THE FREQUENCY OF CHROMOSOMAL ABERRATIONS IN SHEEP FROM THE AREA CONTAMINATED BY DEPLETED URANIUM DURING NATO AIR STRIKES IN 1999

by

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This paper presents the results of cytogenetic studies in sheep from the region of Bujanovac that was contaminated by depleted uranium during the NATO air strikes in 1999. The study was conducted on sheep blood lymphocytes, in order to determine the frequency of chromosomal aberrations and to assess the presence of genetic risk as a result of the possible impact of depleted uranium. Blood samples for lymphocyte cultures were taken at random from the 20 animals of the households in the village of Borovac, near Bujanovac. The animals were chosen because they were pastured, fed, and watered in the NATO bombing area. With the purpose of comparing the results two control groups were cytogenetically analyzed, each consisted of 20 sheep from Zemun and Ovča, two northern localities that were not contaminated with depleted uranium. The established structural chromosomal changes were of breaks and gap types, and their frequencies in sheep of all surveyed localities were within the range of basic level values that are commonly found in the sheep lymphocyte cultures analyses. Significant differences are apparent between the values defined in the sheep from Bujanovac compared to those obtained in the sheep from the northern locality (Zemun), probably as a result of breeding of animals in the farm conditions and their being less exposed to the impact of environmental agents. There were neither elevated values of polyploid and aneuploid cells nor significant differences between the sites. According to earlier known data, depleted uranium was below the detection limit of the method applied both in the soil and feed given to cytogenetically analyzed animals. Based on the low-level changes that are in the range of the basic level changes, commonly observed in sheep lymphocytes control cultures, it cannot be said with certainty that it was depleted uranium that caused the changes, or that it is widespread in the region of Bujanovac.

Key words: depleted uranium, sheep, chromosomal aberration, break and gap, NATO attack in 1999, Bujanovac

INTRODUCTION

The method of cytogenetic analysis of peripheral lymphocytes has been used for more than 50 years to determine the occurrence of increasing frequencies of chromosomal aberrations, indicating the action of ionizing radiation. Permissible level of radiation was also evaluated based on the frequency of chromosomal aberrations, with the intention to protect the exposed individuals against the occurrence of significant genetic changes as well as to protect the gene pool of populations. In addition to numerous studies of radiation effects in laboratory experiments, the effects of the low doses of radiation were mostly studied in humans, occupationally exposed medical workers and nuclear plant personnel [1-3] but also in animals, especially after the Chernobyl accident [4-6]. It was confirmed that chromosomal aberrations in human lymphocytes [7] as well as in other mammals, provide sensitive and reliable method for biological dosimetry. All the cases of individuals exposed to ionizing radiation reveal the elevated frequency of chromosomal aberrations in comparison with controls, even when the doses the organisms were exposed to were lower than the permissible level [7].

During the military attacks in Serbia and Montenegro in 1999 about 500,000 depleted uranium (DU) missiles (3600 kg of uranium oxide of total activity of 18.3 10^{10} Bq) were fired. Eleven locations in South Serbia (Bujanovac, Vranje) and Montenegro had been targeted. The highest contamination measured in soil of the targeted sites was

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200,000-250,000 BqU/kg soil, and once the sites were isolated and decontaminated, the soil top layers of and missile fragments were stored as radioactive waste [8]. Uranium is a primordial radioactive element (half-life 10^9 years) found in nature as a mixture of three radioisotopes, with different relative abundance: ^{238}U (99%), ^{235}U (0.71%), and ^{234}U (0.006%). It is both chemically toxic and radioactive, and as water soluble is easily taken up by plants thus entering food chains and soil/waters systems. Depleted uranium (0.2-0.3% of ^{235}U) is low radioactive waste material, a by-product of uranium processing in nuclear reactors or production of nuclear weapons. Natural and depleted uranium are chemically identical, thus having the same health effects. Depleted uranium came into military use in the early 70's of the 20th century and was used for the first time in the Gulf War in 1991, and later in the wars in the Balkans in 1990 [9].

Health impacts of DU include initial exposure of the environment, local population and animals due to DU particles in air after the explosion and to particles re-suspended from contaminated soils and food/feed, and long term possible exposure from contaminated waters, or by dust containing particles with uranium. Possible cancer effects in humans may appear after the latency period of one to several decades. Health effects of deposited DU are due to alpha particles ionisation effects leading to production of reactive oxygen species. When the concentration of free radicals exceeds the level that could be controlled by effective antioxidant defense, cell membranes, proteins, lipids and nucleic acids are possibly damaged [10] provoking a spectrum of tissue injuries. The effects of depleted uranium are recognized as oxidative DNA damages, neoplastic transformations, mutagenic changes, genome instability, and oncogenesis [11].

