In vitro susceptibility of duck, chicken, and pig erythrocyte lipids to peroxidation

B. Gradinski-Vrbanac[1], Z. Stojević[1], S. Milinković-Tur[1], T. Balenović[2], J. Piršljin[1], M. Zdelar-Tuk[1]

[1]Department of Physiology and Radiobiology, [2]Department of Animal Husbandry, Faculty of Veterinary Medicine, University of Zagreb, Croatia

ABSTRACT: The susceptibility of erythrocyte lipid to *in vitro* peroxidation as measured by TBARS (thiobarbituric acid-reactive substances) and concentration of reduced glutathione (GSH) in whole blood and erythrocyte suspension before and after incubation with hydrogen peroxide was assessed in ducks, chickens and pigs. A high susceptibility of erythrocytes to peroxidation *in vitro* was observed in all animals tested, but this susceptibility was of different intensity. Pig erythrocytes exhibited the lowest resistance to oxidative stress *in vitro* as compared with that in ducks ($P < 0.01$) and chickens ($P < 0.02$). A high level of GSH in the erythrocytes of ducks and chickens offers higher resistance to oxidative stress in comparison with that in the pig erythrocytes.

Keywords: duck; chicken; pig; erythrocytes; thiobarbituric acid-reactive substances; glutathione

All living organisms developed very powerful defence mechanisms against free radicals, mainly those arising from oxygen consumption. The red blood cells (RBC's) are constantly exposed to oxidative injury, but their metabolic activity is capable of reversing the injury under normal conditions. Following an increased oxidative stress when the content of active oxygen species exceeds the cellular antioxidant capacity, irreversible damage to the erythrocytes occurs, resulting in their ultimate loss by hemolysis and/or by removal from the circulation.

Extracellularly produced oxidants can damage the cell membrane before reaching the cytosol protective mechanisms (Kaneko *et al.*, 1997). The peroxidation of membranes usually exhibited as lipid peroxidation has been implicated as one of the primary events in the oxidative damage of the cell (Edwards and Fuller, 1996). At the same time the lowered concentration of reduced glutathione (GSH) has been generally considered to be an index of increased oxidative stress (Kidd, 1997). The importance of GSH in the protection against oxidative injury is best established under conditions when its concentration is reduced.

The erythrocytes of ducks and chickens are nucleated and have a base-line content of GSH that is many-fold higher than that of mammals (Smith, 1974). However, in the duck erythrocytes no catalase activity was detected (Aebi, 1974; Gradinski-Vrbanac *et al.*, 1988) whereas in the pig blood and erythrocytes catalase activity was found to be high (Jones and Masters, 1976; Maral *et al.*, 1977). The erythrocyte catalase level in chicken and pig amounted to 20 ± 2 and 730 ± 80 µg/ml blood, respectively (Maral *et al.*, 1977). In the detoxification of hydrogen peroxide (H_2O_2) catalase may be of great importance in mammals, but in birds at least it appears that catalase does not play a major role in the protection of erythrocytes against H_2O_2. In particular, a low level of catalase may be compensated by a high level of glutathione peroxidase in duck (12 ± 3 µmol NADPH/min per ml of blood). However, the level of glutathione peroxidase also shows a wide dispersion in the different species with 0.3 ± 0.05 of chicken blood, and 2.3 ± 1 µmol NADPH/min per ml in pig blood, respectively.

TBARS are generated from decomposition of lipid peroxide, and their determination gives a reliable estimate of the amount of lipid peroxides present in the membrane.

Accordingly, the aim of our study was to gain better insight into the susceptibility of erythro-

cytes to hydrogen peroxide in three animal species, ducks, chickens and pigs, which are characterised by different intensity of metabolic rate, GSH concentration and catalase activity that may affect its antioxidative status.

MATERIAL AND METHODS

The experiments were performed on blood samples collected from three animal species:

I. 10 hybrids of English breed ducks, "Cherry Valley" of both sexes, 61 days-old, weighing 3.1–3.6 kg, were fed *ad libitum* with commercial mash produced by "Poljoprerada", Hrvatski Leskovac, Croatia. Water was given *ad libitum* as well

II. 8 chickens of avian breed "Ross" of both sexes, 55 days-old, weighing 2.2–2.5 kg, were fed *ad libitum* with PPT-2-finisher for broilers (Poljomix. d.o.o. Bistra, Croatia). Water was given *ad libitum* as well

III. 6 crossbred pigs (Yorkshire × Swedish Landrace) of both sexes, 27 days-old, weighing 6–8 kg, were fed *ad libitum* with starter diet ("Vrbovec" Farm, Vrbovec, Croatia). Water was given *ad libitum* as well

Blood samples were taken from the wing vein of ducks and chickens, and from the anterior *vena cava* of pigs and collected into tubes with heparin as anticoagulant.

