

# Comparative Study Of The Antioxidant Defence Systems In The Erythrocytes Of Australian Marsupials And Monotremes

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## Abstract

A comparison of the erythrocyte (RBC) antioxidant metabolites and enzymes in nine marsupial and two monotreme species was carried out. Reduced glutathione (GSH) concentrations were comparable with those reported for other marsupial and eutherian species. An important finding was that the erythrocytes of the southern hairy nosed wombat regenerated GSH faster than the erythrocytes from its close relative, the common wombat. The activities of glutathione-S-transferase, NADH-methaemoglobin reductase, superoxide dismutase, and glutathione peroxidase (GSH-Px), showed similar levels and extents of variation as those observed in other marsupial and eutherian species. Catalase activities in the marsupials were lower than those measured in the two monotreme species and much lower than those reported in eutherian species. A negative correlation, significant at  $P < 0.05$ , was observed between GSH-Px and catalase activities in the RBC of the marsupials. Since both these enzymes "detoxify"  $H_2O_2$ , there appears to be a reciprocal relationship between the activities of these enzymes in marsupial RBC.

**Keywords:** erythrocytes; monotreme; marsupial; glutathione; catalase; glutathione peroxidase; superoxide dismutase; antioxidant enzymes; methaemoglobin

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## Introduction

Comparative studies on erythrocyte (RBC) metabolism in Australian marsupials and monotremes have revealed some interesting features. These include very low levels of adenosine triphosphate (ATP) in the RBC of the echidna (Kim *et al.*, 1981), remarkably high levels of 2,3-diphosphoglycerate (2,3-DPG) in the RBC of wombats (Agar *et al.*, 1989) and high activities of hexokinase, glyceraldehyde-3-phosphate dehydrogenase, and lactate dehydrogenase in the RBC of the brown antechinus (Agar and Godwin, 1991). It is apparent from such studies that there is a wide variation in the metabolic needs and capabilities of RBC from our native species.

Although informative, none of the studies reported in the literature has examined the antioxidant defence systems in the RBC of marsupials and monotremes. These systems include molecules ranging from small redox molecules to high molecular weight enzymes. In eutherian species, the antioxidant systems protect the RBC against oxidative damage mediated by reactive oxygen species (superoxide anions  $O_2^-$ ; hydrogen peroxide  $H_2O_2$ ; hydroxyl radical  $HO^\bullet$ ), thereby maintaining normal RBC function and survival (Harvey, 1989). A knowledge of the normal functioning of the RBC antioxidant defence systems in marsupials and monotremes would be valuable in their management as it would aid in assessing the health in wild and captive populations. Also it could provide animal models for studying some blood disease in humans.

Agar *et al.* (1989) showed a 6- to 7-fold difference in the activity of glucose-6-phosphate dehydrogenase (G6PD) in the RBC of two closely related marsupials, the southern hairy nosed wombat and the common wombat. Since G6PD is central to the antioxidant defence systems of eutherian RBC (e.g. Beutler, 1984), we have now undertaken studies to test the oxidation sensitivity of RBC from the two species of the wombat.

## Materials and Methods

The monotreme species studied were the platypus (*Ornithorhynchus anatinus*) and the short-beaked echidna (*Tachyglossus aculeatus*). The following marsupial species were also studied: northern brown bandicoot (*Isodon macrourus*), common wombat (*Vombatus ursinus*), southern hairy nosed wombat (*Lasiorhinus latifrons*), black-striped wallaby (*Macropus dorsalis*), tammar wallaby (*Macropus eugenii*), proserpine rock wallaby (*Petrogale persephone*), allied rock wallaby (*Petrogale assimilis*), bridled nailtail wallaby (*Onychogalea fraenata*) and swamp wallaby (*Wallabia bicolor*).

All specimens used in the study were healthy adults. Blood samples (~ 1.0-5.0 ml depending on the size of the animal) were taken from the lateral caudal vein (all wallabies), the superficial branches of the brachial vein (wombats), the venous sinus of the bill (platypus) and by cardiac puncture (bandicoots, echidnas). Samples were collected into tubes containing lithium heparin as the anticoagulant. Human blood samples were obtained from healthy volunteers.

Haemoglobin concentration was measured as the cyanmethaemoglobin derivative. Reduced glutathione (GSH) concentrations in whole blood were measured using the 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) method (Beutler, 1984). GSH regeneration rates were determined according to the method of Kosower *et al.* (1969).