Deleterious effects of uranium were studied in miners [12], occupationally exposed in the weapons industry, and Gulf War veterans [13].

Some studies of the people in the region of South Serbia that had been contaminated by depleted uranium, showed the elevated levels of chromosomal aberrations and rogue cells [14]. The elevated values of chromosomal aberrations were found among the people living in Bosnian areas contaminated by depleted uranium [15].

The study of possible health effects in domestic animals, mostly sheep and cows, reared in area contaminated with DU ammunition, began in 2003 and is still in progress [16-20]. At the same time the soil samples were tested for the presence of DU, heavy metals, and other elements, as well as animal food/feed: grass, wheat, and corn, as well as blood samples from the same animals [20]. These studies showed the presence of some indicators of oxidative stress and severe anemia in sheep and cattle from the area of Bujanovac [16-20].

The aim of this study was to determine the frequency of structural chromosome aberrations and incidence of numerical type changes, polyploid and aneuploid cells in sheep from the region of Bujanovac as a possible effect of DU and assess potential genetic risk from the presence of DU in the region of South Serbia.

MATERIAL AND METHODS

During the spring of 2005 the blood samples collected from 20 sheep that were randomly assigned to six different sites in the region of Bujanovac (Borovac village) in South Serbia.

Blood samples were taken from the jugular vein of 20 clinically healthy sheep (Merino/Svrljig), 3-6 years old. The animals that were taken to pasture and had free access to water in the vicinity of air strikes were randomly selected from 9 households. Blood samples taken simultaneously from the same animals were also used for the study of biochemical parameters and oxidative stress [16, 17, 19, 20]. For the purpose of comparison, a control group consisted of sheep from the two northern localities in Serbia, which had not been directly contaminated by depleted uranium (fig. 1). Blood samples were taken from randomly selected 20 individuals (Merino/Svrljig) from a farm in Zemun, and 20 sheep (Merino/Svrljig) randomly selected from 10 households in the Ovča village. Blood samples from the sheep of both control localities were also taken from the jugular vein. Heparinised blood samples were taken for the purpose of cytogenetic analysis, and the culture of lymphocytes was set up and the chromosome preparation was carried out according to a slightly modified conventional method [21].

Heparinised whole blood samples containing 1.2 10^6 lymphocytes (usually 0.8 ml of whole blood) were added to vials with 9.2 ml Parker 199 medium

Figure 1. Map of Serbia with sampling sites

containing 30% of inactivated calf serum (Serva) and 0.04 mg/ml phytohaemagglutinine (Welcome), so a final count of lymphocytes was 1.2 10^5/ml. Cultures were incubated for 72 hours at 37 °C.

Two hours before harvesting, colcemid (Ciba) was added to the cultures to achieve a final concentration of 0.5 μg/ml. After hypotonic treatment (0.075 M KCl) followed by three repetitive cycles of fixation in methanol/acetic acid solution (3:1, *v*/*v*), centrifugation, and resuspension, the cell suspension was dropped on chilled, grease/free microscopic slides, air dried and stained in 20% Giemsa (Kemika) for 15 minutes. For each animal, minimum of 100 well/spread mitotic figures of chromosomes were examined and number of structural damages of break and gap types and number of polyploid and aneuploid cells were determinated.

Statistical analysis was performed by ANOVA test.

RESULTS AND DISCUSSION

The results of cytogenetic chromosome analysis of sheep from three different localities in Serbia (fig. 1) are presented in tabs. 1 and 2, and figs. 2, 3, and 4. Structural type changes that were observed in the sheep from the region of Bujanovac and the other two studied localities were breaks: the ones of chromatid and chromosome type (izochromatid breaks) and gaps (tabs. 1 and 2, figs. 2 and 3). Structural chromosome changes that results from complex chromosome rearrangements were not identified.