Fresh blood was used for determination of hematological and biochemical parameters. Hemoglobin (g/l) was measured spectrophotometrically using commercially available kits from Herbos d.d. (Sisak, Croatia, Cat. No. TR-1142). The hematocrit (l/l) was determined using a microhematocrit centrifuge (MSE, England). The values of MCHC (mean corpuscular hemoglobin concentration) are calculated from hemoglobin and hematocrit and expressed in g/dl.

RBC's were separated by centrifugation at 1 400 g for 10 minutes and washed three times with cold phosphate buffered saline (PBS; pH 7.4; osmolality 300 Osm/kg). Then RBC's were resuspended in PBS to a hematocrit value of 20%. The susceptibility of erythrocytes to *in vitro* peroxidation was examined by a modified method described by Duthie *et al.* (1990). A cell suspension (2 ml) was added to 2 ml of PBS containing 2 mM sodium azide whereupon 0.44 ml of 0.05% hydrogen peroxide was added. Incubation with hydrogen peroxide was performed at 41°C for duck and chicken erythro-

cytes, and at 37°C for pig erythrocytes in a shaking bath for 1 hour.

The concentrations of TBARS in whole blood (μmol/l) and cell suspension before and after incubation (nmol/gHb) were determined by the method of Trotta *et al.* (1982). It detects malondialdehyde and other thiobarbituric acid-reactive substances generated by free radical-mediated peroxidation of unsaturated lipids (expecting cholesterol and monoenoic or dienoic phospholipids). Absorbance readings at 532 nm were converted to TBARS values using the molar extinction coefficient of 1.5×10^5 (Placer *et al.*, 1963). Concentrations of GSH were determined in whole blood (mmol/l) and in cell suspension of erythrocytes before and after the incubation (μmol/g Hb) by applying the methods of Beutler *et al.* (1966). The changes in TBARS and GSH concentrations were expressed as a percent difference after and before the incubation (Δ [TBARS]/c [TBARS] × 100; Δ [GSH]/c [GSH] × 100).

Data were expressed as means ± SD. All statistical comparisons were made by paired and unpaired Student's *t*-test.

RESULTS

The results of hematological parameters are presented in Table 1. The highest concentration of hemoglobin was recorded in chickens, and it was significantly higher ($P < 0.0001$) than that of ducks. The highest value of hematocrit was determined in ducks, and it was significantly higher than in chickens ($P < 0.0001$) and pigs ($P < 0.0005$). Conversely, the value for MCHC determined in ducks was significantly lower than those obtained for chickens ($P < 0.0001$) and pigs ($P < 0.005$).

In Table 2 the values for concentrations of TBARS and GSH in whole blood are presented. The concentration of TBARS was significantly higher in chickens as compared with that of ducks ($P < 0.05$). The highest concentration of GSH was found in the blood of chickens. The lowest values of GSH were recorded in the pig, and they were significantly different from those determined in ducks ($P < 0.0001$) and chickens ($P < 0.0001$).

Figure 1 demonstrates the results of TBARS and GSH measurements obtained with erythrocytes from the tested species after and before *in vitro* incubation with H_2O_2. The values of these parameters were expressed as percent differences from the

Table 1. Hematological parameters – hemoglobin, hematocrit and mean corpuscular hemoglobin concentration (MCHC) in whole blood of ducks ($n = 10$), chickens ($n = 8$) and pigs ($n = 6$)

