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MICRONUCLEI IN LYMPHOCYTES OF HORSES AND PIGS AFTER *IN VITRO* IRRADIATION

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Within a comprehensive study conducted on the Bosnian-Herzegovinia mountain horse breed, a research on the relative frequency of micronuclei (MN) after in vitro horse blood irradiation was carried out. Experiments on cytogenetic dosimetry were also conducted on pigs.

The results of MN test were presented in terms of MN in binucleate (BN) cells. In control samples the percent of MN is very low in relation to irradiated samples where a higher number of MN cells was asserted, as well as a higher number of MN in BN cells. We could also observe that a number of BN cells with MN is proportionally increased with the radiation dosage. The MN test could provide additional data on the level of radiation damage (depletion) in the lymphocytes of the peripheral blood in domestic animals.

Key words: cytogenetic dosimetry, domestic animals, ionizing radiation, micronuclei

INTRODUCTION

The basic precondition for normal functioning of a living cell is the unimpaired integrity of DNA. Unfortunately, there is a great number of physical, chemical and biological agents which, either directly or indirectly, damage the integrity of this macromolecule, which, in turn, results in a wide range of structural and functional changes, ultimately leading to cell death. One of the challenges posed in front of contemporary man is the identification and prevention of irreversable negative processes in living systems. The very first step in a successful application of a system of radiation protection is high-quality dosimetry of ionizing radiation. The level of damage of the somatic cell (of a tissue or organ), caused by the effect of ionizing radiation can be assessed by a number of parameters (benchmarks). In cases where there is a doubt with regard to exposure to ionizing radiation, the methods of cytogenetic dosimetry are commonly used (IAEA, 2001).

In order to carry out the present research on mutations we chose a strong physical mutagenuous agent, ie. ionising radiation. The cytogenetic analysis of genetoxity of ionizing radiation was carried out by applying the cytohalazin-block micronucleus test. Micronuclei (MN), as a safe indicator of the elimination of the

genetic material, contain acentric fragments of chromosomes or whole chromosomes which have not been found in the main nucleus during the anaphase, so that MN are a reliable biomarker of exposure to clastergene aneugene hazards (Fenech, 1998). Many experiments have proved MN to be a reliable biomarker in biological dosimetry in human-instigated radiation exposures (daCruz *et al*., 1994; Wutke *et al*., 1996). MN test can also be applied in the assessment of cytogenetic damage in populations that inhabit the areas with a high radioactivity level (Chang *et al*., 1997, 1999; Tsai *et al*., 2001), and in groups which are professionally exposed to ionizing radiation (Thierens *et al*., 1996; Cardoso *et al.*, 2001).

By applying cytohalazin B – a blocker of cytokinesis, binucleate cells are generated, exclusively in the second interphase (Obralić, 1992; Ibrulj, 2000; IAEA, 2001; Maluf et al., 2001). MN is formed in the anaphase of the cell cycle. It can be formed from integral chromosomes (aneugene case) or acentric chromosome fragments after puckering (clustogene case) without being integrated into the nucleus of a daughter cell. MN test can successfully be applied on various cell types, starting from lymphocytes, fibroblasts, and peeled off epithelial cells without additional *in vitro* cultivation procedures (Ford et al., 1988). MN test is applied in many laboratories as a substitute for a more complex and far longer analysis of metaphase chromosomes. The micronucleus technique is simpler and faster, but also an equally sensitive method in relation to the test of chromosome aberrations which can be induced in *in vivo* and *in vitro* conditions of exposure to a mutagene. We can also say that the MN test is a more sensitive, and statistically a more reliable technique in relation to the test of chromosome aberrations since the former comprises a significantly greater number of analyzed cells (Norrppa and Falck, 2003). The advantage of the MN test is in its aptitude to detect both clastogene and aneugene effects (Fenech, 2000).

In 2003 Fenech and his associates adopted the main criteria for measuring micronuclei: the MN diameter must be smaller than 1/3 (one third) of the main nucleus; MN must be dyed in the same colour as the main nuclei; MN must be separated, or it can only overlap with the main nucleus on the boundaries (Fenech *et al*., 2003).

In a wider context, it is difficult to set a simple qualitative and quantitative link between the MN and chromosomal aberrations. In respect of quality, the *in vitro* MN test is similar to other cytogenetic tests, and it can serve as an alternative to the test of chromosomal aberrations (IAEA, 2001; Ibrulj, 2000).

