RESEARCH ARTICLE

EFFECT OF LOW DOSE GAMMA IRRADIATION ON NUMBER AND RATIO OF SOME PERIPHERAL BLOOD CELLS IN HATCHED CHICKS

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ABSTRACT

The presented research was carried out to investigate hematological response to low dose gamma radiation in chickens hatched from irradiated eggs. A total of 700 Ross-308 eggs were divided into the experimental group (N=360) and control group (N=340). The experimental group was irradiated one hour before incubation with a cobalt-60 (^{60}Co) panoramic irradiator, and control group was sham irradiated. Blood samples for the red blood cell (RBC) and white blood cell (WBC) counts and differential blood counts were taken on the 1st, 3rd, 5th, 7th and 10th day of life. Blood cell counting was performed using the Natt and Herrick method. Leukocytes were differentiated using amicroscopic examination of stained blood smears and a heterophil:lymphocyte (H/L) ratio was calculated. Our results demonstrate a reduced RBC and WBC indicating negative effects of low-dose radiation on the blood cell counts in chicks during the first week after hatching. Significant decrease in the number of red blood cells on the 5th and 7th day and in the number of white blood cells on the1st and 3rd day, were obtained. Moreover, the number of lymphocytes in one-day-old chicken blood increased, while the number of heterophils decreased in response to radiation, suggesting better stress response in the exposed group. Further research is necessary to investigate the value of H/L ratio as a diagnostic indicator for radiation stress response and to evaluate cells and tissue reactions in specific time frames and across different taxonomic groups challenged by the low radiation dose. Extent of embryonic oxidative damage and recovery mechanisms should be also further investigated.

Keywords: Heterophil, lymphocyte, erythrocyte, chicken embryo, ionizing radiation

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INTRODUCTION

Ionizing radiation has been naturally present in the environment since Earth exists. The effects of ionizing radiation on living cells are proportional to the absorbed dose. Biological effects of ionizing radiation greatly depend on several factors, such as radiation type and dose, exposed species and tissue or age at exposure (Hall and Giaccia, 2019). As a matter of scientific evidence, the adverse health effects in humans and animals have not been clearly discerned in connection with low doses of radiation in the range below 0.5 Gy (ICRP, 2007). Moreover, the research reports and obtained results are fraught with the variations in response and systematic statistical uncertainties (Williams et al., 2010; Calabrese, 2017). Having in mind a growing exposure to ionizing radiation originating from man-made sources used in medicine and industry, it is justifiable to investigate the effect of low dose ionizing radiation on different organisms and age at exposure.

Møller and Mousseau (2015) reported significant interspecific variation between birds negatively affected by radiation in Fukushima. According to authors, species differ in their susceptibility to radiation due to differences in their ability to sustain toxic and genetic effects caused by radiation, which can be linked to an increased level of free radicals and different mechanisms to cope with oxidative stress (Riley, 1994). As a matter of fact, oxidative damage is widely investigated as a link between exposure to ionizing radiation and detrimental effects on organisms, as well as one possible mechanism associated with variation in species' responses to low dose ionizing radiation (Einor et al., 2016). Special attention is paid to radiation effects on embryos because the sensitivity and response to ionizing radiation were found to differ during the early developmental stages, such as preimplantation or organogenesis, and depend upon a dose and developmental stage (Honjo and Ichinohe, 2020).

Beyond this, it has been hypothesized that low levels of ionizing radiation are actually beneficial and can protect against disease or improve physiological processes in exposed animals (Kojima et al., 2000; Arenas et al., 2006; Calabrese et al., 2015; Calabrese et al., 2017). Radiation hormesis, as a scientific phenomenon seen in many laboratory studies (reviewed by Siegel et al., 2018), pointed out on reparative cellular processes that may result in the activation of a number of repair mechanisms of which some have not been elucidated to date (Lampe et al., 2017). Aurengo et al. (2005) described defense mechanisms (activation of antioxidant defense and DNA repair mechanisms, cellular signaling between neighboring or distant cells, arrest of damaged cells before entering the mitosis or programmed cell death if the damage cannot be repaired) as extremely effective in the dose range up to 0.5 Gy and for the dose range to which living beings are naturally exposed (1-20 mSv/year).

