The Impact of Background γ- Radiation on Erythrocyte Nuclear Pathology, The Serotonergic System, and Cytochrome P-450 in Hens (Gallus Gallus Domesticus) from Azerbaijan

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Research Article

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Abstract

High levels of background γ-radiation exist in the suburbs of Baku, Azerbaijan. We examined the impact of radiation on erythrocyte nuclear pathologies, levels of cytochrome P-450, and serotonin-modulating anticonsolidation protein (SMAP) in the tissues of the hens from three settlements with different levels of background radiation. Higher levels of radiation resulted in increased nuclear pathologies, upregulation of tissue SMAP levels, and downregulation of cytochrome P-450.We also carried out controlled dosage studies on Wistar male rats which showed significant upregulation of heat shock proteins with molecular mass 70 kDa (HSP70) in the bone marrow 3 and 5 h later of SMAP intraperitoneal administration. Administration of SMAP to rats 3 h prior to γ-radiation exposure (8 Gy) provided significant protection to somatic cell nuclei. We conclude that SMAP can provide protection from the genotoxic effects of γ-radiation through upregulation of HSP70 or the transformation of chromatin into a condensed, more protective conformational state.

Introduction

The intense development of nuclear energy and the large-scale application of sources of ionizing irradiation in different areas of industry and medicine has resulted in an increase of background radiation in the environment. Furthermore, humans working or travelling in space are exposed to strong γ-irradiation. For these reasons, there is a growing need to understand the effect of ionizing irradiation on living organisms. Studies of the effects of ionizing irradiation on animals have shown damaging effects on the genetic apparatus of cells, such as an increase in frequency of micronuclei and chromosome aberrations (Yagunov et al. 1998). Along with this, the concept of radiation adaptation states that low doses of ionizing irradiation stimulates the activation of radioprotective repair mechanisms that are capable of creating nonspecific resistance towards different stressors including the facilitation of radio resistance (Kauffman 2003).

The above reasons provide an impetus to carry out studies aimed at understanding environmental levels of radiation and their effects on the genetic and physiological systems of free-living organisms. We measured environmental radiation and correlated it with genotoxic effects in hens as inferred from the amount of nuclear pathologies in erythrocytes. We also evaluated physiological effects using cytochrome P-450 and serotonin-modulating anticonsolidation protein (SMAP; Mekhtiev 2000). The cytochrome P-450 system is involved in the oxidation and metabolism of chemical pollutants and recently an indirect role in mediating their biological effects was shown (Oliva et al. 2012). SMAP is a part of the serotonergic system which is directly involved in regulation of adaptive processes that help preventing the adverse effects of oxidative damage (Mustafayev and Mekhtiev 2013, Mustafayev and Mekhtiev 2014, Mekhtiev et al. 2017).

Materials And Methods

The first series of experiments were carried out on domestic hens (*Gallus gallus domesticus*), taken from peoples personal hen houses and exposed to ionizing radiation *in situ*. Specimens were taken from three administrative districts of Baku: Binagady settlement (n=7), Romany settlement (n=7) and Ganly Gol zone (n=6). γ-radiation levels were measured with a “Canberra InSpector 1000 MCA Radiation Isotope Analyzer” (Canberra, USA) spectrometer by touching three different points of soil with the detector and averaging the values. In each settlement, such measurements were taken at other points along parallel lines, 10 m apart from each other. Finally, all measurements were averaged for each studied settlement.

The animals were sacrificed and levels of SMAP and cytochrome P-450 were evaluated in the liver and kidneys of the hens using the indirect solid phase ELISA-test on polysterene plates with moderate adsorption (Sigma, Germany) with the application of specific antibodies (Antibodies Volume II: A Practical Approach, 1989). Protein extracts of liver and kidney, leveled to a concentration 20 μg/ml with Tris-HCl buffer (pH 8.6), were used as antigens. Rabbit polyclonal immunoglobulins to cytochrome P-450 and SMAP were used as the first antibodies, while goat immunoglobulins to rabbit immunoglobulins, conjugated to horseradish peroxidase, were used as the second antibodies. As a substrate for horseradish peroxidase, orthophenilendiamine at a concentration 0.05% in 0.05 M citrate-phosphate buffer (pH 4.5) was used. The reaction was stopped by adding 50 μL of 3 M solution of NaOH into each wells and the results were measured in photometer for ELISA-test “Molecular Devices Spectra Max 250” (MTX Lab Systems, Inc., USA) at wavelength 492 nm in units of optical extinction.