At this point we must mention that within a comprehensive study on the Bosnian-Herzegovinian mountain horse, the investigation of the relative frequency of MN in the peripheral blood of the horse was also carried out following *in vitro* irradiation by various doses of X rays. Experiments were also conducted on the pig, being very convenient for examination from the perspective of cytogenetic dosimetry because of its smaller number of chromosomes and a smaller prevalence of acrocentric chromosomes.

Thanks to a slight modification of the MN test applied for horses and pigs within the present study, it was possible to study the effect of ionizing radiation in

terms of effects of dosage on the appearance of MN, being a reliable indicator of mutagenous action.

MATERIALS AND METHODS

In the present study we used the horse and pig to conduct experiments on. The choice of the horse was deliberate because of scant evidence on cytogenetic dosimetry of this particular animal, while the choice of pigs was conditioned by the convenient caryotic properties.

Horses used for the experiment belong to the autochtonous breed, namely, to the Bosnian -Herzegovinian mountain horse, originating from Borik, near the town of Rogatica. Six horses of both sexes (three males and three females) of the age group from 18 months to 20 years, and of body weight from 250 kg to 600 kg were selected for our experiment. Blood was taken by venepuncture from the *vena jugularis* in sterile heparin vacuum containers.

Blood of five healthy pigs belonging to the Swedish "Landras" breed of both sexes, aged between 4-6 months and body weight from 40 to 50 kilos were used in this experiment. All blood samples of pigs were obtained by puncturing the vein at the joining place of *v.c. cranialis* and *v. axilaris*.

For *in vitro* irradiatiaon of blood samples the *Siemens* X-ray apparatus was used.

The method of micronucleus test suggested by Feench and Morley (1985) was applied. A volume of 0.5 previously irradiated and heparinized blood of horses and pigs was added into flacons with a nutrient base: 7 mL RPMI 1640, 2 mL bovine serum and 0.2 mL phytohemaglutin (PHA). The duration time of cultivation at 38^{o}C was seventy-two hours, while at the 44th hour of incubation we added 6 μg/mL of cytohalasin. Cytohalasin is a B bloker of cytokinesis in the second division, previously dissolved in dymetilsulphoxide (DMSO) in a concentration of 0.5 mg/mL, thereupon diluted in distilled water. In this manner cells become binucleate within a "parent" cell membrane. Upon the expiry of incubation, the cultures were transferred into test tubes and centrifugated for 10 minutes at 1000 turns. The supernatant was removed and 5 mL of hypotonic solution (0.075 M KCl) was added to the sediment. The above content was immediately centrifuged. After separation of the supernatant, 5 mL of cooled fixative (3:1, methanol : glacial acetic acid) was added to the sediment. The fixation procedure lasted 30 minutes at +4^{o}C. Upon fixation, the test tubes were again centrifuged, followed by the separation of the supernatant and addition of a fresh fixative. The intermittent fixation and centrifugation procedures were repeated until the sediment became white. After that, the content of each test tube was suspended with 0.5 mL of the fresh fixative and the suspension was dropped onto cooled glass slides which were later dried at a room temperature. The preparations were dyed with a 5% Gimza solution for 10 minutes, and subsequently rinsed and air dried.

The microscopic analysis of the preparations was carried out by using the Olympus BX 41 light microscope, with a connected digital camera. The incidence

of binucleate lymphocytes with micronuclei was asserted on the basis of 1000 binucleate cells following the above treatment.

The difference in the total number of micronuclei between the horses and pigs in blood cultures irradiated by various doses and of those in control cultures were statistically analysed by the Hi square test.

RESULTS AND DISCUSSION

The results of the analysis of the DNA damage in lymphocytes of horses and pigs exposed to various doses of X irradiation (1, 2 and 3 Gy) by application of the micronucleus test are shown in tables 1, 2 and 3 and figures 1-10.

The frequency of the MN appearance was observed in the blood of 6 horses and 5 pigs in control samples of peripheral blood with the above radiation doses.

