

Original Article:**Assessment of the safety of chicken egg yolk antibody (IgY) consumption by measuring the activity of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) and malondialdehyde concentration as a lipid peroxidation marker in mice****Hoda Zeynalian¹, Faranak kazerouni^{1,*}, Firouz Ebrahimi², Ali Rahimipour¹, Mostafa Bakhshi², Roghaieh Samadi³**¹Department of Laboratory Medicine, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran²Department of Biology, Faculty of Basic Sciences, Imam Hossein University, Tehran, Iran³Department of Laboratory Medicine, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran*Corresponding author: email address: f_kazerouni@sbmu.ac.ir (F. kazerouni)**ABSTRACT**

Production of antibodies in chickens (IgY) has significantly attracted the attention of scientists. Numerous publications have reported the use of IgY in diagnosis, therapy and prophylaxis. Production of antigen-specific antibodies in chicken can help treat and prevent infectious diseases. In this study, the safety of IgY (anti *E. coli* O157:H7) on the antioxidant system of mice was assessed. To that end, three different doses of IgY (0.9375, 1.875 and 3.75 g / kg) were administered through oral route to three treated groups and PBS to the control group. Each group consisted of 18 mice. Fourteen days after administration, blood samples were collected from the mice. Serum malondialdehyde (MDA) level and catalase, glutathione peroxidase and superoxide dismutase activity were measured using commercial kits. Oral administration of IgY against *E. coli* O157:H7 in doses of 0.9375, 1.875 and 3.75 g / kg caused no deaths and showed no toxic effects on mice. In this study, after 14 days of IgY administration, there were no significant changes in the activity of antioxidant enzymes (catalase, glutathione peroxidase and superoxide dismutase) and MDA serum level compared to the control group. This study's findings revealed that oral administration of IgY against *E. coli* O157:H7 does not show any toxic effects and does not disturb the antioxidant system in mice. These findings could be indicative of safety of oral administration of IgY in mice.

Keywords: Egg yolk antibody; Oxidative enzymes; Malondialdehyde**INTRODUCTION**

Despite the significant advances in medical researches during 20th century, infectious diseases are the main cause of mortality in the world [1]. There is one death in each 3 seconds, most of which are caused by infectious diseases [2]. For prevention and treatment of these diseases, antibodies are good options. The use of antibodies in order to protect against the infection is called passive immunity [3]. Researches have shown that IgY antibodies obtained from eggs of

immunized hens by a proper antigen have the potential to be used for passive immunity [4]. Many researchers are using IgY for the evaluation of passive immunity [3]. IgY is a polyclonal antibody which is derived from egg yolk [5] and it has been proven that the antibody is efficient in the prevention and treatment of some diseases in animals [3]. IgY is a promising alternative for antibiotics and has a significant role in the prevention and treatment of diseases [6, 7]. In addition, eggs as the main source of IgY

have many advantages such as cost effectiveness of production [3]. Egg yolk immunoglobulin (IgY) have many advantages over mammalian IgG. IgY antibody do not activate mammalian complement system (6, 8); besides, total amount of IgY obtained per each egg has been reported to be 60-150 mg (and annually 20-40 g) (3) which is much higher than antibody obtained from rabbit in the same time period (9). Human body is composed of different cells. Free radical, especially reactive oxygen species (ROS) are permanently produced in different metabolic pathways [10]. These radicals consist of anion superoxidase, hydroxyl and hydrogen peroxide [11]. In normal condition, there is an equilibrium between the production and removal of free radicals. Any distortion in this equilibrium will result in the increase of oxidative stress and changes in macromolecules, such as DNA, protein and lipids [10, 11] which in turn is associated with pathological changes in the body [12]. Superoxide anion is the first oxygen-derived radical which is degraded to oxygen and hydrogen peroxide via superoxide dismutase enzyme (SOD). The enzyme catalase (CAT) degrades hydrogen peroxide to oxygen and water [13]. Glutathione peroxidase (GPx) converts hydrogen peroxide into water while glutathione will be transformed to its oxidized form, GSSG [14]. Lipids are the main biomolecules affected by ROS. The oxidative degradation of lipids by ROS is called lipid peroxidation which usually occurs in unsaturated fatty acids [15]. Malondialdehyde (MDA) is one of the main products of lipid peroxidation [16]. Measurement of this reactive aldehyde in biological samples can be used as a marker for the oxidative stress level in a live organism. In this study, activity of antioxidant enzymes (glutathione peroxidase, catalase, superoxide dismutase) and malondialdehyde level was measured in mice for the first time after administration of IgY to assess safety of IgY antibody.