Exposure to ionizing radiation is known to have lethal effects in blood cells. While red blood cells (RBCs) are relatively resistant to low doses of radiation, white blood cells (WBCs) respond more often because they are among the most radiosensitive cells in the body and are considered a sensitive indicator of stress (Clark et al., 2009; Ermakov et al., 2009). It is found that a dose as low as 0.3 Gy leads to a reduction in the number of lymphocytes, and larger doses can alter the number of all blood cells (Hall and Giaccia, 2019). The use of heterophil:lymphocyte ratio (H/L) to assess stress across taxa was reviewed by Davis et al. (2008). It is found that social stress, chilling, fasting, bacterial infection or the addition of corticosterone to the diet can affect the H/L ratio (Gross and Siegel, 1983; Gross, 1989).

Hematopoietic system is, however, one of most radiosensitive systems (Smirnova, 2017). Regarding prenatal exposure, although hematopoietic stem cells (HSCs) respond to exposure to gamma radiation reflecting large heterogeneities in responses related to radiation type, dose, and time of exposure, it is known that the hematopoietic stem cells are particularly radiosensitive in comparison with the other fetal cells (Valentin, 2003; UNSCEAR, 2013). However, the transit period from stem cell through multiplication, maturation, and differentiation to fully functioning cell, differs for the various circulatory blood elements, and these differences account for the complex changes in blood count seen after irradiation. The findings obtained by Guo et al. (2015) indicate that continuous exposure to ionizing radiation to a dose range of 0.002 to 0.25 Gy for one month, induces acute and residual injury in hematopoietic stem cells in mice. Detrimental effects on the bone marrow and hematopoietic stem cell were observed within two hours (acute phase) and three months (chronic phase) after the last radiation. The results also revealed that the damage was dose-related, and that low dose exposure might have resulted in different effects on different tissues.

Much of our understanding of the blood cells' response to low dose gamma radiation is currently based upon the assumption that the dose-response curve has no threshold and is linear in the low dose region. However, the question of whether dose response curve linearity exists below 0.5 Gy, remains open (Siegel et al., 2019). Although it seems that low dose radiation stress response plays an important part in determining outcome after exposure (Mikkelsen and Wardman, 2003), mechanistic understanding of the processes associated with exposure to low dose radiation remains uncertain. Moreover, it remains largely unclear why species differ in their resistance to radiation and what effects arise from specific doses. Concerning the subject matter, in this research we aimed to investigate the effect of low dose radiation on the red and white cell count, as well as the effect on WBC differential and the heterophil:lymphocyte ratio in the blood of chickens exposed before incubation.

MATERIALS AND METHODS

Approval of the Ethics Committee

Approval for conducting the experimental study was issued by the Ethics Committee of the Veterinary Faculty, University of Zagreb, under number: 251-61-01/139-15-19.

Eggs, irradiation and dosimetry

The study was performed using 700 normally developing embryos of chicken meat type line Ross-308. Eggs were divided into the radiation group (N=360) and control group (N=340). Eggs in the radiation group were exposed to 0.3 Gy gamma radiation one hour before incubation, and control group was sham irradiated. Eggs were irradiated at the Ruđer Bošković Institute, Zagreb, Croatia with panoramic cobalt-60 (^{60}Co) source (activity about 3 PBq). The dose rate was about 23.84 mGy/s, and a source axis-to-egg axis distance was 291 cm. Dosimetric measurements were performed with an ionization chamber type 2581 and a Farmer Dosimeter type 2570 (NE Technology Limited). The dose is specified as absorbed dose to water (measured free in air). After irradiation eggs were transferred to the automatic egg incubator Victoria (Pavia, Italy) at the "Valipile d.o.o.", Sesvetski Kraljevac, Croatia. Temperature and humidity were controlled during the whole study. On the 19th day of incubation eggs

