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The temporal dynamics of antioxidants and lipid peroxidation in chick embryo livers after low-dose gamma irradiation

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ABSTRACT

The effects were measured of low-doses of gamma radiation within 24 hours after irradiation on the activity of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT) and levels of glutathione (GSH) and lipid peroxide concentrations (malondialdehyde, MDA) in embryonic chick liver. The chick embryos were irradiated with doses of gamma radiation of 0.05, 0.15, 0.3, 0.5 and 0.8 Gy at the dose rate of 0.0117 Gy/s on day 19 of incubation. All parameters were measured 1, 3, 6, 12 and 24 hours after irradiation by spectrophotometry, apart from the level of lipid peroxidation, which was assessed using high performance liquid chromatography (HPLC). A versatile analysis of the data was made and it consisted of: 1) a comparison of the means of antioxidant enzyme activities and GSH and MDA concentrations for each radiation dose and the time after irradiation with the corresponding control samples; 2) research into the temporal dynamics of enzyme activities and concentrations a) for each value of radiation dose and b) when the data for radiation doses were merged, *i.e.* regardless of the radiation doses. A significant increase in GSH-Px activities in time after irradiation was found in dose intervals from 0.05 to 0.5 Gy, which was well described by linear function. This is a highly reliable result, because it was obtained as a result of all the analyses applied. For other parameters, we did not find any dependence of activities and concentrations on time. The analysis of comparison of means of the irradiated and control samples gave statistically significant results for some dose-time pairs.

Key words: antioxidants; chick; embryo; liver; low-dose radiation; gamma irradiation

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Introduction

It has been shown that low doses of ionizing radiation can induce different biological effects, such as oxidative stress (for a mini-review see AZZAM et al., 2012), radiation hormesis, adaptive response, bystander effect, genomic instability (for a review see TANG and LOKE, 2015) and modulation of gene expression (GHANDHI et al., 2015). Furthermore, the induction of antioxidant defense systems, such as glutathione (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) has been also well documented in various tissues of rodents (YAMAOKA, 2006), following exposure to low doses of ionizing radiation.

The effects of exposure to low-dose radiation on antioxidant parameters could differ between species, especially between mammals and birds (COSTANTINI, 2008), and depend strongly on experimental conditions, such as dose and dose rate (WANG et al., 2004). Studies of low dose ionizing radiation effects in poultry have primarily been directed at hatchability, fertility, body mass and liver enzyme activities in chickens (ZAKARIA, 1989; ZAKARIA, 1991; GERRITS and DIJK, 1992; FALIS et al., 2004). However, these studies have shown contradictory data. Although chick embryos have been used as an experimental animal model in various research fields and have served as predictors of response in humans (SUTENDRA and MICHELAKIS, 2007; O'DONNELL and PURI, 2009), data regarding the effects of exposure to a low dose of gamma rays on oxidative stress and lipid peroxidation in chick embryos are relatively scarce.

The data on antioxidant status in chick embryos were mostly gathered for liver, brain, yolk and yolk sac membranes under physiological conditions (GAAL et al., 1995; SURAI, 1999). Thus, SOD, CAT and GSH-Px activity, as well as GSH levels, were found to be higher in embryonic chick livers when compared to embryonic brain and yolk sac membranes at day 19 of incubation.

Since reactive oxygen species (ROS) caused by ionizing radiation leads to lipid peroxidation (AZZAM et al., 2012), the determination of lipid peroxidation in embryonic chick livers can provide important information about oxidative stress or cell damage after exposure to ionizing radiation.

In this study, we investigated lipid peroxidation as the formation of malondialdehyde (MDA) and antioxidants status (GSH-Px, SOD, CAT activity and GSH concentration) in embryonic chick livers over 24 hours after exposure to gamma rays in a wide range of doses, from 0.05 up to 0.8 Gy on the 19th day of incubation. Two different analyses of the data were made: 1) a comparison of the means of antioxidant enzyme activities, and GSH and MDA concentrations for each radiation dose and time after irradiation, with the corresponding control samples; 2) research into the temporal dynamics of enzyme activities and concentrations, a) for each value of radiation dose and b) when the data for radiation doses were merged, *i.e.* regardless of the radiation doses. From studying the temporal dynamics, we expected to find indications about the duration of lipid peroxidation as well as the response of antioxidant enzymes after low-dose gamma irradiation, their time profile, and the time of maximum response. Although the temporal dynamics of a variety of enzymes, including antioxidants, have been used in different studies of plants and animals (KUMARI et al., 2014; MILLER et al., 2014; WEI et al., 2018), to our knowledge, there are no studies of temporal dynamics for embryonic liver tissues after low-dose gamma irradiation. There is another reason why we used several types of analyses in the data processing. Namely, the number of studies in biomedicine with contradictory results is growing, suggesting that many of the conclusions drawn from this research are probably false (COLQUHOUN, 2014; IOANNIDIS, 2005; SIMMONS et al., 2011). This problem, commonly named the statistical or replication crisis, has been considered recently (GELMAN and HILDE, 2017). One of the causes of the low reproducibility of published statistical research, and a source of false positive results is the small sample size frequently used (BUTTON et. al. 2013; CHRISTLEY, 2010; SCHWEIZER and FURLEY, 2016). In reality, it is often very difficult to avoid a small sample size in research. The high