A blood sample was obtained from each hen during the sacrificing process; thin smears were made on histological glass slides, dried and stained by the Romanovsky-Giemsa technique (Sigma, Germany). Nuclear pathologies were counted using a light microscope at X100 magnification in 1000 erythrocytes of each animal.

The second series the studies was carried out on male Wistar rats with body mass 170-200 g. The animals were separated into 2 groups: 1) control group (n=5) animals were administered intraperitoneally with inactive SMAP (inactivated at 35 min in a 60□C water bath) at a dose of 1 mg per 100 g of animal mass; 2) experimental group (n=5) animals were administered with the same dose of SMAP. The animals were sacrificed at 1.5, 3 and 5 h after injection and bone marrow samples were isolated from the femur. Proteins were extracted and western-blotting was carried out with the application of rabbit polyclonal antibodies to heat shock proteins with molecular mass 70 kDa (HSP70). Immune interactions were visualized through exposure of the nitrocellulose membranes with transferred proteins to fluorochrome dyes with following applying photosensitive plates onto the nitrocellulose membranes and further their developing and fixing.

The third series of experiments was carried out on male Wistar rats with body mass 180-210 g. The animals were separated into 3 groups: 1) intact group (n=7); 2) control group (n=7) – the animals were administered with inactive SMAP at a dose 1 mg per 100 g of body mass and 3 h later animals were subjected to γ-radiation at a dose of 8 Gy; 3) experimental group – the animals were administered with same dose of SMAP and 3 h later they were subjected to γ-radiation at a dose of 8 Gy. Four days after

exposure the animals were sacrificed, bone marrow was isolated from femurs, and nuclear pathologies per 1000 immature erythrocytes were scored as described above.

Results obtained from the different series of studies were grouped and inter-group differences were calculated using Student's t-criterion (Rohlf & Sokal 1995).

Anti-SMAP polyclonal immunoglobulins were produced through 5-month immunization of rabbits with purified SMAP at a dose of 300 μg always in mixture with Freund complete adjuvant in 5-7 points on the back, subcutaneously. First three injections were done within timeframe of 14 days, the following injections were done one per month. Ten days later of 3rd and following injections blood samples were taken from ear vein, serum was separated and polyclonal immunoglobulins G were precipitated by adding 100% ammonium sulfate.

Results

The highest level of background γ-radiation was found in the settlement of Romany – 65 μR/h. In the settlement of Binagady, values of background γ-radiation were relatively lower – 30 μR/h. The lowest level of background γ-radiation was observed in Ganly Gel zone (control zone) – 5 μR/h.

In the first series of studies, the results of genotoxic analysis of erythrocytes showed that in the hens from the control zone of Ganly Gel, the number of nuclear pathologies was 6.5±0.6 per 1000 cells. In the hens from the settlements of Binagady and Romany, the levels were 249.7±5.3 per 1000 cells ($p<0.001$) and 450.3±6.5 per 1000 cells ($p<0.001$), respectively (Fig. 1). Most of the pathologically changed nuclei of the erythrocytes had cigar-like shapes.

The results of biochemical monitoring of the three administrative districts of Baku city indicate that elevation of background radiation resulted in the upregulation of SMAP levels in the tissues of hens. In particular, levels of SMAP in the liver (0.149±0.002) and kidney (0.149±0.002) of the animals from Romany significantly exceeded similar indices of hens from the control zone Ganly Gel (0.092±0.006, $p<0.001$ and 0.110±0.001, $p<0.001$, respectively; Fig. 2). Along with this, the levels of SMAP observed in the liver and kidney from hens living in Binagady, which had an intermediate level of radiation, were also intermediate in SMAP elevation compared to the control group (0.107±0.003 and 0.133±0.004, $p<0.001$, correspondently; Fig. 2).

The same pattern was observed with the downregulation of cytochrome P-450. Observed levels of cytochrome P-450 in the liver and kidney of the hens from Romany were 0.068±0.001 and 0.065±0.002, respectively. These values were significantly lower than those observed in hens from the control zone of Ganly Gel (0.097±0.009, $p<0.05$ and 0.1±0.007, $p<0.01$, respectively; Fig. 3). In Binagady the levels of cytochrome P-450 in the liver and kidney of the hens reached 0.073±0.003 ($p<0.05$) and 0.140±0.012 ($p<0.05$), correspondently (Fig. 3).