Haemolysates from washed RBC were pre-pared to assay the activities of the following enzymes: glutathione-S-transferase (GST), NADH-methaemoglobin reductase (NADHMR measured as NADH-ferricyanide reductase), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase. The activities of these enzymes were measured according to the methods described by Beutler (1984), using a Beckman DU-64 recording spectrophotometer at temperatures of 37°C (marsupials) and 32°C (monotremes) (Grigg *et al.*, 1992a,b). Enzyme activities are expressed as EU/g Hb.

The response of marsupial RBC to oxidative stress was investigated using an oxidant drug, acetylphenylhydrazine (APH). The method used was based on that of Maede *et al.* (1989). About 1.0 ml of blood was centrifuged and the plasma and buffy coat removed by careful aspiration. The RBC were then washed twice in cold (4°C) isotonic phosphate buffer consisting of 7.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.9 mM NaH<sub>2</sub>PO<sub>4</sub> and 154 mM NaCl (pH 7.4). Washed RBC were suspended in an equal volume of APH solution (225 mg APH in 100 ml isotonic phosphate buffer) and incubated at 37°C in a shaking water bath. Aliquots were taken at 0, 60 and 180 min of incubation. GSH concentrations were measured as previously described, and methaemoglobin concentration was determined according to the method of Hegesh *et al.* (1970).

Values for all parameters measured were compared by single factor analysis of variance (ANOVA). Comparisons between selected sample-means were conducted using protected t-tests (Glenberg, 1988). A linear function was fitted to the data on catalase and GSH-Px activities in marsupials RBC, to investigate the relationship between the two enzymes in these species. In all cases, significance was assumed if  $P < 0.05$ .

## Results and Discussion

### *GSH levels and regeneration rates*

Mean GSH levels and GSH regeneration rates for the species studied are given in Table 1. GSH concentrations in marsupial RBC have been reported to vary from  $3.18 \pm 0.15$  p mol/g Hb in the eastern wallaroo (Agar *et al.*, 1989) to  $17.67 \pm 2.58$  pmol/g Hb in the rufous hare-wallaby (Agar and Godwin, 1991). The results of the present study fall well within this range and are also comparable with GSH concentrations reported in eutherian species (Suzuki, 1982; Harvey, 1989). GSH regeneration rates were found to vary significantly between the species studied (ANOVA,  $P < 0.05$ ) and were also comparable with those reported in other marsupial and eutherian species (Agar *et al.* 1974, 1975, 1986; Smith *et al.*, 1972; Suzuki, 1982; Wightman *et al.*, 1986). A very interesting observation was that erythrocytes from the southern hairy nosed wombat regenerated GSH at a much faster rate than the RBC from the common wombat. The GSH regeneration test, developed by Kosower *et al.* (1967), is dependent on the functioning of the pentose phosphate pathway (PPP) and reflects a series of enzymic activities: (1) the rate of conversion of glucose to glucose-6-phosphate by hexokinase, (2) the rate of regeneration of reduced nicotinamide adenine dinucleotide phosphate (NADPH) by G6PD and (3) the rate of the reduction of oxidized glutathione (GSSG) to GSH by glutathione reductase.

Table 1. GSH and GSH regeneration rates in marsupials and monotremes (mean  $\pm$  SEM,  $n = 4$  in all cases)

Species	GSH ( $\mu\text{mol g Hb}$ )	GSH regeneration ( $\mu\text{mol GSH/g Hb/min}$ )
Platypus	$2.7 \pm 0.6$	—
Short-beaked echidna	$8.8 \pm 0.7$	$0.29 \pm 0.04$
Northern brown bandicoot	$7.8 \pm 1.9$	$0.14 \pm 0.04$
Common wombat	6.5	0.152
Southern hairy nosed wombat	7.6	0.450
Black-striped wallaby	$7.65 \pm 0.16$	$0.57 \pm 0.04$
Tammar wallaby	$9.9 \pm 0.4$	$0.436 \pm 0.017$
Proserpine rock wallaby	$9.2 \pm 0.5$	$0.144 \pm 0.023$
Allied rock wallaby	$13.8 \pm 0.9$	$0.31 \pm 0.02$
Bridled naitail wallaby	$11.2 \pm 1.3$	$0.21 \pm 0.03$
Swamp wallaby	$9.4 \pm 0.4$	$0.28 \pm 0.05$
Human	$7.8 \pm 0.4$	$0.35 \pm 0.02$

Values varied significantly ( $P < 0.05$ ) between the species studied (ANOVA, except the wombat species where  $n = 1$  and hence were excluded from the statistical treatment).