were transferred to hatching trays located in the same commercial incubator. Newly hatched chicks of both sexes were transferred to the Veterinary Faculty University of Zagreb, Croatia where they were held till the end of experiment. Environmental conditions were maintained at recommended values. Air temperature at placement was 32-35°C then decreased by 2-3 °C per week till 30 days of age when temperature reached and then remained at 20 °C. Relative humidity levels in the first 3 days were approximately 60-65% and around 40-50% for the remainder period. The lighting program up to 7 days of age provided 23 hours light and 1 hour dark, after which light was gradually reduced with at least 4 hours of darkness given till the end of experiment. Water and commercial feed were offered *ad libitum*, according to age requirements.

Sampling and blood cell count

For the purpose of analyzing the blood cells count, 10 chickens were randomized from each group and samples were taken by jugular vein venipuncture into the heparin tubes. Blood tests were performed on the 1st, 3rd, 5th, 7th, and 10th day after hatching. The counts of RBC and WBC were determined in the whole blood immediately after sampling. Total blood cell count was performed manually with a Neubauer hemocytometer (Hadžimusić et al., 2010; Weiss and Wardrop, 2010), while the differential WBC count was performed using the air-dried blood smears stained with May-Grünwald-Giemsa stains and examined under microscope (Hadžimusić et al., 2020). A minimum of 100 leukocytes per slide were sorted into categories: lymphocytes, monocytes, heterophils, basophils, or eosinophils. After the differential, the absolute white blood cell numbers were calculated, as well as the heterophil:lymphocyte ratios obtained by division of the heterophils by the sum of lymphocytes.

Statistical analysis

Statistical analysis was performed using SAS 9.4 software (Statistical Analysis Software 2002-2012 by SAS Institute Inc., Cary, USA). Normality was checked using the Shapiro-Wilk test. Since different animals were tested per day, independent testing of samples between the control and experimental group was performed using the Student's t-test for each sampling day separately. Results are shown as mean values and 95% confidence intervals, using $p<0.05$ as the statistical significance level.

RESULTS

All results are presented in Table 1. As shown in Table 1, the lower red blood cell counts were observed in young chickens in response to radiation. The total number of erythrocytes in the irradiated group was significantly lower ($P<0.05$) compared to the control group on the 5th and 7th day of chick life. Moreover, significantly lower white blood cell counts were found in the experimental group on the 1st and 3rd day of chickens' life. Relative count of heterophil leukocytes and lymphocytes showed significant differences only in one-day-old chicks where the exposed group had a significantly lower relative count of heterophils and a significantly higher relative count of lymphocytes in the total number of leukocytes. During the whole study, monocytes, eosinophils and basophils were not detected or were in a very low percentage, without any significant differences. The heterophil:lymphocyte ratios were significantly different between groups only on the first day of chickens' life where the value in exposed group was significantly lower compared to group of sham irradiated animals.

Table 1 RBC ($\times 10^{12}$/L),WBC (x 10^9/L), relative (%) count of heterophil leukocytes and lymphocyte and heterophile:lymphocyte ratio in blood of chickens hatched from irradiated (Experimental group) and sham irradiated (Control group) eggs