The largest number of structural damage (tabs. 1 and 2, figs. 2 and 3) in sheep from the area of Bujanovac were gaps (fig. 3) and chromatid breaks (0.57%), while chromosomal breaks were 0.19% (fig. 2). As for the sheep from a farm in Zemun, the northern locality, there were also gaps, *i. e*., achromatic lesions (0.38%) accounted for the largest contribution of the chromosomal injures (fig. 3). Although the gaps are not considered as very reliable signs of genetic damage [22], when this type of lesions occurred with high frequency there are opinions that it can be seen as useful indicator of the presence of genotoxic agents in the environment [23]. In the sheep from a farm in Zemun, there was 0.19% of chromatid breaks and 0.09% of chromosomal breaks (fig. 2). Significant statistical difference ($p < 0.05$) could be observed between the values of chromatid breaks identified in the

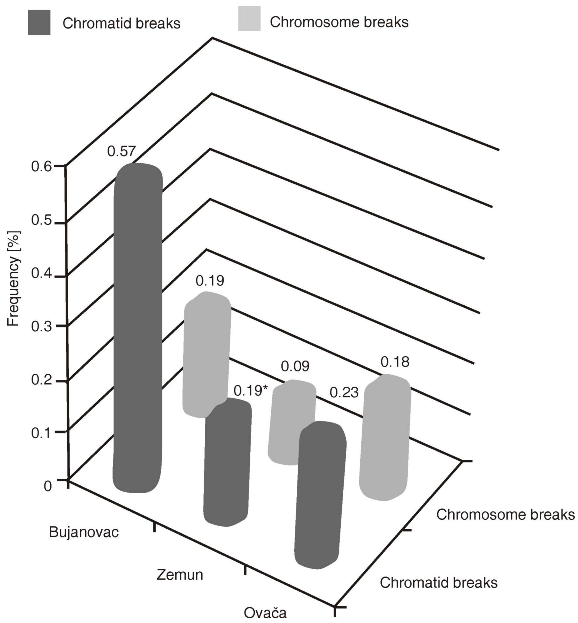

Figure 2. Frequency of chromatid and chromosome breaks (in percentage) in sheep from different localities. Statistical significant differences ($p < 0.05$) appear when comparing chromatid breaks in sheep from the region of Bujanovac and chromatid breaks in the sheep from a farm in Zemun

Table 1. Detected number of different type of chromosomal changes in sheep from three examined localities

Locality	Number of examined individuals	Mitoses examined	Chromatid breaks	Chromosome breaks	Breaks total	Gaps	Polyploid cells	Aneuploid cells
Bujanovac	20	2096	12	4	16	17	32	43
Zemun	20	2074	4	2	6	8	23	30
Ovča	20	2120	5	4	9	12	26	39

Table 2. Results of cytogenic analysis of sheep lymphocytes (Mean + SE) from three different localities: region of Bujanovac, a farm in Zemun and the village Ovča

	Chromatid breaks Mean + SE	Chromosome breaks Mean + SE	Gaps Mean + SE	Polyploid cells Mean + SE	Aneuploid cells Mean + SE
Sheep from the region of Bujanovac	0.6 0.13	0.2 0.09	0.85 0.15	1.6 0.26	2.15 0.34
Sheep from a farm in Zemun	0.2 0.09*	0.1 0.07	0.4 0.15*	1.15 0.28	1.5 0.21
Sheep from the village Ovča	0.25 0.1*	0.2 0.09	0.6 0.17	1.3 0.24	1.95 0.34

* Compared with sheep from the region of Bujanovac, statistically significant difference $p < 0.05$

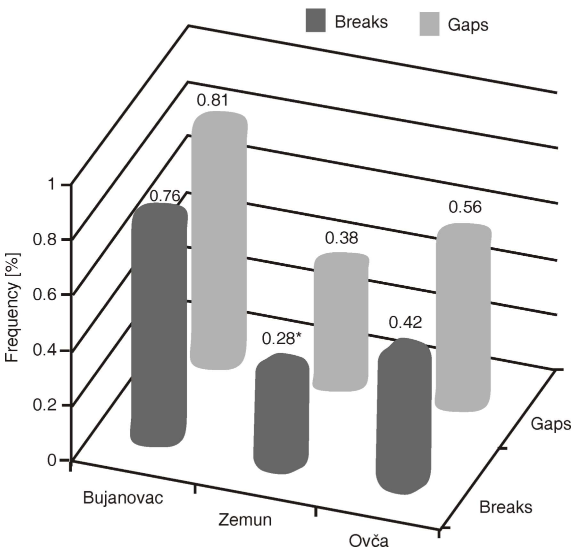

Figure 3. Frequency of breaks (total values in percentage) and gaps (in percentage) in sheep from different localities. Statistical significant differences ($p < 0.05$) appear when comparing breaks in sheep from the region of Bujanovac and breaks in sheep from a farm in Zemun