MATERIALS AND METHODS

Protocol of Immunization of hens

In this study, two laying hens (LSL) were used for immunization against enteropathogenic bacteria, E.coli O157:H7. E.coli O157:H7(10^9 CFU/ml) were inactivated by 1%(v/v) of formaldehyde and after combination with Freund's adjuvant (Freund's adjuvant used was equivalent to the volume of the buffer in which protein was dissolved i.e 200 μ l of adjuvant and 200 μ l of buffer) were administered subcutaneously three times within two weeks intervals. This study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (number:IR.sbm.u.retech.rec.1395.172), our work complied with strict ethics regarding working with animals.

Isolation and Purification of IgY

After separation of the yolk from the white by egg separator, 10 ml PBS was added to 10 ml yolk and was then diluted 4-folds with tap water. To reach the pH 5, HCl 0.05M was used. The solution obtained was stored at -20° C to freeze. Next step was the defreezing of the solution followed by centrifugation at 13500 g at 4° C for 15 minutes. The obtained supernatant (60 ml) was separated, and then 2M NaCl was added per 30ml of supernatant; further, to reach pH 4, HCl 0.5M was added to the solution. The solution was shaken on a shaker for 2h in the lab condition in order to be finely mixed. Then, one more centrifugation step precipitate was collected and added to 3 ml PBS.

SDS-PAGE Electrophoresis and Western Blot Analysis

To evaluate the IgY purity, SDS-PAGE was carried out:

Twenty μ l of IgY and 20 μ l of IgY mixed with sample buffer containing 2 mercaptoethanol were heated in boiling water for 5 mins and were loaded on to the gel wells. Western blot was performed to confirm IgY and detect its purity using anti-IgY antibodies.

Quantification of IgY Concentration

Protein was measured using the modified Bradford method with BSA as standard. The concentration of purified IgY was measured at 595 nm .

Table 1. Protein measurements using Bradford Method

Protein sample	Distilled water	Bradford solution	-
-	100 μ l	1ml	Control sample
10 μ l	90 μ l	1ml	Sample Test

Animals and Experimental Protocols

This study was performed on 24 male mice BALB / C weighing 18-20 grams. Animals were divided into 4 groups containing 6 mice each with 3 treatment groups and 1 control group. Treatment groups received IgY with final concentration of 0.9375 , 1.875 and 3.75 g / kg. The control group received equal volume of PBS. Fourteen days after ingestion of IgY by the treatment groups and PBS by the controls, blood samples were obtained directly from their heart. Blood was collected in sterile micro-tubes and then incubated for 30 mins at 37 ° C. Serum was separated after centrifugation at 5000 rpm for 5 minutes . Commercial kits were used for the measurement of SOD,CAT ,GPX and MDA.

STATISTICAL ANALYSIS

In this study, SPSS software version 2014 was used and data were analyzed using one-way ANOVA. P<0.05 was considered statistically significant.

RESULTS

As shown in figure 1, after performing SDS-PAGE electrophoresis on 9% gel and using commassie blue for staining, band (MW 180 KD) relevant to IgY with proper purity (line 3) was detected. Following the addition of 2-mercaptoethano 1 (2ME), IgY broke into two fragments: a 65 KD and a 25 KD (line 4).



Figure 1. SDS-PAGE electrophoresis:

Line1: protein ladder

Line2: unpurified antibody (without 2-ME in the sample buffer)

Line3: purified antibody (without 2-ME in the sample buffer)

Line4: purified antibody (with 2-ME in the sample buffer)

According to Bradford assay the IgY concentration was calculated to be 12mg/ml.

As it can be seen in figure 2 / table 1, the serum level of MDA in IgY treated groups was not

significantly different from that of the control group.

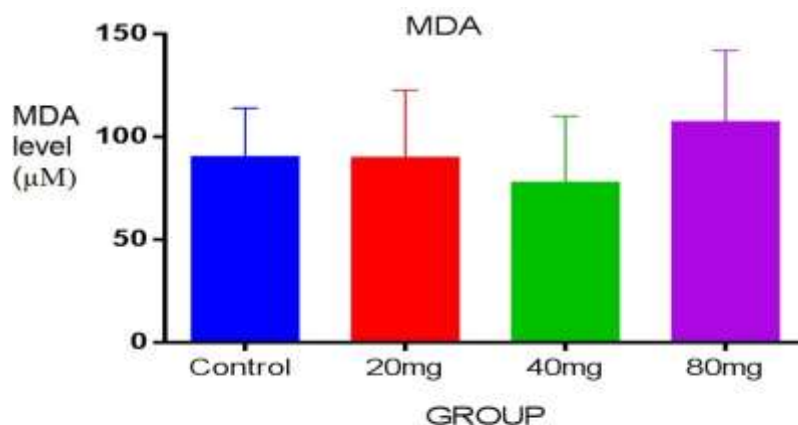


Figure 2. Different doses of IgY (20, 40 and 80 mg) was administered to treated groups of mice and PBS to the control group and 14 days after the administration the level of MDA in the serum was assessed at 530nm (data are presented as mean±SEM)

As shown in figure 3 / table 1, the activity of superoxide dismutase enzyme was not significantly different between the treated groups and the control group.