In principle, one might expect the rate of GSH regeneration to be associated with the activities of the PPP enzymes mentioned above. Indeed the high GSH regeneration rate observed in the southern hairy nosed wombat might be related to the , high activity of G6PD reported in this species (Agar *et al.*, 1989), whilst the opposite was true of the common wombat. However, no simple relationship was detected between G6PD activity (data not shown) and GSH regeneration rate in the other species studied. It may be surmised that the wide differences in GSH regeneration rates between these species are related to differences in the permeability of the erythrocytes to glucose, or to differences in the concentrations of NADPH and/or to some unknown aspect(s) of GSH metabolism in these species.

### Enzyme activities

The activities of GST, NADH-MR, SOD, GSH-Px and catalase in the RBC of the species studied are given in Table 2. Some of the results were striking. To simplify the extensive data obtained, each enzyme will be considered individually with respect to a comparison of its activity in eutherian and marsupial species.

### Glutathione-S-transferase (GST)

GST catalyses the formation of thioether-linked glutathione conjugates between GSH and certain electrophilic substrates such as xenobiotic compounds. These conjugates are transported from the RBC by an ATP-dependent system (Board, 1981). The importance of GST activity in protecting RBC against xenobiotics-induced oxidation, and its involvement in GSH turnover is unknown.

The activity of this enzyme in the RBC of eutherian species ranges from 1.8 EU/g Hb in cattle to 20.1 EU/g Hb in cat (Board and Agar, 1983; Goto *et al.*, 1992; Kurata *et al.*, 1993a,b). Consistent with such a variation in eutherian RBC, we found GST activity to vary considerably between the marsupials and monotremes studied. GST activity was especially low in the RBC of the allied rock wallaby ( $0.77 \pm 0.11$  EU/g Hb) and the southern hairy nosed wombat ( $0.83 \pm 0.13$  EU/g Hb). Low GST activity has also been reported in one other marsupial, the red-legged pademelon ( $0.290 \pm 0.023$  EU/g Hb) (Agar and Spencer, 1993). Why these species should have such low levels of GST in their erythrocytes is unknown.

### NADH-methaemoglobin reductase (NADHMR)

NADH-MR is the enzyme primarily responsible for reducing the Fe(III) in methaemoglobin to Fe(II) to give functional haemoglobin. Its activity in eutherian species ranges from 4.3 EU/g Hb in cattle to 27.67 EU/g Hb in grey-headed fruit bats (Wightman *et al.*, 1986; Harvey, 1989). NADH-MR activities in the RBC of the marsupials and monotremes studied were not too different from those reported in eutherian species, although the short-beaked echidna ( $64 \pm 10$  EU/g Hb) and the northern brown bandicoot ( $52 \pm 7$  EU/g Hb) were found to have very high activities of NADH-MR.

Table 2. Activities of the antioxidant enzymes in the erythrocytes of marsupial and monotremes (mean  $\pm$  SEM, n = 4 in all cases)

Species	GST (EU/g Hb)	(EU/g Hb)	(x 10 <sup>3</sup> EU/g Hb)	(EU/g Hb)	(x 10 <sup>6</sup> EU/g Hb)	
Platypus	12.1 $\pm$ 0.5	64 $\pm$ 10	2.53 $\pm$ 0.15	158 $\pm$ 6	5.5 $\pm$ 0.7	
Short-beaked echidna	8.0 $\pm$ 0.5	52 $\pm$ 7	1.78 $\pm$ 0.01	14.5 $\pm$ 2.5	5.1 $\pm$ 0.3	
Northern brown bandicoot	20.2 $\pm$ 3.0	16	1.79 $\pm$ 0.28	108 $\pm$ 8	-	
Common wombat	3.0	15	1.89	52	3.1	
Southern hairy nosed wombat	0.83	13.8 $\pm$ 1.2	1.33	59	2.9	
Black-striped wallaby	2.9 $\pm$ 0.5	12.0 $\pm$ 0.7	1.61 $\pm$ 0.07	131 $\pm$ 11	-	
Tammar wallaby	6.8 $\pm$ 0.9	12.0 $\pm$ 0.7	1.85 $\pm$ 0.09	70.5 $\pm$ 1.9	1.68 $\pm$ 0.15	
Proserpine rock wallaby	1.24 $\pm$ 0.12	12.7 $\pm$ 0.6	1.85 $\pm$ 0.05	109 $\pm$ 12	0.89 $\pm$ 0.07	
Allied rock wallaby	0.77 $\pm$ 0.11	13.2 $\pm$ 0.2	1.15 $\pm$ 0.19	40.7 $\pm$ 1.3	2.59 $\pm$ 0.24	
Bridled nail-tail wallaby	4.4 $\pm$ 0.6	19.8 $\pm$ 1.6	1.94 $\pm$ 0.16	174 $\pm$ 10	1.18 $\pm$ 0.14	
Swamp wallaby	1.23 $\pm$ 0.13	9.9 $\pm$ 1.0	1.87 $\pm$ 0.08	74 $\pm$ 32	1.5 $\pm$ 0.5	
NADH-MR	SOD	GSH-Px	Catalase			
Human		8.8 $\pm$ 1.1	19.5 $\pm$ 1.4	2.07 $\pm$ 0.09	25.7 $\pm$ 1.3	12.0 $\pm$ 0.7