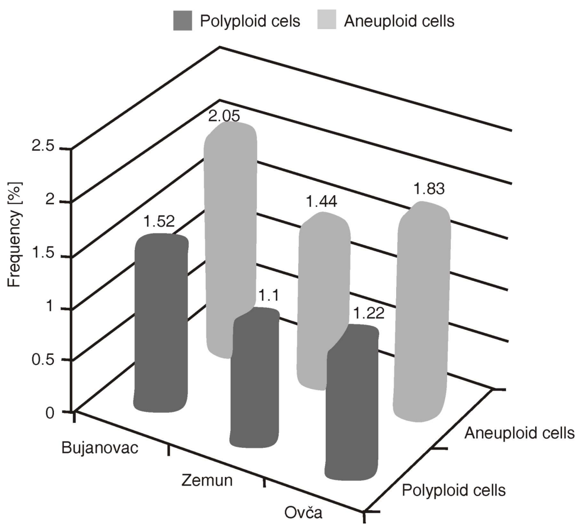

Figure 4. Number of polyploid and aneuploid cells (in percentage) in sheep from three different localities

sheep from Bujanovac and the sheep from the Zemun farm (fig. 2). However, there is no significant difference when it comes to comparing the values expressed in percentage for the gaps obtained in the sheep at these two localities. Statistically significant differences ($p < 0.05$) could be seen in case when mean values of chromatid breaks are compared between the sheep from Bujanovac and the sheep from the farm in Zemun (tab. 2). Statistically significant difference ($p < 0.05$) also appears when comparing the mean value for gaps obtained in sheep from these two sites (tab. 2).

It is possible that the somewhat lower frequency of changes, which was established in sheep from Zemun, was the result of those animal's breeding method, because the animals were not taken to pasture and were not given free access to food and water in the environment, unlike the sheep of the other two analyzed sites. So, these animals could have been significantly protected from the effects of numerous environmental factors.

As for the sheep coming from northern localities of Ovča, it turned out that most of the structural damages were also (fig. 3) gaps (0.56%), while chromatid breaks (0.23%) were more frequent than chromosomal breaks, *i. e.*, 0.18% (fig. 2).

Between northern and southern sites, Ovča and Bujanovac, there was no significant statistical difference when it comes to the values of frequency of any type of chromosomal changes, in case their values are expressed in percentages (figs. 2 and 3). However, considering the mean values, there was a certain difference between the values of chromatid breaks in the sheep from Bujanovac and chromatid breaks in the sheep from Ovča village (tab. 2). No statistically significant differences were obtained when the structural changes found in the sheep from Ovča were compared to the changes defined in the sheep from Zemun (tab. 2, figs. 2 and 3).

There were no statistically significant differences in the number of polyploid and aneuploid cells in ovine lymphocyte cultures of sheep from the different tested sites: Bujanovac, Zemun, and Ovča (tab. 2, fig. 4), which indicated a disturbance in regulation of cell division and a possible carcinogenic effect.

A relatively low frequency of breaks and gaps was established in the sheep at all of three localities. There were no statistically significant differences between the sites of Bujanovac and Ovča, or between Zemun and Ovča, regarding the comparison of presented percentages of detected chromosomal damages. As for the control cultures of sheep lymphocytes, the obtained values can be higher than those established in the sheep from Bujanovac [24, 25].

The values obtained from break and gap frequencies, as well as the possible occurrence of other re-arrangements in the controls of lymphocyte cultures, can be affected by the activity of different chemical agents that are more or less present in the environment, but also by the influence of internal factors inherent to specific genetic structures, which can be different in various species and breeds of animals. Some of them are: aging, inbreeding, and other related internal and environmental factors [1, 14] such as the presence of viral infections [26]. Thus, the basic level of changes can vary, both in the laboratory animals, and – even to a higher degree, in organisms from the environment [27, 28]. In our opinion, only higher values of changes than those detected in this work could clearly indicate the presence and action of genotoxic agents, or a specific agent, on the organisms in the natural environment, regardless of whether it is a chemical substance or radiation.

As for the sites exposed to war and DU contamination in South Serbia and Bosnia, except for some of the performed research dealing with humans [14, 15], no data of cytogenetic testing on animals were done.

According to the findings of the frequencies of breaks and gaps in the areas contaminated with DU in Bosnia [15], the frequency of chromatid and chromosome breaks that we defined in the sheep from Bujanovac, was similar to the incidence of these changes in the region of Sarajevo, which had not been directly exposed to DU contamination.