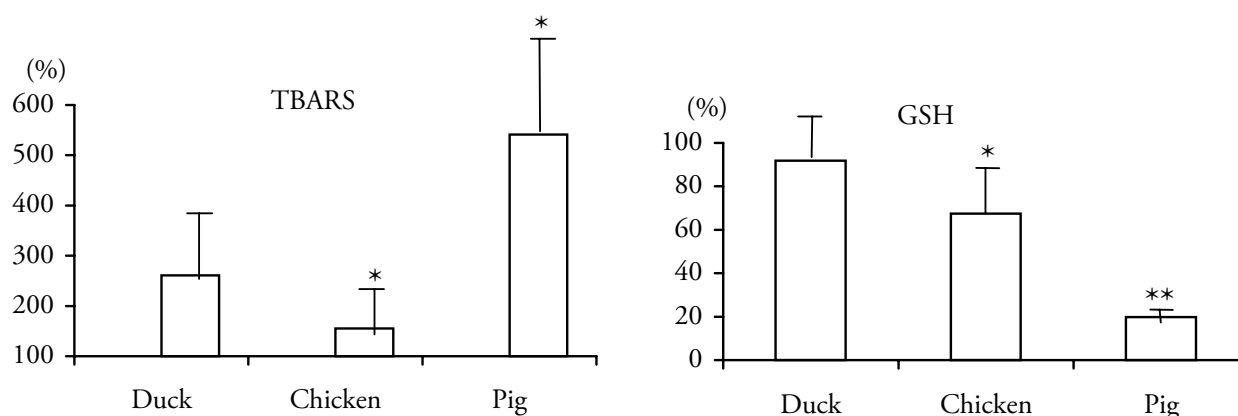| Hemoglobin (g/l)		
Duck	96.8 ± 10.8	
Chicken	179.4 ± 22.9	vs duck ***
Pig	102.3 ± 28.1	
Hematocrit (l/l)		
Duck	0.405 ± 0.031	vs chicken *** and pig **
Chicken	0.304 ± 0.020	
Pig	0.319 ± 0.022	
MCHC (g/dl)		
Duck	24.0 ± 2.8	vs chicken *** and pig *
Chicken	57.9 ± 5.4	
Pig	31.7 ± 6.8	

*$P < 0.005$; **$P < 0.0005$; ***$P < 0.0001$

Figure 1. Levels of thiobarbituric acid-reactive substances (TBARS – left panel) and reduced glutathione (GSH – right panel) in erythrocyte suspension expressed as percent differences of values obtained after and before incubation with hydrogen peroxide (H_2O_2)

TBARS in ducks compared with chickens and pigs were significantly different at *$P < 0.02$
GSH in ducks compared with chickens was significantly different *$P < 0.02$, and with pigs at **$P < 0.0001$

values obtained after and prior to incubation. The TBARS values were increased in all three tested species, and were highest in pigs. The values obtained for pigs were significantly different from those obtained for ducks ($P < 0.01$) and chickens ($P <$ 0.02). The greater loss of glutathione (about 92%) was observed in duck erythrocytes, and it was significantly greater than that determined in chicken (about 68%) and pig (about 20%) erythrocytes ($P < 0.02$; $P < 0.0001$, respectively).

Table 2. The concentrations of thiobarbituric acid-reactive substances (TBARS in µmol/l) and glutathione (GSH in mmol/l) in whole blood of ducks (*n* = 10), chickens (*n* = 8) and pigs (*n* = 6)

TBARS (µmol/l)		
Duck	2.81 ± 0.71	
Chicken	3.84 ± 0.91	vs duck *
Pig	3.38 ± 0.62	
GSH (mmol/l)		
Duck	1.73 ± 0.26	
Chicken	2.12 ± 0.41	vs duck * and pig **
Pig	0.62 ± 0.07	vs duck and chicken **

*$P < 0.05$; **$P < 0.0001$

DISCUSSION

It is well known that a variety of physiological factors, but also ageing, breed differences, climate, and stress may affect the values of some hematological parameters (Tumbleson and Scholl, 1981; Sturkie, 1986). As shown in Table 1, differences exist not only between avian and mammalian species but also between different avian species. Thus, the concentrations of hemoglobin and MCHC determined in duck erythrocytes were significantly lower than those in chicken erythrocytes ($P < 0.0001$). The value of MCHC in duck erythrocytes was also significantly lower as compared with pig erythrocytes ($P < 0.005$). The low MCHC in ducks indicates that the unite volume of packed RBC's contains less hemoglobin. These values are also lower than those found in Pekin ducks (Gaehtgens *et al.*, 1981).

The life span of avian erythrocytes is shorter, their body temperature is higher, and their metabolism is more intense when compared with the respective parameters in mammals (Sturkie, 1986). Accordingly, the intensive avian metabolism will need more oxygen, and thus the birds are exposed to a higher oxidative stress due to an increased level of reduced oxygen species. Consistent with this is quite a high concentration of GSH we recorded in ducks and chickens. In the mammalian blood the GSH concentration was found to be twice to three (or more) times lower than that found in birds (Smith, 1974; Phelps *et al.*, 1989). This is in agreement with our finding in pigs (Table 2). Regardless