price of one measurement, difficulties in replication of experimental conditions, the large number of parameters and/or number of measured values for each parameter, which results in a huge number of measurements, make multiple repetitions of such measurements almost impossible and/or too expensive, and/or lead to a long, consuming time process (*i.e.* in our study 5 compounds × 5 time points × 5 different values of radiation dose × 8 measurements of each trial = 1,000 measurements). Solutions for overcoming the replication crisis are attracting the attention of researchers (FORSTMEIER et al., 2016; SCHOOLER, 2014). In the present paper, we give a way of increasing the reliability of final results by conducting as many different independent statistical analyses of data as possible. We base our statement on the basic theorem of the theory of probability - the probability of two or more independent events (LANE et al., 2019). Let P_1 (P_1 <1), P_2 (P_2 <1), …, P_n (P_n <1) as probabilities for wrong conclusions obtained in independent analyses 1, 2, …, n, respectively. Then, the probability P to make a mistake in the conclusion obtained from all analyses is $P = P1 \cdot P2 \cdot \ldots \cdot Pn$. It follows that P<<1 and P<<P1, P<<P2, … P<<Pn, *i.e.* the greater the number of analyses made, the much lower the probability is for error in the final conclusion.

Material and methods

The study was reviewed and approved by the Ethics Committee of the Faculty of Veterinary Medicine of the University of Zagreb (Class: 640-01/11-17/43; Record Number: 251-61-01/139-11-2).

Experimental protocol. Fertilized eggs produced by a commercial flock of COBB 500 were used in this trial. All the eggs were placed in the same commercial incubator (Victoria, Pavia, Italy), capacity of 22100 eggs, for a period of 18 days. The incubator had automatic temperature control (37.8 °C), humidity (60-62% relative humidity), and incubation rack turning. On the 19th day of incubation, 200 eggs were randomly taken and transported to the Ruđer Bošković Institute, Zagreb, Croatia, for irradiation, in a portable chicken egg incubator with temperature control (37 °C) and humidity (80%). Additionally, 40 eggs were taken but remained unexposed to gamma-radiation, and were used as a control group. The non-irradiated eggs were retained in the same place for the same period of time as the irradiated eggs.

Irradiation and sample preparation. Chick embryos were irradiated with doses of 0.05, 0.15, 0.3, 0.5 and 0.8 Gy gamma radiation from a panoramic ^{60}Co source of activity about 3 PBq, at the Ruđer Bošković Institute, Zagreb, Croatia (MILJANIĆ and RANOGAJEC-KOMOR, 1997). The dose rate was about 0.0117 Gy/s, and the source axis-to-egg axis distance was 2.91 m. Dosimetric measurements were performed with a Farmer ionization chamber, type NE 2581 (Nuclear Enterprise Technology Limited, UK) and a Farmer Dosimeter, type 2570 (Nuclear Enterprise Technology Limited, UK). The dose was specified as an absorbed dose to water (measured free in the air). After exposure to gamma radiation, all the irradiated eggs and non-irradiated eggs (control group) were transported to the Faculty of Veterinary Medicine of the University of Zagreb. At the Faculty of Veterinary Medicine of the University of Zagreb, the eggs were kept in the same incubator at a temperature of 37 °C and humidity of 80-88%.

Chick embryos (n = 8 per dose) were sacrificed 1, 3, 6, 12 and 24 hours after irradiation. The liver was immediately removed, washed in a cold saline, weighed and quick-frozen in liquid nitrogen, and placed in a deep-freezer at -80 °C until analysis of antioxidant parameters. Eight chick embryos were used for each of the time intervals per dose. Liver tissue was homogenized on ice in 0.14 mol/L KCl using a Schütt homgenplus homogenizer (Schütt Labortechnik, Göttingen, Germany) at 2,800 rpm for 30 s. The tissue mass to buffer ratio was 1:5 (w/v). The liver homogenate was centrifuged at 20,000g for 30 minutes to prepare the supernatant using refrigerated centrifuge Sigma 3K15 (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The supernatant was used to measure GSH-Px, SOD and CAT activity as well as lipid peroxide and GSH concentration.

Evaluation of oxidative stress. Lipid peroxidation was estimated by measuring malondialdehyde (MDA). The concentration of MDA was determined by the high performance liquid chromatography (HPLC) method with UV detection described by GROTTO et al. (2007) using TSP-130 system (Thermo Separation Products, Inc., Thermo Fisher Scientific, Inc., Waltham, MA, SAD) with the reversed phase analytical column protected with a guard column (Waters Symmetry® C18 column, 5 µm, 150 mm × 4.6 mm *i.e.*), maintained at 40 °C. 1,1,3,3-tetraethoxypropane was used for calibration (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). The mobile phase was a mix of 50 mM phosphate buffer and methanol (50:50, v/v), and the flow rate was maintained isocratically at 1 mL/min. The UV was programmed at 532 nm. The retention time was 2.9 min.