The given results do not show an increase in the number of polyploid and aneuploid mitosis in sheep lymphocyte cells. In addition no statistically significant differences were found in the process of comparing the values obtained in analyzing the animals from different localities; It is to be noted that some polyploid cells that can normally be seen in a lymphocyte cultures and aneuploid mitoses may also occur as a result of the method of chromosome preparation.

Some signs of oxidative stress and anemia, which were obvious in the analyses of the animals from the area of Bujanovac [16-20] and a slightly elevated frequency of chromosomal changes, such as breaks and gaps found in sheep, might have been caused by some different influences and by other genotoxic agents that are present in trace amounts in the environment.

In the vicinity of Bujanovac, where the sheep subjected to cytogenetic analysis were farmed, as a result of anthropogenic pollution, which is common in the environment, and where a combustion of fossil fuels, oil products, fertilizers, pesticides, *etc*. takes place, the elevated concentrations of heavy metals were found, especially Cd, Hg, and Pb, in both soil and feed [20]. The analysis showed that the content of Pb and Hg in the feed of animals that were subjected to cytogenetic analysis, although below the national permitted level was still high, while the content of Cd exceeded the permitted limit [20]. The content of Cd was highly elevated, most notably in wheat [20]. The use of phosphate fertilizers and burning of fossil fuels are cited as the possible cause of this phenomenon [20]. Apart from being highly reactive and toxic, it was confirmed that cadmium possesses genotoxic properties [29-31]. Fertilizers and smelting of zinc and lead can also enrich soil with cadmium. In addition to high contents of cadmium, some studies [32] revealed the increasing contamination by lead, zinc and arsenic in the southern regions of Serbia. The analyses performed in 2005 [20] showed that grass included the lead concentrations that were at least an order of magnitude greater than those of cadmium and mercury, but still below the permissible levels. Many researches also point out a genotoxic potential of mercury [29] and lead [29, 30, 33]. However, detoxification of lead by adult animals is more efficient and lead is less harmful for essential physiological functions than cadmium or mercury.

Considering the possible effects of depleted uranium, it has to be pointed out that the official report [34] regarding the level of radioactivity in Serbia stated that there were no elevated levels of natural and anthropogenic radionuclides in aerosols, soils, plants, food, food feed, drinking water, and rivers. The results of measured radioactivity in the exposed area indicate that although the region had been exposed to DU ammunition, it did not affect the soil and food significantly, mainly due to the fact that the contamination was limited to the target places or shells did not explode and therefore, possible contamination can only be related to the effect of corrosion [8, 34-36]. The study on the environmental radioactivity level in 2005 [37] showed no evidence of DU contamination, since the ratio of uranium isotopes confirmed its natural origins. However, the previous studies showed a higher content of Chernobyl originated cesium in vegetation than in other parts of the country [37]. Studies of the trace elements in the blood of domestic animals, cows and sheep in the region of Bujanovac, as well as the content of natural and anthropogenic radionuclides and some heavy metals in feed [20], also showed that the activities of natural radionuclides in feeds were within the average values reported for the region, while the activities of ^{210}Pb and $^{235/238}$U were below the limit of detection. This is in accordance with the previous researches revealing no widespread contamination by depleted uranium in the area of Bujanovac [35-37].

The results of cytogenetic analyses indicate that in spite of a slightly higher frequency of chromatid breaks in the sheep from Bujanovac, compared with the level of those changes in the sheep from other localities (fig. 2, tab. 2), this value was in the range of basic values that are usually observed in control cultures of sheep blood lymphocytes [24, 25].

Therefore, it cannot be confirmed that DU contributed to creation of these chromosomal damage identified. All other values of the frequency of structural changes in sheep that were obtained at Bujanovac, Zemun, and Ovča locations, were placed in the range of values normally found in control cultures of sheep lymphocytes [24, 25], *i. e.*, the values of basic changes, which vary considerably, both in humans [7] and in animals [27, 28].

It seems more likely that the variation of the frequency of base level changes is caused by different environmental impacts or their additive effect. In doing so, we should not neglect the above analysis of the soil and feed in the region of Bujanovac [20, 37] and identified elevated Cd content in feeds, high levels of Pb and Hg, although below the permissible limit, as well as other external and possible internal factors [1, 26].

The results presented neither the elevated levels of polyploid and aneuploid cells in the sheep of the region of Bujanovac, in comparison to other two analyzed localities – Zemun and Ovča, nor significant statistical differences between the values of these changes, the ones that might indicate the possible carcinogenic effect.

