetal calf serum, 0.2 ml of phytohaemaglutinine (PHA) and 0.8 ml of blood. Samples were prepared in duplicate for each animal and dosage. Lymphocytes were cultivated at 38°C for 48 hours. Following 45 hours of cultivation all samples were supplemented with 0.2 ml of 0.05% colchicine. Over the following three hours, all dividing cells were arrested in metaphase. Three hours following colchicine treatment (48 hours after beginning of cultivation), all cultures were transferred into tubes and spun at 1000 rpm for 10 minutes. Supernatant was discarded and sediment mixed with fresh hypotonic solution 0.075 M KCl.

Treatment with hypotonic solution lasted for 20 minutes at 38°C. This treatment increases cell volume and improves chromosome distribution. Warm hypotonic solution improves efficiency by increasing the rate of transmembrane water transport and membrane softening, which promotes its expansiveness.

Following this procedure, the cell suspension was spun again. Thereafter, fixing solution was added to the sediment. Fixing solution contains ethanol and glacial acetic acid in 3:1 ratio and is used precooled at +4°C. During fixation, excess water is removed from cells and they were fixed. Cold fixing solution improves chromosome outlines. Repetitive sequential washes in fixing solution with centrifugation (10 minutes at 1000 rpm) result in white sediment (cell suspension). Finally, 0.5 ml of fixing solution was added onto white sediment and thoroughly mixed with a pipette. Suspension is dripped on slightly slanted chilled slides (-20°C) from an elevated position. Slides were dried at room temperature and stained with 5% Giemsa for 10 minutes. The stained slides were washed with running water and with distilled water afterwards.

Chromosomal damage and mitotic index were analyzed in 48-hour cultures. Mitotic index (MI) represent the number of metaphases per 1000 analyzed cells. Statistical analysis of the data was performed using variance analysis and post-hoc multiple tests (LSD test, Scheffe's test). Cytogenetic analysis of the slides was performed using research light microscope Olympus BX41 equipped with digital camera. Changes were recorded using immersion objective (x 1000). Clearly visible metaphases were examined for each one of the tested irradiation dosages. In the control samples, 0.1 Gy and 0.2 Gy irradiated samples, we examined 1600 metaphases for each group. A total of 958 metaphases were examined in the samples irradiated with 0.5 Gy. The observed aberrations were statistically analyzed using variance analysis and post-hoc multiple tests (LSD test, Scheffe's test) by SPSS 15.0 for Windows program.
Results and Discussion / Rezultati i diskusija

There are no available data providing information about the karyogram of Bosnian and Herzegovinian mountain horse which is our autochthonous horse breed. In order to form baseline control for the identification and the analysis of chromosomal aberration following blood irradiation we have constructed a normal horse karyogram. Comparison of the constructed karyogram with karyograms of other horse breeds described in literature (Richer et al., 1990) revealed no observable differences. Diploid number of chromosomes is \(2n = 64,XY\) (\(2n = 64, XX\)) which includes 62 autosomal and one pair of sex chromosomes. Diploid chromosome set consists of 13 pairs of metacentric and sub-metacentric chromosomes and 18 pairs of acrocentric chromosomes. Sex chromosomes in female specimens comprise two sub-metacentric \(X\) chromosomes while male karyogram features has one sub-metacentric \(X\) and one acrocentric \(Y\) chromosome. It is worth emphasizing that horse karyotype analysis is quite complex due to a high

![Figure 1. Metaphase figure and karyogram of a horse (male)](image1)

![Figure 2. Metaphase figure and karyogram of a horse (female)](image2)
number of chromosomes and an abundance of acrocentric chromosomes (18 pairs), whose morphology complicates the analysis of chromosomal aberrations (dicentric chromosomes and acentric fragments in particular). Basic attributes of normal horse karyogram are presented in Figures 1 and 2.

Mitotic index was determined as a relative frequency (%) of cells in mitosis per 1000 examined cells in 48-hour horse blood cultures irradiated with different dosages. The results are presented in Table 1 and Figure 3.

Table 1. Mitotic index of cultivated horse lymphocytes following the irradiation /

<table>
<thead>
<tr>
<th>Dosage (Gy)</th>
<th>Control / Kontrola</th>
<th>0.1</th>
<th>0.2</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>MI (%)</td>
<td>15.2</td>
<td>14.8</td>
<td>14.1</td>
<td>8.4*</td>
</tr>
</tbody>
</table>

*p < 0.05

Variance analysis ANOVA established significant differences among the examined groups (F = 34,800; p<0.05).