of the level of glutathione in erythrocytes of the species tested in our study, supplementation of the incubation medium with H_2O_2 led to a decreased concentration of glutathione following the increase in lipid peroxidation (Figure 1). However, these changes were not of equal intensity. Following the incubation the highest decrease in GSH was recorded in duck erythrocytes (92%). It is well known that no catalase activity was established for duck erythrocytes (Aebi, 1974; Gradinski-Vrbanac *et al.*, 1988), and that catalase is a key enzyme for H_2O_2 degradation. Indeed, Gaetani *et al.* (1989) and Scott *et al.* (1991, 1993) demonstrated increased susceptibility of erythrocytes to H_2O_2 in acatalasemic mice and glucose-6-phosphate dehydrogenase deficient humans. When GSH was added to these erythrocytes, neither change in the methemoglobin concentration nor in the lipid peroxidation occurred. However, after catalase was added, the same authors observed a significant decrease in susceptibility of erythrocytes to oxidation. The role of GSH in erythrocytes is not diminished by the latter finding. It is still exceptionally important in the protection of the proteinaceous membrane (Reglinski *et al.*, 1988), lipid peroxidation (Thomas *et al.*, 1990; Miyazaki and Motoi, 1992), and gelation of free heme (Shiviro and Shaklai, 1987). Miyazaki and Motoi (1992) reported that the monomeric glutathione peroxidase (M-GSH-PX) has a high activity towards phospholipid hydroperoxides, and thus, it can protect the phospholipid membranes against

oxidative injury. In the liver of ducks and chickens the portion of M-GSH-PX in total glutathione peroxidase is 12% and 27%, respectively, and less than 1% in mammals.

The erythrocytes of chickens lost 68% of GSH after the incubation (Figure 1) resulting in lower susceptibility of their RBC's to peroxidation. It is assumed that such an effect was not only due to a high concentration of GSH in erythrocytes but also it could be ascribed to a higher activity of M-GSH-PX (Miyazaki and Motoi, 1992) as well as to the presence of catalase (Gradinski-Vrbanac et al., 1988). Following the incubation, the lowest decrease of GSH (20%) was observed in pig erythrocytes. This could be explained by a high activity of catalase (Maral et al., 1977). Although the pig erythrocytes lost only 20% of GSH after the incubation (Figure 1), the extremely high lipid peroxidation was determined. This peroxidation was about 441% higher than that before the incubation. Such high susceptibility of erythrocytes to peroxidation is detectable in vitamin E-deficient animals (Cynamon et al., 1985). Vitamin E is a critical membrane protector because it terminates the chain reaction by interrupting the propagation cycle. Even after the propagation cycle is terminated by vitamin E, the presence of membrane iron would promote conversion of lipid hydroperoxide (LOOH) to alkoxy (LO·) or peroxy (LOO·) radicals, which can reinitiate the chain reaction (Hebbel, 1986). Although we did not determine vitamin E in our study, the effect of dietary vitamin E (it amounted to 30 mg/kg diet for ducks and chickens, and 120 mg/kg diet for pigs) could not be excluded. One of the possible explanations for such different susceptibility of erythrocyte lipids to peroxidation is a different content of lipids in the membrane of different animal species (Engen and Clark, 1990; Avellini et al., 1993).

In conclusion, we emphasize that the pig erythrocytes exhibited the lowest resistance to oxidative stress *in vitro*, whereas duck and chicken erythrocytes exhibited a higher level of resistance to peroxidation. A high level of GSH found in duck and chicken erythrocytes corresponded with higher resistance to the oxidative stress.

Acknowledgement

REFERENCES

Aebi H. (1974): In: Bergmeyer H.U.: Methods of Enzymatic Analysis. 2nd ed. Vol. *2*, Verlag Chemie, Academic Press, Inc. New York and London. 673 pp.

Avellini L., Spaterna A., Reboldi G.P., Gaiti A. (1993): Defence mechanisms against free radical-induced damage in sheep, cattle and dog erythrocytes. Comp. Biochem. Physiol., *106B*, 391–394.

Beutler E., Duron B., Kelly M. (1963): Improved method for the determination of blood glutathione. J. Lab. Clin. Med., *62*, 882–888.

Cynamon H.A., Isenberg J.N., Nguyen C.H. (1985): Erythrocyte malondialdehyde release *in vitro*: A functional measure of vitamin E status. Clin. Chim. Acta, *151*, 169–176.

Duthie G.G., Robertson J.D., Maughan R.J., Morrice P.C. (1990): Blood antioxidant status and erythrocyte lipid peroxidation following distance running. Arch. Biochim. Biophys., *282*, 78–83.

Edwards C.J., Fuller J. (1996): Oxidative stress in erythrocytes. Comp. Haematol. Int., *6*, 24–31.