The second series of studies employed three exposure times (1.5, 3 and 5 h) between the intraperitoneal administration of SMAP and bone marrow sampling to determining HSP70 levels in the experimental group of rats. The highest observed level of upregulation of HSP70 was observed at 3 and 5 h exposure (Fig. 4). Western-blot analysis did not reveal the presence of HSP70 in the liver samples taken from the control animals.

In the third series of studies, control rats were given inactive SMAP, and exposed to γ-radiation at a dose of 8 Gy. We observed a sharp (20 fold) increase in nuclear pathologies – 55.3±3.2 per 1000 immature erythrocytes – relatively to their level in the control animals that received no radiation exposure – 2.6±0.4 per 1000 cells ($p<0.001$, Fig. 5). The observed pathologically changed nuclei of the erythrocytes mostly had cigar-shaped or bean-shaped configurations. On the other hand, in rats that received an administration of SMAP prior to γ-radiation exposure, nuclear pathologies were reduced by 41% in comparison to the values of the group receiving inactivated SMAP (32.8±1.9 and 55.3±3.2 per 1000 cells, correspondently, $p<0.001$, Fig. 5). Administration of inactive and active SMAP to the animals was done 3 h prior to γ-irradiation exposure, based on the results of the second series of studies indicating that the highest upregulation of HSP70 occurred within this timeframe.

Discussion

As demonstrated previously, biochemical studies on rats (Mekhtiev 2000) and electrophysiological studies on the identified command neurons of snails (Mekhtiev et al. 2004) showed SMAP has a linear relationship with serotonin. This was identified first in the rat brain cortex and thereafter purified from the whole brain (Mekhtiev 2000).

Mass-spectroscopy analysis of SMAP revealed it to be composed of three proteins – dihydropyrimidinase-related protein 2 (DRP2; its other name – collapsin response mediator protein 2, CRMP2; Nakamura et al. 2020), actin and tubulin, which are bound tightly to each other by calcium-mediated bonds (Garina et al. 2018). The calcium nature of these bonds was shown by their sensitivity to the effects of 40 mM EDTA, which causes disruption and splits SMAP into the component proteins (Mekhtiev, *unpublished data*). The existence of such strong bonds between these proteins clarifies the observed behavior of SMAP as a single protein in protein fractionation by gel-chromatography (it is eluted as a single peak from the column Sephadex G-150), electrophoresis under non-denaturating conditions and western-blotting. As actin and tubulin are structural proteins of the cells, they, apparently, do not have regulatory activity and the observed nucleus-protective activity is, obviously, realized solely by DRP2.

On a whole, the results show upregulation of SMAP level in the tissues of the hens living in the districts with high levels of background γ-radiation along with a significant increase of nuclear pathologies in their erythrocytes and significant downregulation of cytochrome P-450 in their tissues. Furthermore, administration of SMAP to rats prior to their exposure to high doses of γ-radiation caused a significant decrease in the number of nuclear pathologies in their immature erythrocytes.

The results of controlled experiments carried out on rats, demonstrate that induced upregulation of SMAP through its administration to the animals provides significant protection of the nuclear apparatus of somatic cells from the damaging impact of high doses of γ-irradiation. These results are consistent with our earlier data showing the anti-mutagenic activity of SMAP. In those studies, administration of SMAP to fish prior to exposure to high levels of polyaromatic hydrocarbons and heavy metals decreased the level of mutagenic effects (micronucleus analysis; Schmid 1975) in somatic cells by 50% relatively to their levels in control animals (Mekhtiev & Movsum-zadeh 2008).

The above conclusion explains the significant upregulation of SMAP observed in the liver and kidney of the hens from settlements Binagady and Romany. SMAP provides protection for the animals to the high levels of γ-radiation background. It should be noted that despite the protective levels of SMAP, the high levels of γ-radiation at these settlements exerted strong adverse effects on the animals in the sense of sharp increases of nuclear pathologies in the erythrocytes and significant changes of the level of cytochrome P-450 in their tissues.