The results of post-hoc multiple tests demonstrated significant difference between 0.5 Gy irradiated samples and all other samples. At the same time, no significant difference was observed among the control samples and samples irradiated with 0.1 Gy and 0.2 Gy. X-rays were found to alter mitotic index regardless of the tested dosage. The number of metaphases in irradiated samples equilibrated against the control decreased with the increase in dosage. The number of metaphase lymphocytes in samples irradiated with 0.1 Gy and 0.2 Gy did not significantly differ from the control. At the same time, the change in mitotic index in 0.5 Gy irradiated samples was significant experiencing decrease from 15.2% in the control to 8.4%.
This result suggests that X-rays exert a negative influence over the mitotic index, which declines with the increase in dosage. Increase in the received irradiation dosage causes accumulation of unfavorable changes that cells cannot sustain, thus numerous cells yield to selection and undergo lysis. It was observed that the cell abundance decreases with the increase in dosage. When the health risk assessment is concerned, damages sustained by cells are of greater concern. Such damages undergo clonal expansion and are passed on to the following generations of cells. At certain level they most probably cause transformation of normal into neoplastic cells, i.e. tumor development (Ibrulj, 2000).

It is well known that ionizing irradiation induces chromosomal aberrations observable in various human and animal tumors. Many neoplasms are characterized by visible chromosomal aberrations along with certain somatic conditions. Numerous discussions were dedicated to the significance of chromosomal aberrations and their potential role in the etiology of malignant transformation. The incidence of chromosomal aberrations and tumors increases with the increase of irradiation dosage which suggests an etiological link between chromosomal aberrations and malignant transformation. Numerous experiments conducted on plants and animals revealed chromosomal aberrations in dividing cells that were exposed to irradiation as an observable response to irradiation or injuries (Makino, 1975).

The results of the analysis of chromosomal damage in the cultured lymphocytes of the Bosnian and Herzegovinan mountain horse following in vitro irradiation with a low dosage X-rays are summarized in Table 2 and Figures 4-9.

Table 2. Summarized data on chromosomal damage in horse lymphocytes following in vitro irradiation

<table>
<thead>
<tr>
<th>Dosage (Gy)</th>
<th>Number of metaphase figures</th>
<th>Chromatid aberrations</th>
<th>Chromosomal aberrations</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gap / Break / Acentric fragments / Dicentrics / Quadriradial translocation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1600</td>
<td>6 5 2 1</td>
<td>-</td>
<td>0.88</td>
</tr>
<tr>
<td>0.1 Gy</td>
<td>1600</td>
<td>7 5 4 2</td>
<td>-</td>
<td>1.13</td>
</tr>
<tr>
<td>0.2 Gy</td>
<td>1600</td>
<td>10 5 10 8 1</td>
<td>1</td>
<td>2.13</td>
</tr>
<tr>
<td>0.5 Gy</td>
<td>958</td>
<td>7 4 24 18 1</td>
<td>1</td>
<td>5.74</td>
</tr>
</tbody>
</table>

Chromosome analysis in non-irradiated samples revealed spontaneous chromosomal aberrations, which is consistent with the available data (Pertti et
vonKoskull, 1976; Dolphin, 1978; Kubelka, 1985; Lloyd et al., 1987). The frequency of spontaneous chromosomal aberrations in our research was 0.88% while reports on this value in animals vary. The frequency of spontaneous chromosomal aberrations in pig lymphocytes was 0.77% (Slijepčević, 1991), 1.40% in goats (Hasanbašić, 1991) and 1.62% in cattle (Hasanbašić et al., 1998).

Also, the data on spontaneous chromosomal aberrations in humans fluctuate. However, the data by Zaharov are the most reliable (after Obralic, 1992). According to an examination of 60 thousand people of both sexes, 0 – 70 years of age, he established a frequency of spontaneous aberrations of 1.02%.

Following *in vitro* irradiation of horse blood we confirmed that ionizing irradiation increases the chromosomal aberrations load per metaphase. Also, chromosomal aberrations are clearly dependant on the irradiation dosage. Chromosome type aberrations are superior to the other types since the aberrations found in peripheral blood lymphocytes following the irradiation were mainly of chromosome type. In biological dosimetry, the occurrence of dicentric chromosomes, acentric fragments and ring chromosomes is observed with particular attention (Galloway, 1994; Hasanbašić, 1991; Slijepčević, 1991).

In this research, chromatid type aberrations (gaps and breaks) did not significantly depart from the condition found in non-irradiated lymphocytes. This finding is consistent with literature data (Bender et al., 1985; Hasanbašić, 1991; Slijepčević, 1991) (Figures 4 and 5).

The number of chromosome type aberration increased with the increase in irradiation dosage. Also, acentric fragments (Figure 6) were always somewhat more abundant than dicentric chromosomes (Figure 7). Our results concur with those found in pigs, goats and cattle (Hasanbašić, 1991; Slijepčević, 1991).
1991; Hasanbašić et al., 1998), and in humans as well (Kašuba, 1995). The finding of a ring chromosome (Figure 8) at an early stage of our research at a dosage of 0.2 Gy may be explained by the advanced age of the particular experimental animal (20 years). It is well established that age may influence the level of chromosomal aberrations since it also influences the metabolic condition of an animal as well as the condition of enzymatic repair processes. The occurrence of quadriradial translocation in the same sample at 0.5 Gy dosage may be explained in the same manner (Figure 9).
We noted a significant increase in chromosomal aberrations frequency following the irradiation of blood with 0.5 Gy. The same observation was reported by Anderson et al. (2000). They observed a significant increase in chromosomal aberrations frequency in the first human lymphocytes division following the irradiation with 0.5 Gy.