Enzymes activities varied significantly ( $P < 0.05$ ) between the species studied (ANOVA, except the wombat species where  $n = 1$  and hence were excluded from the statistical treatment)

Activities of NADH-MR reported here in the common wombat ( $16 \pm 4$  EU/g Hb) and the southern hairy nosed wombat ( $15 \pm 5$  EU/g Hb) were approximately half of the activities ( $38.3 \pm 6.45$  and  $36.5 \pm 5.5$ , respectively) previously measured by Agar *et al.* (1989). Whether these differences reflect normal intraspecies variation in NADH-MR activity or not is unknown, although wide individual variations in NADH-MR activity in eutherians have been reported (Harvey, 1989). Nevertheless, the present results support the previous observation by Agar *et al.* (1989) that NADHMR activity does not differ significantly between the two wombat species.

#### ***Superoxide dismutase (SOD)***

SOD catalyses the dismutation of superoxide anions to form hydrogen peroxide and oxygen. Previous investigations of SOD activity in RBC have shown that it is quite uniform among a variety of animal species, ranging from  $551 \pm 236$  EU/g Hb in the grey-headed fruit bat to  $3058 \pm 542$  EU/g Hb in cattle (Maral *et al.*, 1977; Suzuki, 1982; Wightman *et al.*, 1986; Agar *et al.*, 1989; Kurata *et al.*, 1993a). The results of the present investigation fall within this range. Thus, in addition to extending such studies to include a variety of marsupial and monotreme species, the present findings also confirm the uniform activity of SOD observed in other animals. The universal role of SOD, therefore, appears to be that of protecting RBC from oxidative damage through its action on super-oxide anions generated primarily from the reaction between haemoglobin and molecular oxygen.

#### ***Glutathione peroxidase (GSH-Px)***

GSH-Px contributes to the "disposal" of hydrogen peroxide by converting it to water in a reaction which also oxidizes GSH to its disulfide (GSSG). GSH-Px activity varies significantly between different eutherian and marsupial species (Harvey and Kaneko, 1975; Maral *et al.*, 1977; Suzuki, 1982; Wightman *et al.*, 1986; Agar and Godwin, 1991; Agar and Spencer, 1993; Kurata *et al.*, 1993b). In the present study, considerable variation was also observed in the RBC of the species studied. The highest activities of GSH-Px were found in the bridled nailtail wallaby ( $174 \pm 10$  EU/g Hb), the platypus ( $158 \pm 6$  EU/g Hb) and the black-striped wallaby ( $131 \pm 11$  EU/g Hb). These activities resemble those reported in ruminant species (Maral *et al.*, 1977; Suzuki, 1982).

GSH-Px activity was also found to vary between members of the same genus. For instance, in the genus *Macropus*, GSH-Px activity in the black-striped wallaby was significantly higher than that in the tammar wallaby ( $P < 0.01$ ). Similarly, in the genus *Petrogale*, GSH-Px activity in the proserpine rock wallaby was significantly higher than that in the allied rock wallaby ( $P < 0.01$ ). GSH-Px activity is known to vary considerably between humans (Beutler and Matsumoto, 1975) and sheep (Atroschi *et al.*, 1981) and such variations have been attributed to genetically determined polymorphisms and/or blood selenium levels since selenium is an integral part of the GSH-Px enzyme; the present findings of GSH-Px variation within a genus might also be attributed to such factors, although this requires further investigation.