Earlier studies, carried out on the fish *Alburnoides bipunctatus eichwaldi*, a sedentary fish that lives in the rivers flowing through the territory of Azerbaijan, showed that a significant upregulation of SMAP in the tissues, accompanied by downregulation of cytochrome P-450, enhances adaptation of the animals to the impact of the toxin phenol. The concentration of phenol in the river water exceeded by 3 times the maximum permissible concentration (MPC). However, a higher concentration of phenol that exceeded the MPC by 4-fold resulted in a noticeable downregulation of SMAP in the fish tissues. This was likely due to the exhaustion of the animal's adaptation potential (Mustafayev & Mekhtiev 2014). Based on these earlier studies, we conclude that the observed upregulation of SMAP in the tissues of the hens, living in settlements Binagady and Romany, reflects their adaptation to high doses of γ-radiation and, finally, their survival.

Our earlier studies, carried out on the animals of different species, including mice, rats and rabbits, showed that administration of SMAP significantly upregulates the level of HSP70 in their tissues (Ismailova & Mekhtiev 2018; Allahverdiyeva et al. 2019). HSP70 belongs to a group of chaperon proteins that are engaged in recovering the structure of the proteins damaged and denatured by the impact of various toxins (Sharp 1999, Daugaard et al. 2007). The results of the second series of the present studies and the data of other researchers show that 3- and 5-hour exposure times induce upregulation of HSP70 after SMAP administration or after exposure to heat shock (Wang et al. 2003). For this reason, in the third series of experiments the rats were subjected to the damaging effects of high dose of γ-radiation exactly 3 h after SMAP administration. These data provide an understanding for the possible molecular mechanisms of the protective activity of SMAP through upregulation of HSP70 which helps to alleviate the impact of γ-radiation and, consequently, decrease the level of nuclear pathologies of immature erythrocytes. At the same time, it indicates that SMAP provides for the adaptation and survival of the hens of settlements Binagady and Romany through the upregulation of HSP70 in the face of high levels of background γ-radiation.

Additionally, the results of our study and previous work allow us to hypothesize the existence of an alternative mechanism whereby SMAP protects cellular nuclei. Perhaps SMAP induces conformational changes to chromatin structure in which a condensed, and consequently more protective state, reduces the impact of radiation and prevents the formation of pathological changes to cellular nuclei. The possibility of such a protective mechanism is indicated, on one hand, by our earlier studies on the anti-mutagenic activity of SMAP in the model of pollutants-induced mutagenesis in the erythrocytes of fish (Mekhtiev and Movsum-zadeh 2008), and, on the other hand, by the evidence of a sharp increase of mutations in decondensed chromatin of somatic cells while passing through the phase of transcriptional activity (Jinks-Robertson 2014).

Declarations

Data Availability: Whole data and material, used in the article, are available.in the Department of Molecular Basis of Integrative Activity, Academician Abdulla Garayev Institute of Physiology, NAS of Azerbaijan Republic

Animal Research: The studies on animals were carried out with adhering international adopted ethical guidelines.and after approval of the protocols by National Committee of Bioethics.

Consent to participate: All participating coauthors give their consent to participate as coauthors of the article.

Consent for publication: All participating coauthors give their consent to publish this manuscript.

Plant Reproducibility: The research did not include studies on plants.

Clinical Trial Registration: The research did not include studies on humans.

Conflicts of interest/Competing interests: There is no conflict of interests among the coauthors of the article.