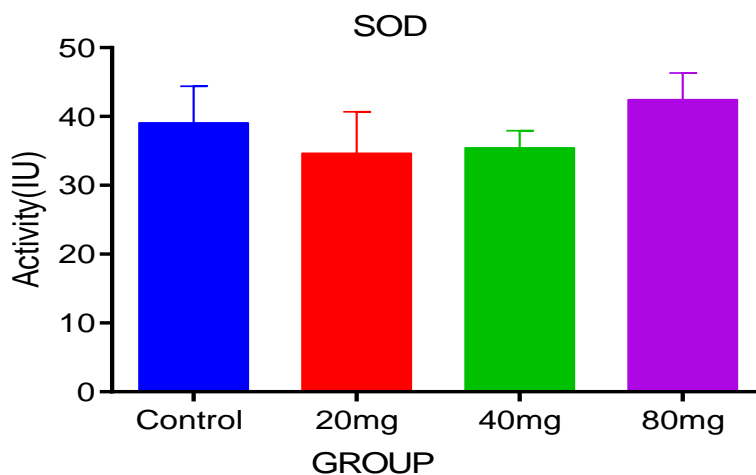


Figure3. Different doses of IgY (20,40 and 80 mg) was administered to treated groups of mice and PBS to the control group 14 days after the administration the activity of SOD enzyme was assessed at 420nm (data are presented as mean±SEM)

Based on the results obtained, the activity of catalase enzyme in treated groups with different doses of IgY (20,40 and 80 mg) showed no significant difference compared to the control

group, but enzyme activity at the highest dose of 3.75 g / kg (80 mg) was significantly different compared to 1.875g/kg (40mg)group(*P<0.05). (figure 4 / table 1).

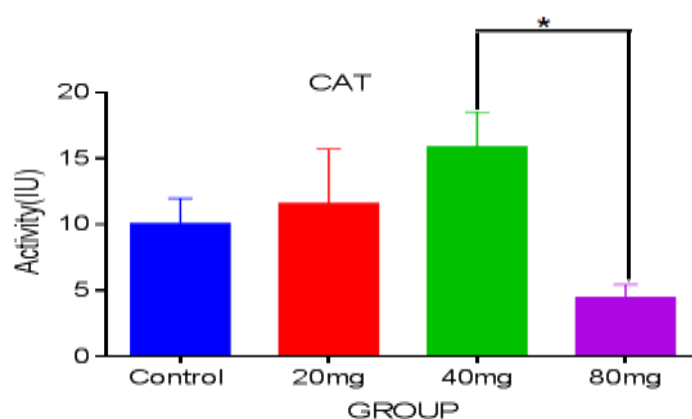


Figure 4. Different doses of IgY (20,40 and 80 mg) was administered to treated groups of mice and PBS to the control group ,14 days after the administration activity of CAT enzyme was assessed at 405nm (data are presented as mean±SEM)

As given in figure 5 / table 1, the activity of GPX enzyme in IgY treated groups was not significantly different from the control group.

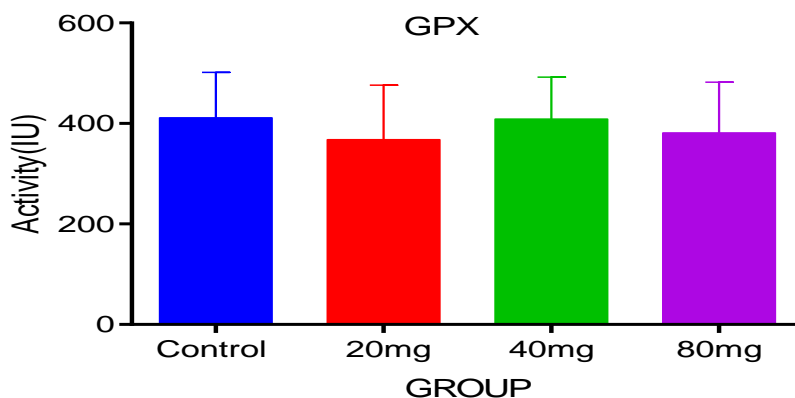


Figure 5. Different doses of IgY (20,40 and 80 mg) was administered to treated groups of mice and PBS to the control group ,14 days after the administration activity of GPX enzyme was assessed at 412nm (data are presented as mean±SEM)