Thus, the results of cytogenetic analysis could not fully confirm that the low value of chromosomal changes (within the basic level of changes) in the sheep of Bujanovac area was caused by the presence and activity of depleted uranium. The mentioned results are much more consistent with the data obtained by researches [8, 20, 34-37] that revealed no sign of widespread contamination by depleted uranium in the mentioned area.

CONCLUSIONS

Chromosome structural changes that were the subject of the analysis carried out on sheep from the region of Bujanovac contaminated with DU during NATO air strikes in 1999, and on sheep belonging to other two analyzed control localities (Zemun and Ovča) revealed breaks of chromatid and chromosome type (izochromatid breaks) and gaps. Structural chromosome changes that result from complex chromosome rearrangements were not identified. The results of cytogenetic analysis, in spite of statistically significant differences ($p < 0.05$) that appear in comparison between the locality of Bujanovac and a northern locality of Zemun, show the frequency of breaks (and gaps) in the sheep of all examined localities within the range of basic level changes (normally occuring in the sheep control cultures). It is assumed that lower frequency of breaks (and gaps) observed in the sheep from Zemun farm was mainly due to the method of breeding, as these animals were less exposed to the agents always present in the environment which was not the case with the sheep from the households of Ovča and Bujanovac. The results did not show any elevated level of polyploid and aneuploid cells in the sheep from the region of Bujanovac in comparison to other two analyzed localities – Zemun and Ovča. In addition, there were no significant statistical differences between the values of these changes, the ones that might indicate potential carcinogenic effect. The value of the frequency of chromosomal breaks and gaps found in the sheep from Bujanovac was within the range of basic values, which are changeable, while the previous studies revealed that DU was not detected in the soil and feed for animals (the ones that were the subject of this cytogenetic analysis), but it was confirmed that the content of Cd was above the permitted level, and that the content of Pb and Hg was elevated, although within the permissible limits. The cytogenetic analysis of blood lymphocytes of the sheep from the region of Bujanovac did not show any increased frequency of chromosomal aberrations that might provide a more profound indication of the action and widespread presence of DU.

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AUTHOR CONTRIBUTIONS

Theoretical analysis and experiments were carried out and the manuscript was written by S. L. Fišter and S. Z. Jović.

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УЧЕСТАЛОСТ ХРОМОЗОМСКИХ АБЕРАЦИЈА КОД ОВАЦА СА ПОДРУЧЈА ЗАГАЂЕНОГ ОСИРОМАШЕНИМ УРАНИЈУМОМ ЗА ВРЕМЕ НАТО БОМБАРДОВАЊА 1999

У овом раду приказани су резултати цитогенетског истраживања на овцама са подручја Бујановца које је било загађено осиромашеним уранијумом (ДУ) за време НАТО бомбардовања 1999 године. Студија је спроведена на лимфоцитима крви оваца како би се утврдила учесталост хромозомских аберација и проценило присуство генетичког ризика као последица могућег деловања ДУ. Узорци крви за културу лимфоцита узети су по принципу случајног узорка од 20 животиња из домаћинства у селу Боровац у близини Бујановца, а животиње су одабране јер су вођене на пашу, храниле се и појиле на подручју НАТО бомбардовања. Ради поређења резултата, цитогенетски су анализиране две контролне групе од по 20 оваца са два северна локалитета: из Земуна и Овче, која нису била загађена ДУ. Установљене су структурне хромозомске промене типа гапа и прекида, а њихова учесталост код оваца са свих испитаних локалитета је била у оквиру основног нивоа вредности које се уобичајено налазе у контролама култура ломфоцита оваца. Статистички значајне разлике су се јавиле између вредности забележених код оваца из Бујановца у поређењу са вредностима добијеним код оваца једног северног локалитета (Земун), вероватно као последица самог начина гајења на фарми и њихове мање изложености утицају агенаса спољашње средине. Нису установљене повишене вредности полиплоидних и анеуплоидних ћелија, нити су се јавиле статистичке значајне разлике између локалитета. Према ранијим подацима, ДУ је био испод границе детекције примењене методе у земљишту и храни која је давана цитогенетски анализираним животињама. На основу ниског нивоа промена који је у опсегу нивоа основних промена које се уобичајено уочавају у контролним културама ломфоцита оваца, не може се са сигурношћу потврдити да је ДУ изазвао ове промене, нити да је широко распростањен у региону Бујановца.

Кључне речи: осиромашени уранијум, овца, хромозомска аберација, прекид и гап, НАТО напад 1999, Бујановац