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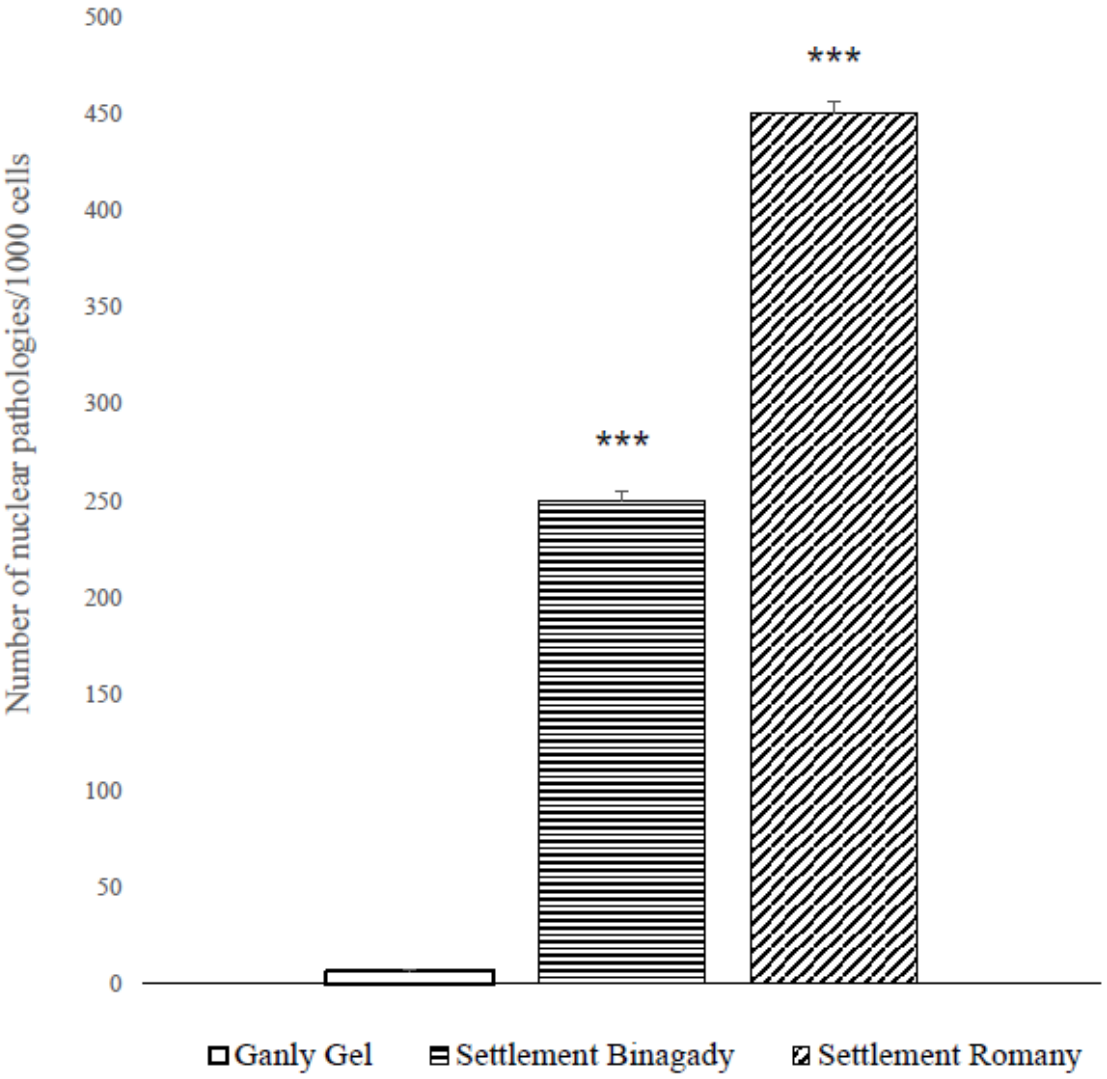
Figures