		Mean and 95% Confidence interval (in parentheses)				
		AGE (days)				
Parameter		1	3	5	7	10
RBC (x 10^{12}/L)	C	1.07 (0.98-1.16)	0.92 (0.83-1.01)	0.95 (0.86-1.04)	0.99 (0.90-1.08)	1.01 (0.92-1.10)
	E	0.98 (0.89-1.07)	0.83 (0.74-0.92)	0.82* (0.73-0.91)	0.82* (0.74-0.91)	0.89 (0.80-0.98)
WBC (x 10^9/L)	C	6.44 (5.70-7.17)	5.29 (4.55-6.02)	5.33 (4.59-6.06)	6.22 (5.48-6.95)	6.74 (6.00-7.47)
	E	5.19* (4.45-5.92)	3.98* (3.24-4.71)	5.11 (4.37-5.84)	5.55 (4.81-6.28)	6.27 (5.53-7.00)
Lymphocyte (%)	C	29.70 (22.28-37.11)	33.10 (25.68-40.51)	56.90 (49.48-64.31)	84.00 (76.58-91.41)	77.20 (69.78-84.61)
	E	40.30* (32.88-47.71)	33.50 (26.08-40.91)	58.00 (50.58-65.41)	77.30 (69.88-84.71)	77.50 (70.08-84.91)
Heterophil (%)	C	70.30 (63.08-77.90)	66.00 (58.80-73.61)	42.60 (35.51-50.32)	15.50 (8.97-23.79)	22.30 (15.50-30.32)
	E	59.70* (52.52-67.33)	65.60 (58.40-73.21)	40.60 (33.53-48.34)	22.60 (15.80-30.61)	21.80 (15.02-29.83)
H/L	C	3.45 (2.81-4.24)	2.39 (1.77-3.22)	0.89 (0.40-1.98)	0.18 (0.004-8.41)	0.46 (0.10-2.15)
	E	1.78* (1.19-2.66)	2.59 (1.96-3.41)	0.77 (0.30-1.94)	0.30 (0.02-3.16)	0.30 (0.02-3.23)

Values marked with * are significantly different ($P<0.05$) between groups
C – control group; E – experimental group; H/L - heterophil:lymphocyte ratio

DISCUSSION AND CONCLUSION

The total numbers of blood cells and the relative proportions of each type vary considerably among species and breeds (Wirth-Dzięciołowska et al., 2008; Weiss and Wardrop, 2010). Organisms also differ in their susceptibility to ionizing radiation, although the ecological basis for such differences remain poorly understood (Moller and Mousseau, 2007; Møller et al., 2015). Embryonic and fetal hematopoiesis has been studied over the last few decades (Edmonds, 1964; Edmonds, 1966; Ceresa-Castellani and Leone, 1969; Small and Davies, 1972.), but still there are no clear answers on how low doses gamma radiation affect the developing hematopoietic system in birds.

The standard automated cell counting is not an acceptable method for blood analysis in birds due to the specific morphological structure of avian blood cells, i.e., nucleated red blood cells and platelets. Despite major disadvantages and limitations, the manual blood cell counting technique is still the best available method of avian blood analysis (Carisch et al., 2019). Several studies confirmed large variations in the manual blood counts; reported coefficients of variation are between 20 and 40% (Har et al., 2005; Weiss and Wardrop, 2010). A disadvantage of Natt-Herrick solution is the difficulty to differentiate thrombocytes from lymphocytes, thus creating significant counting errors (Carisch et al., 2019). In order to improve the accuracy of cell counting, several methods were used, namely, an experienced operator, same brand of properly cleaned instrumentation and material, uniform distribution of cells in the observation area, more fields and cells counted and obtaining the results from at least 3 repetitions (Walberg, 2001).

In our research, the hematologic response to low dose radiation was significantly different in groups of exposed chickens in the first week of their life. More research is needed to fully understand ionizing radiation damage and repair capacity of fertilized hen egg, and moreover, to investigate low doses effects on the bone marrow capacity to maintain hematopoiesis. Because of insufficient scientific publications and research data in the context of low dose effects in poultry, we compared our findings to those found in the available studies in vertebrates. Our results are, in that regard, in accordance with the research on dogs carried by Nold et al. (1987). They prenatally exposed dogs to 1.5 Gy gamma radiation and found a transient reduction of peripheral blood leukocytes up to 24 weeks of age due to the bone marrow damage. Similar marrow damage was observed in the postnatally exposed dogs, accompanied by a significant decrease in peripheral white and red blood cell counts. However, their bone marrow partially recovered, same as hematologic parameters by 24 weeks of age. Compared to the prenatally exposed dogs where the radiation effects were persistent, observed was a relatively greater sensitivity of the fetal marrow as compared to the neonatal bone marrow. As the research has demonstrated, prenatal exposure to ionizing radiation has a strong effect on a hematopoietic stem cell, while circulating mature cells are less sensitive to ionizing radiation. These results are in line with a previous finding of the radiosensitive blood precursor cells and consequently impaired erythropoiesis and migration of the RBC to the bloodstream (Dainiak et al., 2003).