#### ***Catalase***

Catalase is another enzyme which "disposes of" hydrogen peroxide, converting it to water and oxygen. Its activity in eutherians RBC ranges from  $\sim 1.64 \times 10^4$  EU/g Hb in sheep to  $19.2 \times 10^4$  EU/g Hb in rhesus monkeys (Maral *et al.*, 1977; Suzuki, 1982; Kurata *et al.*, 1993b). The present results are the first reported on the catalase activity of RBC from marsupial and monotreme species. Compared with catalase activities in eutherians, the values in marsupials were generally lower ranging only from  $\sim 0.89 \times 10^4$  EU/g Hb in the proserpine rock wallaby to  $3.1 \times 10^4$  EU/g Hb in the common wombat. In fact, catalase activity could not be detected in the RBC of the black-striped wallaby and the northern brown bandicoot. Monotreme catalase activities were higher than those in marsupials and were comparable with the levels in some eutherian species. Catalase activities in monotremes, however, were still lower than those in humans, cattle, rabbits and most rodents. Conversely, both marsupials and monotremes, like eutherians, have higher RBC catalase activities than those reported in avian species (Maral *et al.*, 1977). Thus in order of increasing activities of RBC catalase, there appears to be the following pattern: avian species > marsupials > monotremes > eutherians. Whether or not such a pattern pertains to the evolution of catalase and the importance of its function in erythrocytes, is unknown and requires further investigation.

### ***Relationship between GSH-Px and catalase***

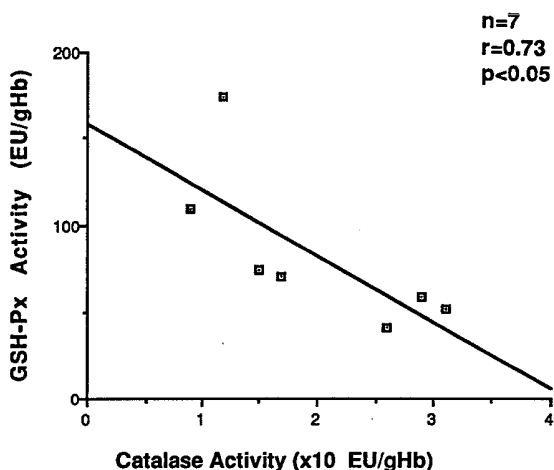
As mentioned previously, both of these enzymes "detoxify" hydrogen peroxide, thereby protecting RBC from oxidative damage. There is much conflicting evidence in the literature, however, regarding the relative contributions of these enzymes to this process in eutherian RBC (Cohen and Hochstein, 1963; Kirkman and Gaetani, 1984; Gaetani *et al.*, 1989). To our knowledge, the actual relationship between GSH-Px and catalase activities has not been investigated until now. When the RBC of marsupials studied were treated as a group, rather than as individual species, a negative correlation ( $r = 0.73$ ,  $P < 0.05$ ) was found between GSH-Px and catalase activities (Fig. 1); in other words, when GSH-Px activity is high, catalase activity is low, and vice versa. Thus there appears to be a balance between the activities of these enzymes in erythrocytes of marsupials. Since both enzymes "detoxify" hydrogen peroxide, it seems plausible that RBC of a particular species has one of these enzymes with a high activity and the other serves as a type of "reserve" that affords extra protection against this and other reactive species. Further studies on these enzymes in marsupials RBC may contribute to our understanding of the relative roles of these enzymes in detoxifying hydrogen peroxide and other peroxides in eutherian RBC.

### ***Response of erythrocyte to in vitro oxidant stress***

Incubation of eutherian RBC with APH leads to the formation of methaemoglobin and concomitant depletion of GSH over time (Maede *et al.*, 1989). Similar responses of RBC to APH were also observed in the present investigation. At the end of 3 hr of incubation, GSH levels were found to be as low as 32% of the control values in Tammar wallaby to as high as 89% in Proserpine rock wallaby; the values in other species fell in between. The extent of methaemoglobin formation also varied markedly among the species. Although the GSH depletion was similar in the two species of the wombat, the rates of methaemoglobin formation were different; it was 34% in the RBC of the common wombat and 23% in those of southern hairy nosed wombats. Thus, the RBC of the common wombat appear to be more susceptible to methaemoglobin formation compared with those of the southern hairy nosed wombat. Further studies are needed to confirm our findings on the differences in the susceptibility of erythrocytes to oxidants in the wombats.

In conclusion, wide variations in the activities of the enzymes associated with antioxidant defence systems have been observed in the marsupials and the monotremes.

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**Fig. 1.** Relationship between GSH-Px and catalase activities in the erythrocytes of marsupials.

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