Assay of antioxidant parameters. The GSH-Px activity was measured using the RANSEL commercial kit (Randox Laboratories Ltd., Ardmore, Diamond Road, Crumlin, Co. Antrim, United Kingdom). The GSH-Px activity assay method is based on the ability of GSH-Px to catalyze the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, oxidized glutathione is immediately converted to a reduced form with concomitant oxidation of NADPH to $NADP^+$ (PAGLIA and VALENTINE, 1967). The decrease in absorbance at 340 nm was measured. GSH-Px activity was expressed in terms of U/g protein.

The SOD activity was measured by the method of FLOHE and OTTING (1984). The SOD activity was determined on an SABA auto analyzer with RANSOD reagent (Randox Laboratories Ltd., Ardmore, Diamond Road, Crumlin, Co. Antrim, United Kingdom). The method used for determination of SOD activity is based on the formation of superoxide radicals from xanthine by the action of xanthine oxidase, which react with 2-(4-iodophenyl)3-(4-nitrophenyl)5-phenyltetrazole chloride to produce formazan red stain. The activity of SOD was measured as the grade of inhibition of this reaction, expressed in terms of U/mg protein.

CAT activity was measured by the method of JOHANSSON and BORG (1988). The method was based on the reaction of the enzyme with methanol in the presence of an optimal concentration of hydrogen peroxide. The formaldehyde produced was measured by spectrophotometry with 4-amino-3-hydrazino-5-mercapto.1, 2, 4-triazole (Purpald) as a chromogen at 470 nm. CAT activity was expressed in terms of U/g protein.

The concentration of GSH was determined by the method of BEUTLER et al. (1963). Briefly, this method is based upon the development of a relatively stable yellow color when 5,5'-dithiobis-(2-nitrobenzoic acid) is added to sulfhydryl compounds. The absorbance was read at 412 nm. The GSH level was calculated as µmol/g protein. Protein concentration was measured using the method of LOWRY et al. (1951), with bovine serum albumin as a standard. All parameters were expressed per g of protein.

Statistical analyses. The data were analyzed in three ways: a) by comparing the means of enzyme activities (GSH-Px, CAT and SOD) and concentrations (GSH and MDA) for each radiation dose and time after irradiation with the corresponding control samples; b) analyzing the dependence of the enzyme activities as well as GSH and MDA concentrations on time after irradiation for a fixed value of radiation dose (radiation dose is a parameter), and c) when the data for the radiation doses were merged, *i.e.* regardless of the radiation dose.

Differences in the means between treated and control samples. The differences between the means of the treated and the control samples were tested using the *t*-test and nonparametric Mann-Whitney test. The normality of the data was checked using the Kolmogorov-Smirnov test, which gave positive results for almost all data, and the Shapiro-Wilk test, which proved that the majority of data were normally distributed. Differences were tested at statistical significances of $P<0.05$ and $P<0.01$.

Dependence of the activities and the concentrations on time after irradiation for a fixed value of radiation dose. We analyzed the dependence of the activities and concentrations on

time for a fixed value of radiation dose, graphically and statistically. The graphic analyses consist of investigation of 3D graphs, where the x-axis was time, the y axis radiation dose, and the z axis the activity or concentration, and a series of 2D graphs. The 2D graphs presented the dependence of the activity or concentration (y axis) on time (x axis) for each antioxidant and MDA, and for each radiation dose. From the data presented on the graphs we calculated the coefficients of the correlation and corresponding P value, as the result of testing the following null hypotheses: there is no dependence of the activity or concentration on time after irradiation for certain values of the radiation dose. We undertook regression analysis only for GSH-Px, in order to describe the temporal dynamics of its activity.

Dependence of the activities and the concentrations on time after irradiation regardless of radiation dose. We performed an additional test of the results obtained in the previous subsection. Namely, for all the antioxidants and MDA, we merged the data with different radiation doses, including the control data, for each time after the irradiation. After the calculations of means, we obtained the dependence of concentrations on time, regardless of radiation doses. These data were analyzed as the previous ones.

Results

Differences in the means between the irradiated and control groups. The calculated means, standard deviation of the data, standard errors of the mean and the results of the *t*-test of differences between the means of antioxidant enzyme activities, as well as GSH and MDA concentrations 1, 3, 6, 12, 24 hours after irradiation, and doses of 0.05, 0.15, 0.3, 0.5 and 0.8 Gy, and the control groups, are presented in Tables 1-5. As the *t*-test and Mann-Whitney test gave almost the same results for testing the null hypothesis and similar P values, we have only shown the P values of the *t*-test in Tables 1-5.

The GSH concentration in the livers of chick embryos had significantly decreased at 6 h after gamma irradiation with a dose of 0.3 Gy ($P<0.01$), 0.5 Gy ($P<0.05$) and 0.8 Gy ($P<0.05$) compared with the control group. However, at 24 h after irradiation the concentration of GSH had significantly increased in groups irradiated with doses of 0.05 Gy ($P<0.05$), 0.15 Gy ($P<0.01$) and 0.3 Gy ($P<0.05$), compared to the control group (Table 1).