Engen R.L., Clark C.L. (1990): High-performance liquid chromatography determination of erythrocyte membrane phospholipid composition in several animal species. Am. J. Vet. Res., *51*, 577–580.

Gaehtgens P., Schmidt F., Will G. (1981): Comparative rheology of nucleated and non-nucleated red blood cells. I. Microrheology of avian erythrocytes during capillary flow. Pflügers Arch., *390*, 278–282.

Gaetani G. F., Galiano S., Canepa L., Ferraris A.M., Kirkman H.N. (1989): Catalase and glutathione peroxidase are equally active in detoxification of hydrogen peroxide in human erythrocytes. Blood, *73*, 334–339.

Gradinski-Vrbanac B., Timet D., Kardum P., Kecerin B. (1988): Akatalazemija u eritrocitima nekih ptica (Acatalasemia in erythrocytes of some avian species). Vet. Arhiv, *58*, 91–94.

Hebbel R.P. (1986): Erythrocyte antioxidants and membrane vulnerability. J. Lab. Clin. Med., *107*, 401–404.

Jones G.L., Masters C.J. (1976): On the comparative characteristics of mammalian catalases. Comp. Biochem. Physiol., *55B*, 511–518.

Kaneko J.J., Harvey J.W., Bruss M.L. (1997): Clinical Biochemistry of Domestic Animals. 5th ed. Academic Press, San Diego, London, Boston, New York, Sydney, Tokyo, Toronto. 185, 168.

Kidd P.M. (1997): Glutathione: Systemic protectant against oxidative and free radical damage. Alternative Med. Rew., *2*, 155–176.

Maral J., Puget K., Michelson A.M. (1977): Comparative study of superoxide dismutase, catalase and glutathione peroxidase levels in erythrocytes of different animals. Biochem. Biophys. Res. Comm., *77*, 1525–1535.

Miyazaki S., Motoi Y. (1992): Tissue distribution of monomeric glutathione peroxidase in broiler chicks. Res. Vet. Sci., *53*, 47–51.

Phelps D.T., Deneke S.M., Baxter D.F., Fanburg B.L. (1989): Erythrocytes fail to induce glutathione in response to diethyl maleate on hyperoxia. Am. J. Physiol., *257*, (Lung. Cell Mol. Physiol. 1), L272–L276.

Placer Z.A., Cushman L.L., Johanson B.C. (1963): Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. Anal. Biochem., *16*, 359–364.

Reglinski J., Hoey S., Smith W.E., Sturrock R.D. (1988): Cellular response to oxidative stress at sulfhydryl group receptor sites on the erythrocyte membrane. J. Biol. Chem., *263*, 12360–12366.

Scott M.D., Zuo L., Lubin B.H., Chiu D.T.Y. (1991): NADPH, not glutathione, status modulates oxidant sensitivity in normal and glucose-6-phopsphate dehydrogenase-deficient erythrocytes. Blood, *77*, 2059–2064.

Scott M.D., Wagner C.T., Chiu D.T.Y. (1993): Decreased catalase activity is the underlying mechanism of oxidant susceptibility in glucose-6-phosphate dehydrogenase-deficient erythrocytes. Bichem. Biophys. Acta, *1181*, 163–168.

Shviro Y., Shaklai N. (1987): Glutathione as a scavenger of free hemin. A mechanism of preventing red cell membrane damage. Bichem. Pharmacol., *36*, 3801–3807.

Smith J.E. (1974): Relationship of *in vivo* erythrocyte glutathione flux to the oxidized glutathione transport system. J. Lab. Clin. Med., *83*, 444–450.

Sturkie P.D. (1986): Avian Physiology. Springer-Verlag, New York, Berlin, Heidelberg, Tokyo. 109–116.

Thomas J.P., Maiorino M., Ursini F., Girotti A.W. (1990): Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. *In situ* reduction of phospholipd and cholesterol hydroperoxides. J. Biol. Chem., *265*, 454–460.

Trotta R.J., Sullivan S.G., Stern A. (1982): Lipid peroxidation and haemoglobin degradation in red blood cells exposed to t-butyl hydroperoxide. Biochem. J., *204*, 405–415.

Tumbleson M.E., Scholl E. (1981): In: Diseases of Swine. 5th ed. The Iowa State University Press, Ames, Iowa, U.S.A. 27–30.

Corresponding Author

Dr. sc Bojana Gradinski-Vrbanac, Department of Physiology and Radiobiology, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10 000 Zagreb, Croatia
Tel. +385 1 239 01 71, fax +385 1 21 46 97, e-mail: ivan.vrbanac@zg.tel.hr