Research of Lucas and Denington (1957), where erythrocytes were not significantly changed in the groups of chickens exposed to doses from 0.5 Gy to 1 Gy, was in contrast to our research, where we found significantly decreased number of erythrocytes on the 5th and 7th day of chick's life. However, in their research, exposed animals

were 6-12 weeks old chicks, thus the observed differences between our results most likely resulted from an age-dependent response in the embryo as compared to adults. On the other hand, blood test results in rats exposed to several doses ranged from 0.1 Gy to 1 Gy obtained three hours, 24 hours, 48 hours, and seven days after irradiation showed that doses up to 0.5 Gy increased the mean number of erythrocytes in the blood, while doses above 0.5 Gy caused a sharp decrease in erythrocyte counts (El-Shanshoury et al, 2016). Moreover, the above-mentioned authors found that the erythrocyte recovery rate also increased with an increase in ionizing radiation for doses up to 0.5 Gy, while higher doses reduced recovery capacity of the red blood cell in exposed rats. Comparing to our results where a significant decrease in red blood cells count was observed at dose as low as 0.3 Gy, we can conclude that even low dose of ionizing radiation can be considered a potential health risk, but different thresholds in different species/age suggesting the need for more research.

As Rodrigues-Moreira et al. (2017) recently described, low-dose irradiation induced long-term oxidative stress and decreased self-renewal capacity in hematopoietic stem cells, thus our results may be due to the oxidation sensitivity of red blood cells. It is known that erythrocyte membranes are rich in polyunsaturated fatty acids and continuously exposed to high concentrations of oxygen (Clemens and Waller, 1987). Early theories on the mechanisms of action of ionizing radiation on erythrocytes assumed an electrolyte imbalance (Kollmann et al., 1969), but today it is known that free radicals can damage erythrocyte membranes and cytoskeleton affecting cell viability (Leyko and Bartosz, 1985).

Persistent oxidative stress, as the late effect of low-dose irradiation on blood cells, may also explain leukocyte response in our research. We found a significantly lower number of leukocytes on the 1st and 3rd day of chick's life, compared to the control group. These results are in line with observations from many studies that confirm negative response of WBC profile to different stressors (Gross, 1990; Shini et al., 2008). In the research carried by Lin et al. (1996), significant decreases in the total white blood cells were observed for weeks in mice exposed to 0.05 Gy, 0.5 Gy, and 1 Gy. An attempt to explain the blood changes resulting from both irradiation and time is given by El-Shanshoury et al. (2016), who exposed rats to several doses of ionizing radiation ranged between 0.3 and 1 Gy and investigated the effects over few weeks. Their results are in accordance with our results, as we both found a significant reduction in WBC counts first week after exposure, which they confirmed at all dose levels compared to the control group.

However, our results for the heterophils: lymphocytes ratio were inconsistent with several studies where the number of lymphocytes in chicken blood samples decreased and the number of heterophils increased in response to stressors (Gross and Siegel, 1983; Gross, 1990; Shini et al., 2008). Our results showed just the opposite result in one-day-old chicks, and furthermore, the H/L value was closer to reference value 1 (Milinković Tur and Aladrović, 2012) suggesting the lower stress response in chicks newly hatched from eggs exposed to 0.3 Gy.