GSH-Px activity was statistically significantly higher at 12 h and 24 h after gamma irradiation with the doses of 0.15 Gy ($P<0.05$) and 0.3 Gy ($P<0.05$), compared with the control group (Table 2).

CAT activity had significantly increased 1 h after irradiation with the doses of 0.5 Gy ($P<0.01$) and 0.8 Gy ($P<0.01$) compared to the control group, while at 3 and 6 h, the CAT activity of the group irradiated with a dose of 0.8 Gy had decreased statistically significantly ($P<0.05$), as well as at 24 h after irradiation with doses of 0.05, 0.15 and 0.3 Gy ($P<0.05$) (Table 3).

SOD activity had significantly increased 1 hour after irradiation with doses of 0.5 and 0.8 Gy, and 12 h after irradiation with doses of 0.15, 0.3, 0.5 and 0.8 Gy ($P<0.01$) compared to the control group. On the other hand, at 3 h after irradiation, SOD activity had significantly decreased in groups irradiated with doses of 0.15, 0.3 and 0.5 Gy ($P<0.05$), as well as 0.8 Gy ($P<0.01$) when compared to the control group. Furthermore, SOD activity had significantly decreased at 24 h after irradiation in the groups irradiated with doses of 0.5 Gy ($P<0.05$) and 0.8 Gy ($P<0.01$), when compared with the control group (Table 4).

MDA concentrations in the livers of chick embryos had significantly increased at 3 h after gamma irradiation with doses of 0.05, 0.15, 0.3 and 0.8 Gy ($P<0.01$), as well as at 12 h after gamma irradiation with a dose of 0.3 Gy ($P<0.01$). Note that the control group at 3 h had an unusually low mean and the standard deviation. However, the MDA concentration had significantly decreased 1 h after irradiation with a dose of 0.5 Gy ($P<0.05$), as well as 24 h after irradiation with doses of 0.15 Gy and 0.3 Gy ($P<0.01$) compared with the control group (Table 5).

Table 1. Glutathione (GST) concentrations in a chick embryo liver after gamma-irradiation with doses of 0.05, 0.15, 0.3, 0.5 and 0.8 Gy and a dose rate of 0.0117 Gy/s on day 19 of incubation

GSH						
Dose (Gy)	Control	0.05	0.15	0.3	0.5	0.8
Time	1 hour					
Mean (μmol/g protein)	35.6	29.1	29.1	24.3	40.1	34.4
SD (μmol/g protein)	21.0	13.4	13.2	12.3	11.5	11.4
SEM (μmol/g protein)	7.4	4.7	4.7	4.3	4.1	4.0
t-test		0.473	0.469	0.21	0.6	0.884
Time	3 hours					
Mean (μmol/g protein)	48.7	47.0	47.6	38.45	37.1	38.8
SD (μmol/g protein)	14.9	19.3	13.0	26.5	17.5	14.6
SEM (μmol/g protein)	5.2	6.8	4.6	9.4	6.2	5.2
t-test		0.849	0.879	0.36	0.178	0.202
Time	6 hours					
Mean (μmol/g protein)	39.4	33.1	30.8	22.5	30.4	28.2
SD (μmol/g protein)	6.1	15.9	10.5	7.6	6.1	7.8
SEM (μmol/g protein)	2.2	5.6	3.7	2.7	2.2	2.8
t-test		0.329	0.068	2×10^{-4}**	0.011*	0.007*
Time	12 hours					
Mean (μmol/g protein)	29.8	30.9	30.2	39.5	22.5	29.4
SD (μmol/g protein)	8.1	5.3	8.2	26.2	8.8	9.5
SEM (μmol/g protein)	2.9	1.9	2.9	9.3	3.1	3.4
t-test		0.734	0.906	0.345	0.11	0.945
Time	24 hours					
Mean (μmol/g protein)	25.9	47.3	48.6	47.9	27.7	32.0
SD (μmol/g protein)	7.8	18.5	11.5	19.9	7.1	7.4
SEM (μmol/g protein)	2.8	6.5	4.1	7.0	2.5	2.6
t-test		0.014*	4×10^{-4}**	0.017*	0.631	0.126

SD - Standard deviation; SEM - standard error of the mean; P values - *P<0.05; **P<0.01) of *t*-tests for each value of the measured parameters (doses and time after radiation)

Table 2. Glutathione peroxidase (GSH-Px) activity in a chick embryo liver after gamma-irradiation with doses of 0.05, 0.15, 0.3, 0.5 and 0.8 Gy, and a dose rate of 0.0117 Gy/s on day 19 of incubation