Table 1. Micronuclei in the lymphocytes of horses (cumulative overview)

Gy dose	Number of analysed cells	MN number	BN cells with MN %
0.00	6 000	41	0.68
1	6 000	293	4.88
2	6 000	426	7.1
3	6 000	508	8.46

Table 2. Micronuclei in the lymphocytes of pigs (cumulative overview)

Gy dose	Number of analysed cells	MN number	BN cells with MN %
0.00	5 000	29	0.58
1	5 000	179	3.58
2	5 000	346	6.92
3	5 000	381	7.62

Table 3. X^2 test for control and irradiated blood samples of horses and pigs

Gy dose	0.00	1	2	3
X^2 test	0.312	11.19**	0.1093	2.518

Statistically significant difference in relation to animal species:
** $p<0.05$ and 0.01

The frequency of binucleate (BN) cells with MN was asserted upon analysis of 1000 BN cells for each dose. By applying *in vitro* MN test we have confirmed our expectations that the MN appearance indicates an evident dependence on the radiation dose. The analysis of preparations in control samples has shown the

presence of MN in BN cells. A higher number of BN cells with MN in control samples is probably a consequence of the age of the examined horses (ranging from 18 months to 20 years) which is in a significant correlation with the frequency of MN presence. In their research related to the effect of age and MN appearance. Bonassi *et al*. (2001) ascertained that the factor of age could be reflected in a progressive increase of spontaneous chromosome instabilities along with the accumulation of DNA damage as a result of decreasing repair capacity of DNA with age. The effects could be accounted for by an increase of chromosome losses with age. Hence, future researches should be based on a well-balanced age structure of the examined animals. The discrepancy in respect of control values of MN in horses and pigs could also be a consequence of the unknown molecular interactions between cytohalazin B and the cell structures while the backgrund of the mutagenous action of this powerful chemical agent has still remained unaccounted for (IAEA, 2001; Ibrulj, 2000; Slijepčević 1990).

Through the microscopic analysis an increase of MN number was noticed irrespective of the radiation dose. There was a correlation between increased doses and increased numbers of BN cells with micronuclei, but also the MN number in BN cells. Regardless of the radiation dose, apart from the BN cell with one MN, the appearance of those with two or three MN of a different size was also noticed. The presence of a higher number of mononuclear cells with MN was noticed, but also of the polinucleate cells with/without the presence of MN which were not specifically registered. In BN and polynucleate cells some nuclei were completely separated, but they were most commonly associated with "nucleate bridges" in the form of a thin chromatin filament (Figures 1-8).