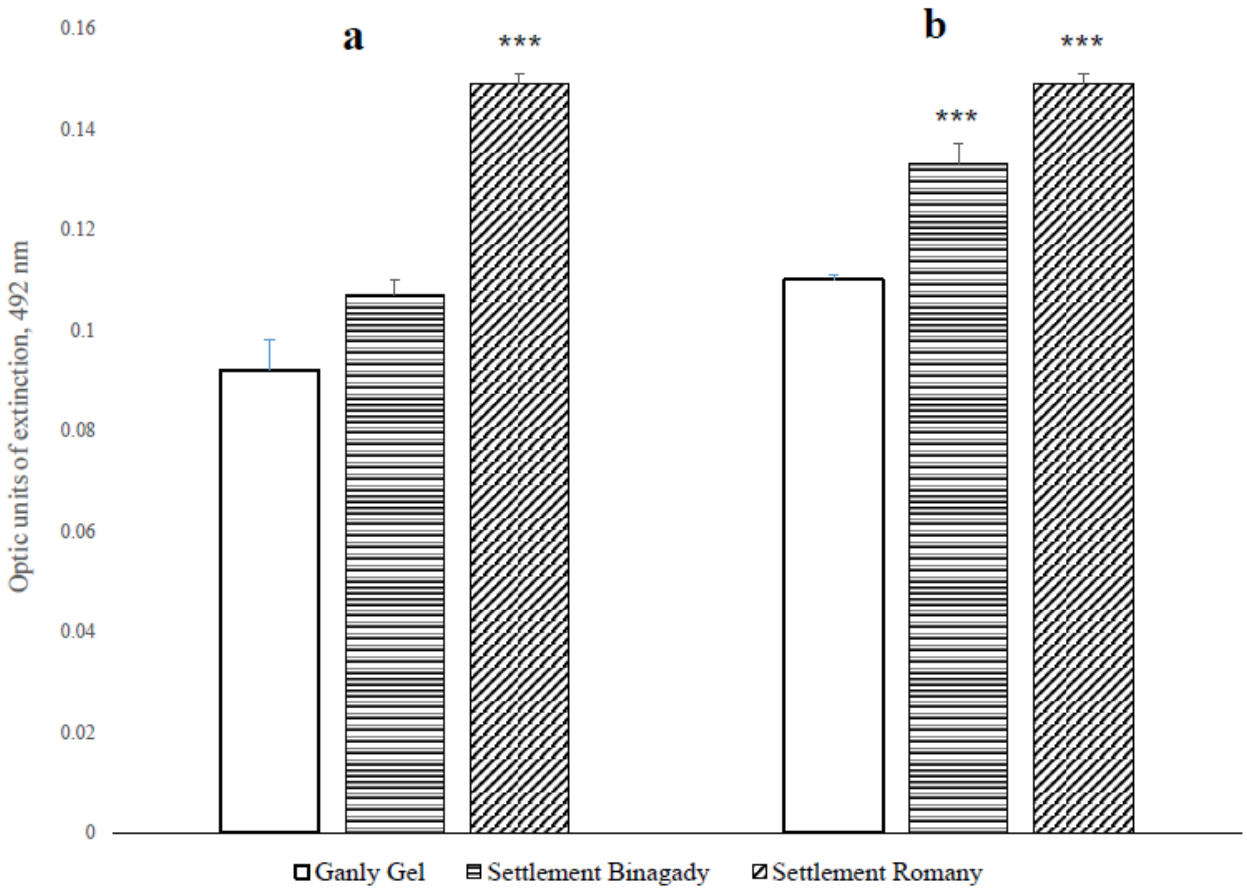


Number of nuclear pathologies per 1000 erythrocytes

Ganly Gel	Binagady	Romany
7	230	466
6	260	432
7	256	456
4	237	470
8	260	442
7	255	436
6.5	249.6667	450.3333
0.6	5.258	6.52
	t=45.95	t=67.78
	p<0.001	p<0.001

Figure 1

Levels of nuclear pathologies in the erythrocytes of the hens living in Binagady and Romany. *** - p<0.001