GSH-PX						
Dose (Gy)	Control	0.05	0.15	0.3	0.5	0.8
Time	1 hour					
Mean (U/g protein)	361	330	352	312	345	387
SD (U/g protein)	85	65	91	109	103	63
SEM (U/g protein)	30	23	32	38	36	22
t-test		0.426	0.84	0.341	0.742	0.493
Time	3 hours					
Mean (U/g protein)	353	406	383	396	310	275
SD (U/g protein)	122	50	77	68	87	88
SEM (U/g protein)	43	18	27	23	31	31
t-test		0.282	0.56	0.388	0.439	0.169
Time	6 hours					
Mean (U/g protein)	418	416	440	428	369	383
SD (U/g protein)	48	66	60	85	71	45
SEM (U/g protein)	17	23	21	30	25	16
t-test		0.951	0.436	0.777	0.124	0.159
Time	12 hours					
Mean (U/g protein)	358	394	437	442	414	366
SD (U/g protein)	65	35	50	67	66	126
SEM (U/g protein)	23	12	18	24	23	44
t-test		0.193	0.017*	0.023*	0.112	0.876
Time	24 hours					
Mean (U/g protein)	440	587	542	558	471	422
SD (U/g protein)	66	147	85	93	48	54
SEM (U/g protein)	23	50	30	33	17	19
t-test		0.026*	0.019*	0.011*	0.311	0.55

SD - Standard deviation; SEM - standard error of the mean; P values - *P<0.05; **P<0.01) of *t*-tests for each value of the measured parameters (doses and time after radiation)

Table 3. Catalase (CAT) activity in a chick embryo liver after gamma-irradiation with doses of 0.05, 0.15, 0.3, 0.5 and 0.8 Gy and a dose rate of 0.0117 Gy/s on day 19 of incubation

CAT						
Dose(Gy)	Control	0.05	0.15	0.3	0.5	0.8
Time	1 hour					
Mean (U/g protein)	1324	1316	1058	923	2807	2171
SD (U/g protein)	591	362	413	194	591	372
SEM (U/g protein)	209	128	146	69	209	131
t-test		0.975	0.315	0.102	3×10^{-4}**	0.004**
Time	3 hours					
Mean (U/g protein)	1774	2264	1522	1409	1806	1088
SD (U/g protein)	511	390	553	427	370	435
SEM (U/g protein)	181	138	196	151	131	154
t-test		0.059	0.362	0.144	0.886	0.012*
Time	6 hours					
Mean (U/g protein)	1631	1360	1670	1105	1850	1091
SD (U/g protein)	559	387	495	489	575	156
SEM (U/g protein)	198	137	175	173	203	55
t-test		0.278	0.799	0.065	0.453	0.03*
Time	12 hours					
Mean (U/g protein)	1168	1580	1200	1310	1098	993
SD (U/g protein)	552	308	266	215	179	368
SEM (U/g protein)	195	109	94.1	76	63	130
t-test		0.068	0.876	0.484	0.728	0.44
Time	24 hours					
Mean (U/g protein)	1911	1062	1189	786	1516	1259
SD (U/g protein)	443	385	305	312	323	307
SEM (U/g protein)	157	136	115	110	114	116
t-test		0.001**	0.002**	4×10^{-4}**	0.061	0.004

SD - Standard deviation; SEM - standard error of the mean; P values - *P<0.05; **P<0.01) of *t*-tests for each value of the measured parameters (doses and time after radiation)

Table 4. Superoxide dismutase (SOD) activity in a chick embryo liver after gamma-irradiation with doses of 0.05, 0.15, 0.3, 0.5 and 0.8 Gy, and a dose rate of 0.0117 Gy/s on day 19 of incubation

SOD						
Dose(Gy)	Control	0.05	0.15	0.3	0.5	0.8
Time	1 hour					
Mean (U/g protein)	11528	9781	11146	9838	17401	19222
SD (U/g protein)	2871	2221	1731	2329	1848	2798
SEM (U/g protein)	1015	785	612	823	654	989
t-test		0.195	0.752	0.217	3×10^{-4}**	2×10^{-4}**
Time	3 hours					
Mean (U/g protein)	15351	19184	11632	10570	10269	9460
SD (U/g protein)	4100	3088	2132	984	2053	2048
SEM (U/g protein)	1449	1091	754	345	726	724
t-test		0.053	0.039*	0.012*	0.011*	0.005**
Time	6 hours					
Mean (U/g protein)	14276	15329	15798	14923	13063	12076
SD (U/g protein)	1533	2717	1844	2566	2180	1176
SEM (U/g protein)	542	961	652	907	771	416
t-test		0.356	0.094	0.55	0.219	0.006
Time	12 hours					
Mean (U/g protein)	11329	12087	14920	16355	16264	15740
SD (U/g protein)	2169	1430	2439	2274	2766	2388
SEM (U/g protein)	767	505	862	804	978	844
t-test		0.414	0.006**	3×10^{-4}**	0.001**	0.001**
Time	24 hours					
Mean (U/g protein)	15290	13649	14114	15327	11661	10626
SD (U/g protein)	3687	1526	2163	3734	1470	1962
SEM (U/g protein)	1304	540	764	1 320	520	694
t-test		0.275	0.45	0.984	0.029*	0.007**