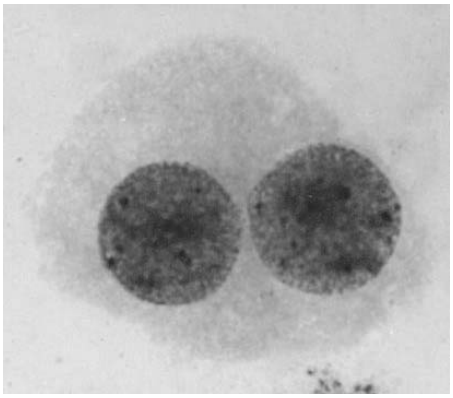

Figure 1. Tipical BN cell

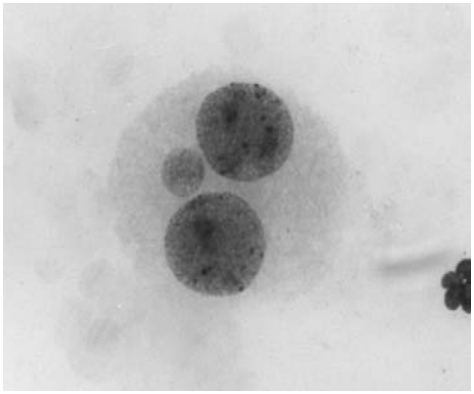

Figure 2. BN cell with MN

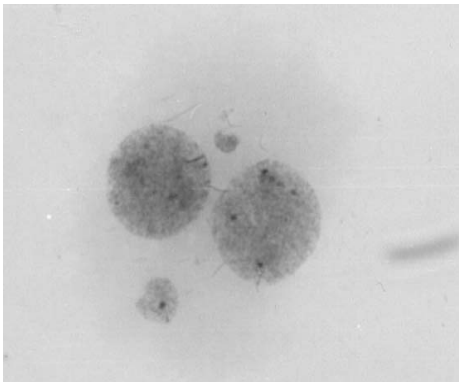

Figure 3. BN cell with two MN

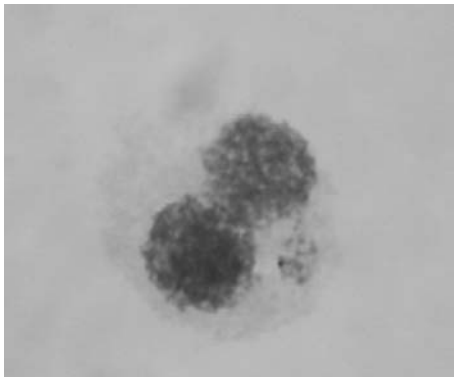

Figure 4. BN cell with MN and chromatin filaments

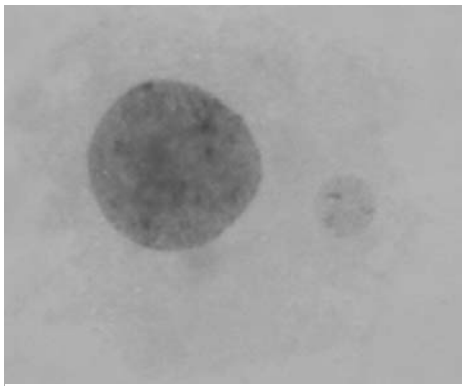

Figure 5. Mononuclear cell with MN

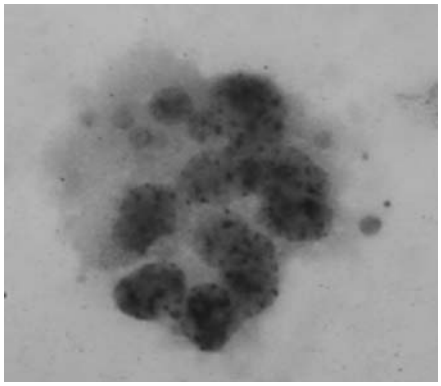

Figure 6. Polinuclear cell with diferent size MN

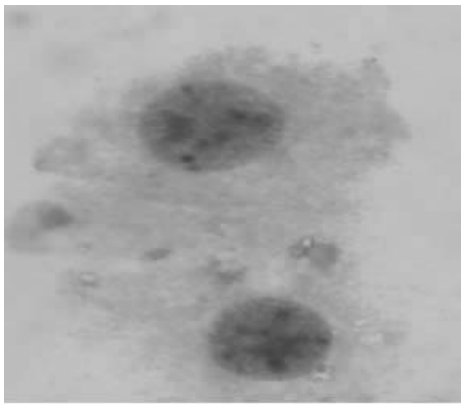

Figure 7. BN cell with more MN

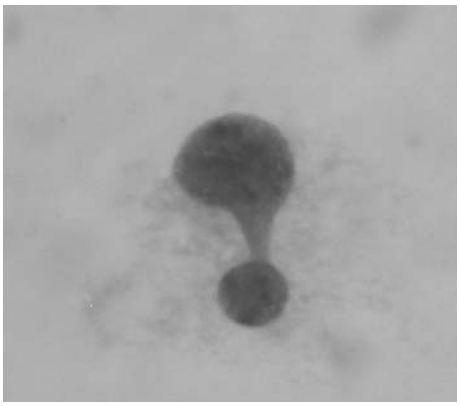

Figure 8. BN cell with joined unequal nuclei

The difference between horses and pigs in respect of the BN cell number with MN in the control cell population is not statistically, significant nor is it in the cell populations irradiated by doses of 2 Gy and 3 Gy. A statistically significant difference ($p<0.05$ and 0.01) was noticed with the dose of 1 Gy. The results show that by increasing the radiation dose the MN frequency in BN cells also increases, followed by the saturation of MN frequency with the application of 2 Gy and 3 Gy doses. It was noticed that the frequency of the micronucleus presence in lymphocytes of the peripheral blood in horses and pigs after *in vitro* irradiation is dose dependent. It was also observed that X-rays disturb the kinetics and separation of the genetic material. Numerous experiments indicate that MN are derived from the acentric fragments although it has been in general accepted that the formation of MN involves those derived from the genesis of asymmetrical alternate aberrations, either of chromosome or chromatid type (Hasanbašić *et al*., 2005). Many authors state a high level of control values of MN in comparison with the same for chromosome aberrations. This difference was observed in the analysis of MN and acentric fragments from human lymphocytes and those of pigs. Also, a lower level of MN at higher radiation doses in comparison with the frequency of acentric fragments in pigs is accounted for by the fact that a dose increase brings about the saturation of MN frequency, resulting in the association of two or more pairs of acentric fragments which form one micronucleus (Maluf *et al*., 2001; Obralić, 1992; Slijepčević, 1990).