SMAP
Liver

Ganly Gel	Binagady	Romany
0.092	0.107	0.149
0.006	0.003	0.002

p<0.001

SMAP
Kidney

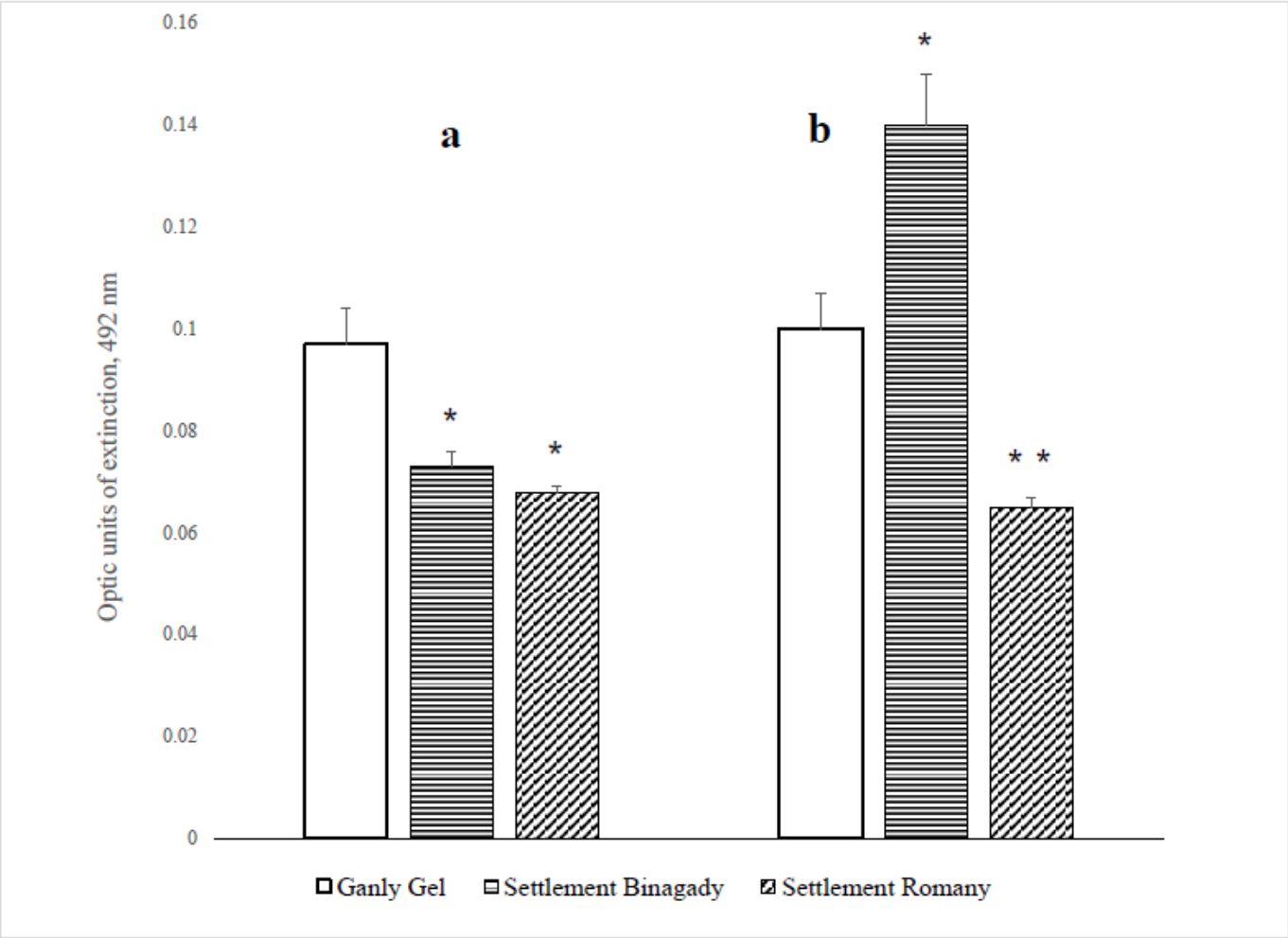
Ganly Gel	Binagady	Romany
0.11	0.133	0.149
0.001	0.004	0.002

p<0.001 p<0.001

Ganly Gel	Settl. Bina	Settl. Romany	
0.092	0.107	0.149	Liver
0.11	0.133	0.149	Kidney
0.006	0.003	0.002	
0.001	0.004	0.002	

Figure 2

Levels of SMAP in the tissues of the hens living in Binagady and Romany. *** - p<0.001



Cytochrome P-450 Liver			Cytochrome P-450 Kidney		
Ganly Gel	Binagady	Romany	Ganly Gel	Binagady	Romany
0.097	0.073	0.068	0.1	0.14	0.065
0.007	0.003	0.001	0.007	0.01	0.002
	p<0.05	p<0.05		p<0.05	p<0.01