SD - Standard deviation; SEM - standard error of the mean; P values - *P<0.05; **P<0.01) of *t*-tests for each value of the measured parameters (doses and time after radiation)

Table 5. Lipid peroxide concentration (malondialdehyde, MDA) in a chick embryo liver after gamma-irradiation with doses of 0.05, 0.15, 0.3, 0.5 and 0.8 Gy and a dose rate of 0.0117 Gy/s on day 19 of incubation

MDA						
Dose (Gy)	Control	0.05	0.15	0.3	0.5	0.8
Time	1 hour					
Mean (μmol/g tissue)	36.5	43.2	26.5	28.9	21.2	29.1
SD (μmol/g tissue)	14.9	12.5	8.6	8.5	4.6	18.1
SEM (μmol/g tissue)	5.3	4.4	3.0	3.0	1.6	6.4
t-test		0.345	0.123	0.233	0.024*	0.392
Time	3 hours					
Mean (μmol/g tissue)	17.3	26.5	35.4	34.8	21.1	24.9
SD (μmol/g tissue)	4.0	3.8	9.9	10.9	7.7	5.1
SEM (μmol/g tissue)	1.4	1.3	3.5	3.8	2.7	1.8
t-test		4×10^{-4}**	0.001**	0.002**	0.236	0.005**
Time	6 hours					
Mean (μmol/g tissue)	33.5	40.4	25.4	23.2	32.0	29.9
SD (μmol/g tissue)	15.0	9.3	7.5	4.2	9.0	9.8
SEM (μmol/g tissue)	5.3	3.3	2.7	1.6	3.4	3.5
t-test		0.287	0.203	0.099	0.808	0.552
Time	12 hours					
Mean (μmol/g tissue)	40.0	32.4	49	59.5	33.9	39.4
SD (μmol/g tissue)	11.6	6.0	16.5	12.7	14.4	13.2
SEM (μmol/g tissue)	4.1	2.1	5.8	4.5	5.1	4.7
t-test		0.121	0.23	0.006**	0.366	0.921
Time	24 hours					
Mean (μmol/g tissue)	41.1	44.9	18.0	15.9	29.3	29.4
SD (μmol/g tissue)	12.0	11.6	5.8	2.4	9.7	10.7
SEM (μmol/g tissue)	4.3	4.1	2.1	0.9	3.4	3.8
t-test		0.528	0.001**	4×10^{-4}**	0.05	0.058

SD - Standard deviation; SEM - standard error of the mean; P values - *P<0.05; **P<0.01) of *t*-tests for each value of the measured parameters (doses and time after radiation)

Table 6. Values of coefficients of correlation for the dependences of activities or concentrations on time after irradiation for the measured values of radiation doses and control groups

Dose (Gy)	Control	0.05	0.15	0.3	0.5	0.8
GSH						
Correl. Coeff.	-0.774	0.457	0.470	0.772	-0.698	-0.357
P value	0.071	0.362	0.346	0.072	0.123	0.488
GSH-PX						
Correl. Coeff.	0.696	0.904	0.956	0.946	0.949	0.571
P value	0.125	0.013*	0.002**	0.004**	0.005**	0.236
CAT						
Correl. Coeff.	0.391	-0.534	-0.263	-0.473	-0.627	-0.375
P value	0.444	0.275	0.615	0.344	0.183	0.463
SOD						
Correl. Coeff.	0.307	-0.094	0.484	0.714	-0.268	-0.361
P value	0.554	0.859	0.331	0.111	0.607	0.482
MDA						
Correl. Coeff.	0.580	0.374	-0.259	-0.202	0.542	0.289
P value	0.227	0.465	0.620	0.701	0.266	0.578

P values indicate the statistical significance of the values of the coefficients of the correlation

Table 7. Values of the parameters *a* (slope) and *b* (y-intercept) of linear functions presented in Fig. 1, which describe the temporal dynamics of GSP-Px activities within 24 hours after radiation with doses of 0.05, 0.15, 0.31 and 0.5 Gy. The goodness of fit of the data is described by R^2.

Dose (Gy)	0.05	0.15	0.3	0.5
a [(U/g protein)/h]	8.2	7.5	9.0	6.4
b (U/g protein)	346	361	344	322
R^2	0.82	0.92	0.89	0.90

Table 8. Values of the coefficients of correlation for the dependences of activities or concentrations on time after irradiation, regardless of radiation dose values

	GSH	GSH-PX	CAT	SOD	MDA
Correl. Coeff.	0.115	0.959	-0.803	0.261	0.191
P value	0.828	0.002**	0.055	0.617	0.717

P values indicate the statistical significance of the values of the coefficients of the correlation

Dependence of antioxidant-enzyme activities as well as GSH and MDA concentrations on time after irradiation for fixed values of radiation dose. The results for the coefficients of the correlation of the dependence of antioxidant enzyme activities, as well as GSH and MDA concentrations, on time for fixed values of radiation doses, and the corresponding P values, are shown in Table 6. These results show that the activities of SOD, CAT and concentrations of GSH and MDA for the controls and radiated samples, including the GSH-Px activities of the control samples and at 0.8 Gy, do not depend on time, *i.e.* their concentrations and activities are constant over time. The GSH-Px activities of irradiated samples for doses of 0.05, 0.15, 0.3 and 0.5 Gy show a statistically significant correlation with time, *i.e.* a significant increase over time. The measured points of the GSH-Px activities (circle) and their linear approximations (lines) are represented in Fig. 1. Values of the parameters of linear functions, slope a and y-intercept b, together with the goodness of fit of the data expressed by R^2, are shown in Table 7. The meaning of the slopes a of the linear functions is the increase rate of GSH-Px activities. The R^2 values ranged from 0.82 to 0.92 (mostly about 0.9).