The investigation of MN in the present study was conducted on a small number of horses (of different ages) and pigs. Ionizing radiation induced the formation of MN in lymphocyte cultures of horses and pigs.The basic mechanism of MN formation is clastogenesis and aneuploidygenesis. The level of disturbance varies with the radiation dose. In order to assess precisely the relation of the radiation dose and the number of induced MN so that a dose-effect curve could be drawn in conditions of *in vitro* irradiation, which could successfully be applied for assessment of the radiation dose after *in vivo* irradiation, it is necessary to take a greater number of peripheral blood samples of horses and pigs. It is also necessary to introduce a method in cytogenetic laboratories with the aim of assessing rapidly the absorbed radiation dose which is indispensable in accidental conditions. The micronucleus test can provide additional information on the level of radiation damage in the peripheral blood lymphocytes. In respect of cytogenetic dosimetry MN and chromosome aberrations should be considered as two distinctive consequences since each of them has its advantages and constraints. The best results are obtained by combining the two techniques of cytogenetic dosimetry.

In considering these results it is obvious that the micronucleus test is not an alternative or substitute for the analysis of chromosome aberrations. Every known clastogen disturbs a normal progression of cells in the cell cycle. The disturbance level varies with the increase of radiation dose. With the increase of acentric fragments per cell, there is an increasing likelihood that one MN will be formed from several acentric fragments. Therefore, we have a good reason to state that with higher radiation doses the ratio 1:1 = (equals) AF: MN decreases (Slijepčević, 1990). With the high doses of ionizing radiation the MN frequency is

far smaller in human lymphocytes in peripheral blood (Obralić, 1992). In our experiment, which was carried out on horses and pigs, it was observed that with high radiation doses amounting to 2 Gy and 3 Gy, the result was saturation of MN frequency.

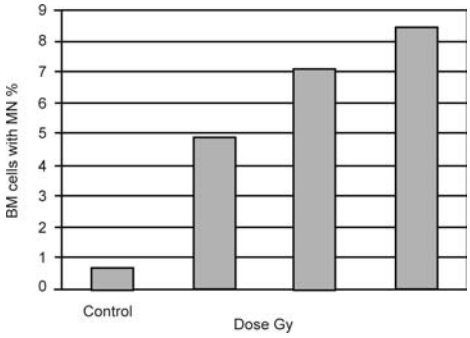

Figure 9. Relative frequency of binucleate (BN) lymphocytes of horses with micronuclei (MN) after *in vitro* irradiation of blood

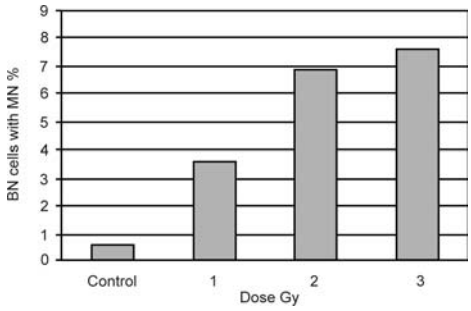

Figure 10. Relative frequency of binucleate (BN) lymphocytes of pigs with micronuclei (MN) after *in vitro* irradiation of blood

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MIKRONUKLEUSI U LIMFOCITIMA KONJA I SVINJA NAKON *IN VITRO* OZRAČIVANJA

HASANBAŠIĆ DANICA i RUKAVINA DUNJA

SADRŽAJ

U sklopu opsežne studije, rađene na bosanskohercegovačkom brdskom konju, vršena su istraživanja relativne učestalosti mikronukleusa (MN) nakon *in vitro* ozračivanja periferne krvi konja. Također, pored konja, sa stanovišta citogenetičke dozimetrije kao eksperimentalna životinja korištena je i svinja.

Rezultati MN testa predstavljeni su brojem MN u binuklearnim (BN) ćelijama. U kontrolnim uzorcima procenat MN je veoma nizak u odnosu na ozračene uzorke, gdje je konstatovan veći broj ćelija sa MN, kao i više MN u BN ćelijama. Zapaženo je da se broj BN ćelija sa MN povećava sa porastom doze zračenja. MN test bi mogao pružiti dodatne informacije o stepenu radijacionog oštećenja u limfocitima periferne krvi domaćih životinja.