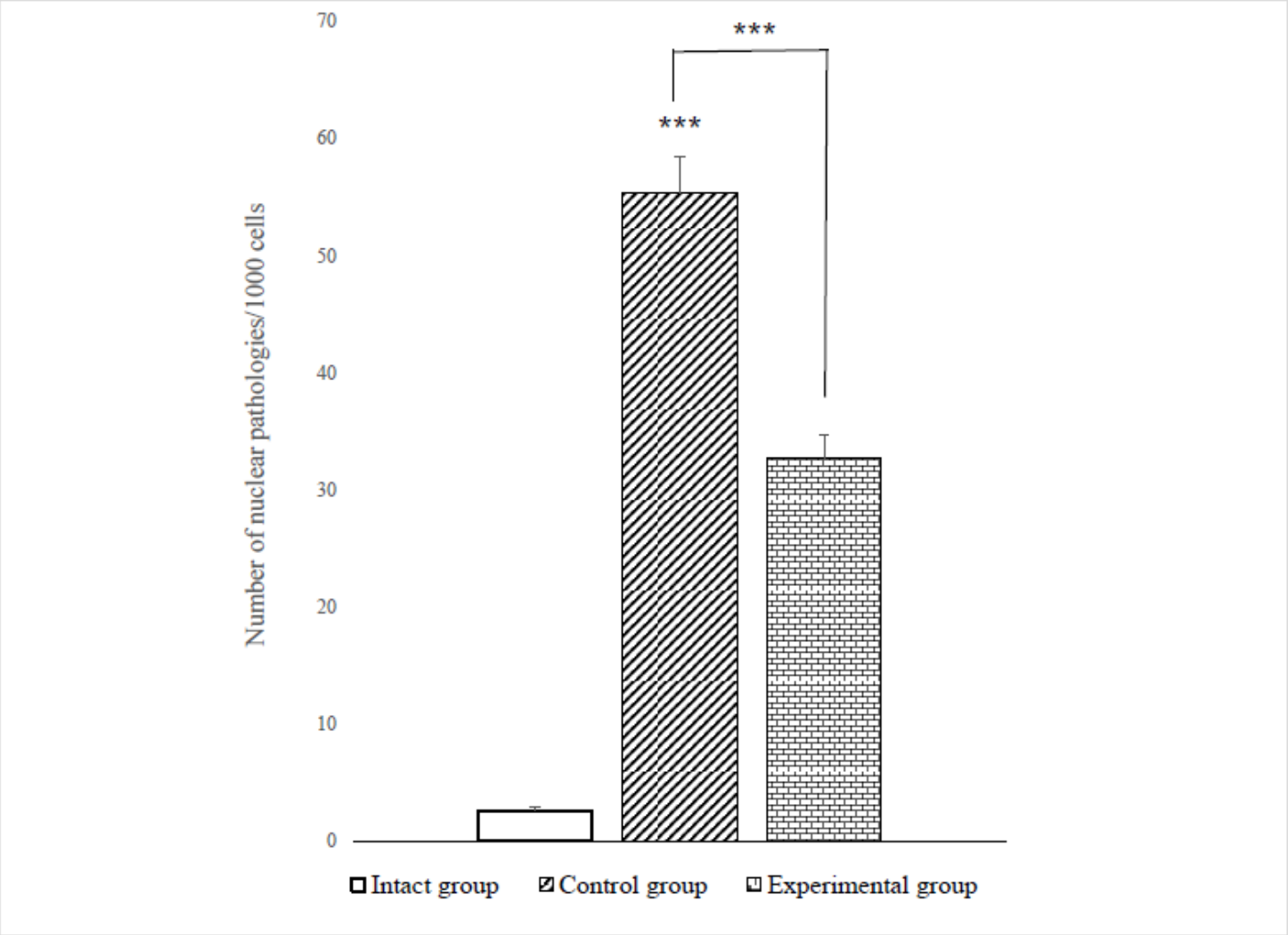
0.097	0.073	0.068
0.1	0.14	0.065
0.007	0.003	0.001
0.007	0.01	0.002

Figure 3

Levels of cytochrome P-450 in the tissues of the hens living in Binagady and Romany. * - p<0.05, ** - p<0.01

Figure 4

Western blotting of the protein extract of the rat bone marrow sampled 1.5, 3 and 5 h after intra-peritoneal administration of SMAP.



Nuclear pathologies under gamma-irradiation (8 Grey)					
Intact	Non-active	SMAP+Irradiation			
1.4	56.1	36.2			
2.3	64.4	36.7			
1.2	55.4	30.9			
3.3	63	32.4			
4	44.6	22.4			
2.7	48.4	34.6			
3.4		36.5			
2.61429	55.3167	32.8143			
0.396	3.188	1.925			
	t=16.298	t=6.042			
	p<0.001	p<0.001			
		Decrease by 41% relatively to the control group			

Figure 5

Levels of nuclear pathologies in the immature erythrocytes of the rats administered with SMAP 3 h prior to their exposure to γ-irradiation at a dose 8 Gy