Dependence of the activities and concentrations on time after irradiation regardless of radiation dose. The results for the coefficients of the correlation of dependence of activities and concentrations on time, regardless of the radiation dose, and corresponding P values, are shown in Table 8. Expectedly, only GSH-Px increased significantly ($P<0.002$).

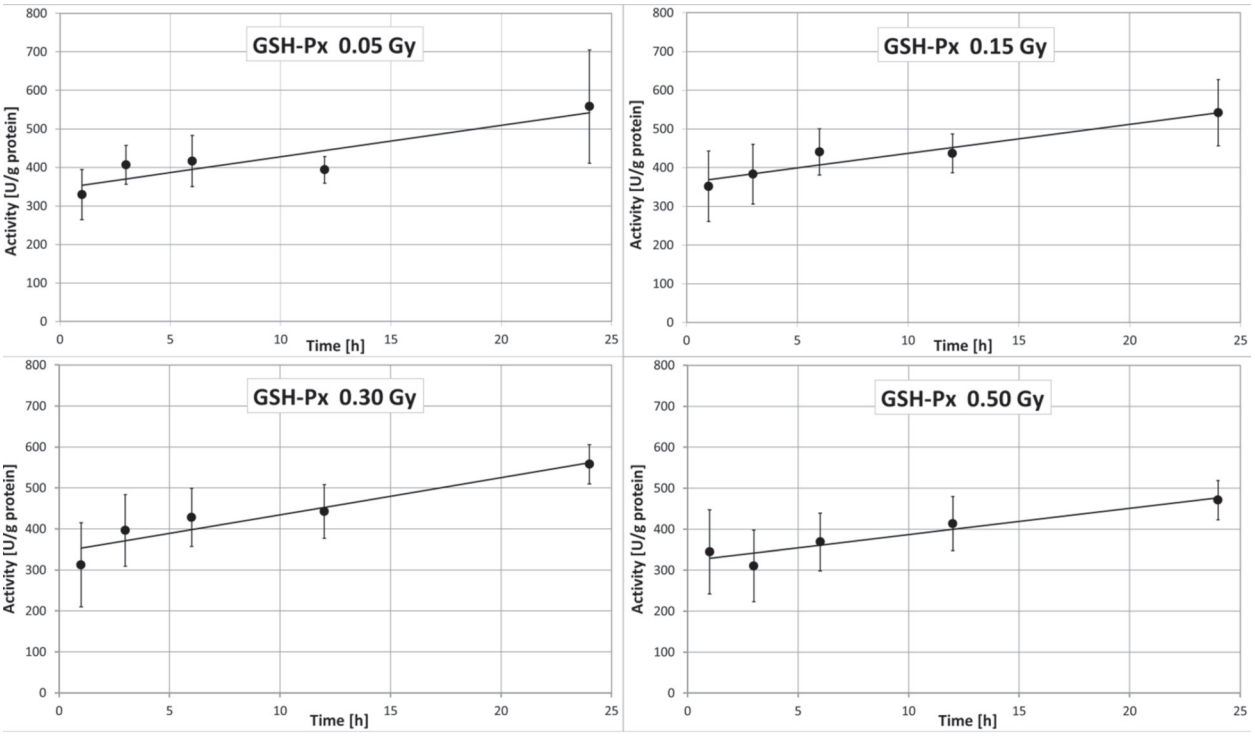

Fig. 1. The temporal dynamics of GSH-Px activities in a chick embryo liver after gamma irradiation with doses of 0.05, 0.15, 0.3 and 0.5 Gy. Data are represented by filled circles. The error bars of the data are plus-minus standard deviation. Lines are linear functions obtained by linear regression.

Discussion

In this study, we analyzed antioxidants (SOD, CAT and GSH-Px enzyme activities, as well as GSH concentration) and lipid peroxidation (MDA concentration) in chick embryo livers after exposure to low doses of gamma radiation on day 19 of incubation, using two different statistical approaches. Our results of temporal dynamics analysis show that the activities of SOD, CAT and the concentrations of GSH and MDA for both control and radiated samples, including GSH-Px activity of the control groups and at a dose 0.8 Gy, did not depend on time, *i.e.* they were constant in time. It follows that any variation in activities and concentrations from the constant are statistical fluctuations (random deviations) of the data measured, including those we found to have statistically significant difference from the controls.