Change in ploidy was also noted, mainly in the form of tetraploidy which is the result of mitotic spindle dysfunction. This numerical aberration was not recorded separately. Variance analysis (ANOVA) confirmed significance of the differences (F = 71.349; p<0.05). LSD test established significant differences between the samples irradiated with 0.2 Gy and 0.5 Gy and the control samples and the samples that received 0.1 Gy. Scheffe's test showed significant difference only between the 0.5 Gy irradiated group and all the other analyzed groups.

Analysis of the cultivated peripheral blood lymphocytes has proven to be a very convenient method for the analysis of chromosomal aberrations. The results confirm extreme mutagenic and carcinogenic activity of the applied agent. Uncontrolled utilization of nuclear power, fast technological development and the use of X-rays in medical diagnostics and therapy contribute to the dangers of human and animal exposure to ionizing irradiation as well as environmental contamination.

References

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HROMOZOMSKA OŠTEĆENJA I PROMJENE MITOTSKOG INDEKSA U LIMFOCITIMA BOSANSKOHERCEGOVAČKOG BRDSKOG KONJA NAKON IN VITRO OZRAČIVANJA NISKIM DOZAMA X ZRAKA

Dunja Rukavina, Danica Hasanbašić, E. Suljkanović, Amela Katica

U radu je istraživan uticaj niskih doza X zraka na pojavu hromozomskih oštećenja i promjene mitotskog indeksa (MI) u kulturi limfocita bosanskohercegovačkog brdskog konja nakon in vitro ozračivanja. Rezultati su statistički obrađeni korišćenjem analize varijanse i post-hoc multiplih testova (LSD test, Scheffes' test).

Rezultati analize MI su ukazali da X zrači negativno utiču na MI, smanjujući njegove vrijednosti sa povećanjem doze zračenja. Utvrđeno je da se skupina zračena na 0,5 Gy statistički značajno razlikovala u odnosu na ostale ispitivane skupine (p<0,05). Analiza hromozomskih oštećenja pokazala je da jonizujuće zračenje povećava broj hromozomskih oštećenja po metafazi, da hromozomske aberacije pokazuju jasnu ovisnost o dozi zračenja. Postojanje signifikantnih razlika utvrđeno je korišćenjem analize varijanse (p<0,05). Rezultati LSD testa potvrdili su postojanje značajnih razlika između skupina zračenih na 0,2 Gy i 0,5 Gy u odnosu na kontrolnu skupinu i skupinu zračenu na 0,1 Gy, dok su rezultati Scheffes' testa pokazali da se signifikantno razlikuje jedino skupina zračena na 0,5 Gy.

Ključne reči: hromozomska oštećenja, citogenetska dozimetrija, konj, jonizujuće zračenje

HROMOSOMNYE POVREJDENI I IZMENENI MITOTICHESKOG INDEKSA V LIMFOCITAX BOSNIYSKOGERCHEVOINSKOGO GORNYY LOCHADY POSLE IN VITRO OBSLUHVENI NIZKIMI DOZAMI X LUCHEEY

Дуня Рукавина, Даница Хасанбашич, Э. Сульканович, Амела Катица

В работе исследовано влияние низких доз X лучей на явление хромосомных повреждений и изменения митотического индекса (МИ) в культуре лимфоцитов боснийско-герцеговинской горной лошади после in vitro облучения. Результаты статистически обработаны пользованием анализа варианта и post-hoc мультиплых тестов (LSD test, Scheffes' test).

Результаты анализа МИ указали, что X лучи отрицательно влияют на МИ, уменьшая их стоимости с увеличением дозы излучения. Нами утверждено, что группа, излученная на 0,5 Gy статистически значительно различалась в отношении остальных испытанных групп (p<0,05). Анализ хромосомных повреждений показал, что ионизирующее излучение увеличивает число хромосомных повреждений по метафазе, что хромосомные аберрации показывают ясную зависимость о дозе излучения, и, что хромосомные типы аберраций превосходящие в отношении других типов аберраций. Существование значимых разниц утверждено пользованием анализа варианта (p<0,05). Результаты LSD теста подтвердили существование значительных разниц между группами, излученных на 0,2 Gy и 0,5 Gy в отношении кон-
трольной группы и группу, излученную на 0,1 Гу, пока результаты Scheffes' теста показали, что значимо различается только группа, излученная на 0,5 Гу.

Ключевые слова: хромосомные повреждения, цитогенетическая дозиметрия, лошадь, ионизирующее излучение