Possible explanation may be the fact that leukocyte response can be distinctive for different stressors (Maxwell, 1993), or due to considerable interspecific variation among breeds in susceptibility to radiation effects (Møller and Mousseau, 2015). However, one possible explanation for our result is also the adaptive response described as the ability of a cell, tissue, or organism to better resist stress damage by prior exposure to a lesser amount of stress (Crawford

and Davies, 1994). Even adaptive response is observed in all organisms in response to a number of different cytotoxic agents, the molecular processes that take place in fertilized egg in the embryo's early stage are yet to be investigated. However, increased toxic activity of heterophils, activation of the immune system and a better response to oxidative stress are already proposed as possible underlying mechanisms of radiation-induced adaptive response (Ermakov et al., 2009). It is in accordance with our previous studies where 0.3 Gy low dose of gamma radiation increased humoral immunity and some antioxidant enzymes in chickens hatched from eggs irradiated before incubation and on the 19th day of incubation (Vilić et al., 2009; Vilić et al., 2010).

Summing up all arguments and results, our research provided information on the erythrocyte and leukocyte response in chicks irradiated *in ovo* to assess the low dose radiation effects on blood cells in commercial poultry breed. Our result indicates negative effects of low dose radiation on the blood cell counts in chicks during the first week after hatching. Significant decrease in the number of red blood cells on the 5th and 7th day, and the number of white blood cells on the 1st and 3rd day were obtained. Moreover, the number of lymphocytes in one-day-old chicken blood increased, while the number of heterophils decreased in response to radiation. Consequently, the H/L value was closer to the reference value, suggesting better stress response in the exposed group. As both, hormonal profile and H/L ratio can be used as a method of stress assessment, it could be useful investigating them together with leukocytes profiles to evaluate the value of H/L as a diagnostic indicator for radiation stress response. Extent of embryonic oxidative damage and potential recovery mechanisms should be further investigated, as well as leukocyte response in relation to radiation dose and a developmental stage of exposed animals.

CONFLICTS OF INTEREST

Authors declare no conflict of interest.

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UČINAK NISKE DOZE GAMA ZRAČENJA NA BROJ I OMJER POJEDINIH ĆELIJA PERIFERNE KRVI KOD IZLEGLIH PILIĆA

SAŽETAK

Predstavljeno istraživanje je izvedeno sa ciljem ispitivanja hematološkog odgovora na nisku dozu gama zračenja kod pilića izleglih iz ozračenih jaja. Ukupno 700 Ross-308 jaja je podijeljeno u eksperimentalnu (N=360) i kontrolnu (N=340) grupu. Jaja iz eksperimentalne grupe su ozračena jedan sat prije inkubacije sa kobalt-60 (^{60}Co) panoramskim izvorom zračenja, dok kontrolna grupa nije ozračena. Uzorci krvi na crvene (RBC) i bijele (WBC) krvne ćelije i diferencijalnu krvnu sliku su uzeti 1, 3, 5, 7. i 10. dan života. Za brojanje krvnih ćelija je korištena metoda po Nattu i Herricku. Leukociti su diferencirani korištenjem mikroskopskog pregleda obojenih krvnih razmaza pri čemu je izračunat omjer heterofila i limfocita (H/L). Dobiveni rezultati pokazuju sniženi broj RBC i WBC kao negativan učinak djelovanja niske doze zračenja na broj krvnih ćelija kod pilića u prvoj sedmici nakon izlijeganja. Uočeno je znatno smanjenje broja eritrocita 5. i 7. dana i leukocita 1. i 3. dana. Broj limfocita kod pilića starih jedan dan je povišen, dok je broj heterofila snižen kao odgovor na zračenje što ukazuje na bolji odgovor na stress u ozračenoj grupi. Neophodno je vršiti daljnja istraživanja H/L omjera kao dijagnostičkog indikatora odgovora na stres zračenjem, kao i u cilju evaluacije reakcija ćelija i tkiva na nisku dozu zračenja unutar određenih vremenskih perioda i različitih taksonomskih grupa. Obime oksidativnog oštećenja embriona i mehanizme oporavka je potrebno dalje istražiti.

Ključne riječi: Heterofil, limfocit, eritrocit, pileći embrio, jonizirajuće zračenje