A possible reason why we did not find temporal dynamics within 24 hours is that this time interval could be too short to see the time dynamics of antioxidants and lipid peroxidation. This assumption could be confirmed by the results of OTSUKA, et al. (2006), who described increased gene expression of CAT and Mn-SOD in mice after 23 days of exposure to a dose of 0.5 Gy gamma radiation. In addition, the same authors reported that the enzymatic activity of catalase corresponded to the gene expression level.

We found that the GSH-Px activities of the samples irradiated with doses of 0.05 to 0.5 Gy showed a statistically significant increase over time. This result is in accordance with both statistical approaches applied. The only question is why we did not find a statistically significant difference over at least 24 hours for 0.5 Gy (Table 2), taking into account the increase in activity over time. First, in Table 7, we can see that the increase in the rate of activity (slope *a*) was lower for 0.5 Gy than for other radiation doses. From the parameters of the line at 0.5 Gy, given in Table 7, we calculated that predicted activity at 24 hours was 476 U/g. This differs from the control value by only 35 U/g. This difference is much lower than the common double SEM (57 U/g) of the control and measured value at 0.5 Gy, and at 24 h. This means that the statistical power of the analysis of testing differences between treated and control samples is not sufficient to see such a relatively weak increase, but also that the temporal dynamics analysis has higher statistical power (it "sees" higher values of activities over time).

The GSH-Px activity in different organs of rats, mice or rabbits after low-dose irradiation also showed an increase over 24 h (AVTI et al., 2005; YAMAOKA, 2006; PATHAK et al., 2007). However, when comparing our results with birds and mammals, one has to be aware of the fundamental differences in lung ventilation during embryonic development between oviparous and viviparous species (THOMPSON, 2007), as well as lipid metabolism, especially at the end of a chick's embryonic development. On day 17 of development, GSH-Px activity in an embryo chick liver is higher by 4.0, 2.8, 2.0,1.7,1.3 and 1.2 times compared to the brain, skeletal muscle, heart, yellow sac membrane, kidney and lung, respectively (SURAI et al., 2016). One reason for the tendency of GSH-Px activity to increase is their role in the embryonic chick liver of removing hydrogen peroxide and lipid hydroperoxides particularly at the end of embryonic development (SURAI, et al., 2016; YIGIT et al. 2014). Namely, embryonic chick livers on the 19th day of incubation contain a very high content of unsaturated fatty acids (NOBLE 1986; NOBLE and COCCHI 1990; SPEAKE et al., 1998) which are prone to oxidation.

Conclusion

In conclusion, our results, using different statistical approach, indicate that a small number of samples is not enough to find statistically significant differences between means, but it is necessary to determine a consistent trend or pattern of such results. If they have a random pattern, by definition of randomness, a new unpredictable data pattern is expected in a repeated experiment, *i.e.* replication of these "significant" differences are impossible. Therefore, we consider our finding showing an increase in activities of GSP-Px at radiation doses from 0.05 Gy to 0.5 Gy within 24 hours in embryonic chick livers to be a very reliable result. Since GSH-Px activities end at 24 hours with

an increase, obviously this increase would have to continue after 24 hours. This opens up several new interesting topics for future research, such as the time of the maximum activity, its values, the search for the whole range of temporal dynamics (increase and the decrease tails), and an estimate of the ratios of benefit to damages for different doses of radiation, and maybe finding the complete model of the temporal dynamics of the activity.

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SAŽETAK

U ovom je radu istražen učinak malih doza gama-zračenja na aktivnost glutation-peroksidaze (GSH-Px), superoksid-dismutaze (SOD), katalaze (CAT) te koncentraciju glutationa (GSH) i malondialdehida (MDA) u jetri kokošjih zametaka. Kokošji su zametci ozračeni dozom od 0,05, 0,15, 0,3, 0,5 i 0,8 Gy gama-zračenja i brzinom doze od 0,0117 Gy/s 19. dana inkubacije. Aktivnost GSH-Px, SOD, CAT i GSH određena je spektrofotometrijski dok je koncentracija MDA određena tekućinskom kromatografijom visoke učinkovitosti (HPLC). Svi su pokazatelji određivani 1, 3, 6, 12 i 24 sata nakon ozračivanja a rezultati su obrađeni različitim statističkim metodama pri: 1) usporedbi prosječnih vrijednosti svih pokazatelja pokusne s odgovarajućom kontrolnom skupinom; 2) određivanju vremenske dinamike za sve vrijednosti promatranih pokazatelja kada su bile: a) ovisne o dozi zračenja i b) neovisne o dozi zračenja tj. kada su vrijednosti pokazatelja svih doza bile međusobno spojene. Aktivnost GSH-Px u ozračenih kokošijih zametaka bila je značajno povećana primjenom svih statističkih metoda u rasponu doza od 0,05 Gy do 0,5 Gy te je imala linearnu funkciju. Rezultati ostalih pokazatelja nisu pokazali promjene vrijednosti tijekom vremena promatranja.

Ključne riječi: antioksidansi; kokošji zametak; jetra; mala doza zračenja; gama-zračenje