Table 2. Comparison of enzymes activity and MDA serum level in treated groups and the control group

-	Control	20mg Igy	40mg Igy	80mg Igy
GPX	465.525 ± 102.704	367.500 ± 108.558	408.558 ± 83.285	381.225 ± 101.242
Catalase	10.08±1.889	11.6±4.119	15.9±2.632	4.467±0.981
MDA	90.283±23.68	60.83±19.085	48.11±16.034	107.34±34.555
SOD	39.05 ± 5.357	34.64 ± 6.039	35.425 ± 2.514	42.433 ± 3.891

DISCUSSION

Polyclonal antibodies and their components obtained from the serum of immunized animals have many applications in the treatment of diseases. However, in recent years oral administration of antibodies for the treatment of diseases has gained many attentions. The use of IgY antibody from laying birds is one of the efficient strategies for the treatment of viral and bacterial infections. The egg yolk is rich in a polyclonal antibody called IgY. Oral administration of IgY antibody for protection of cow and human against enteropathogenic agents such as enterotoxigenic *E.coli*, Corona virus, *Salmonella tarda*, *Yersinia rockeri* has been investigated. In 1998 Yokoyama et al could protect cows against *Salmonella dublin* and *Salmonella typhimurium* by the oral administration of edible IgY. In 2007, Boxter et.al used these antibodies in the treatment of *Clostridium difficile* toxin [7]. IgY is an immunogene when it is injected (17). Therefore, to evaluate the safety of egg yolk antibody on mice, oral IgY was administered since it is not immunogene when used orally. In the present study, treatment with the IgY anti *E. coli* O157:H7 did not decrease the water and food consumption. Mice administered with the IgY anti *E. coli* O157:H7 at doses of 0.9375; 1.875; 3.75; mg/kg did not show signs of toxicity (convulsion, diarrhea). The body weight and mortality of the animals treated with IgY anti *E. coli* O157:H7 did not show any significant changes when compared with the control group. Based on this study's findings, except for catalase activity which in the highest dose (80 mg) of Igy was significantly lower than the group which received 40mg/kg IgY, other enzymes and malondialdehyde level as the most important marker of cell damage showed no significant difference between the study groups and the control group. In the study conducted by Sudjarwo et al, anti-HIV IgY was used in 5 different doses (in the range of 0.9375-15g/kg) . Mice treated with these different doses showed no reduction on water and food consumption, nor any symptoms of convulsion and diarrhea. They also observed no mortality in the treated mice.

Sudjarwo et al also observed that the weight of vital organs (lung, heart, spleen, kidney and liver) in the test and the control groups were not significantly different. The macroscopic structure of the organs (the color and texture) also showed no significant difference among the groups [18]. In the present study in agreement to Sudjarwo et.al's work none of the IgY treated mice showed decrease in food and water consumption or poisoning signs such as convulsion and diarrhea. There were no weight loss or mortality in the studied groups which indicates that IgY has no toxic effect even following administration of the highest dose of 3.75 g/kg. Oxidative stress is an imbalance between production of free radicals, so-called oxidants or reactive oxygen species (ROS) and antioxidants. This imbalance leads to damage of important biomolecules and cells, with potential impact on the whole organism. In order to prove the safety of IgY, three different doses of this immunoglobulin (0.9375, 1.875 and 3.75 g/kg), similar to the first three doses of Sudjarwo's study were used and after 14 days some oxidative stress markers such as malondialdehyde, glutathione peroxidase, superoxide dismutase and catalase were measured in mice sera . The results showed that MDA level and the activity of catalase, glutathione peroxidase and superoxide dismutase in the treated animals was not significantly different compared to the control group. Based on the results of the present study, catalase activity in the group which received 80mg/kg IgY was significantly lower than the group which received 40mg/kg IgY which is perhaps an indication of dose dependent effect of IgY on catalase activation. However, since other markers such as malondialdehyde level and activity of the other two enzymes were not different between the tests and control group, the repetition of the experiment with larger number of mice and higher concentrations of IgY is suggested. In conclusion, this study's findings revealed that oral administration of IgY against *E.coli* O157:H7 does not show any toxic effects and does not disturb the antioxidant system in mice. These findings could be indicative of safety of oral administration of IgY in mice.

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“The authors declare no conflict of interest”

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