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Original article

Antioxidant systems and lymphocyte proliferation in the horse, sheep and dog

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Abstract – To better define the species-specific antioxidant systems and to ascertain the influence of the intracellular redox status on the immune system of different animal species, we determined lymphocyte glutathione peroxidase (GSHPx) activity, plasmatic glutathione levels (GSH) and the effect of H_2O_2 on the responsiveness of lymphocytes to proliferative stimuli. Among the three species considered, sheep presented the lowest plasmatic GSH and the highest lymphocyte GSHPx activity. On the contrary, dogs showed an inverted pattern (high GSH – low GSHPx). Horses displayed intermediate values for both parameters analysed. The effect of H_2O_2 on the proliferative capacity of lymphocytes was the same for all three species; the 200 μM dose in particular was strongly inhibiting. Each species, however, showed different rates of inhibition: sheep exhibited the highest sensitivity to the antiproliferative effect of H_2O_2. Our results confirmed that high H_2O_2 concentrations (200 μM) are noxious for the cellular functions of all animals; however this effect is mediated by a rigorously species-specific relationship between the intracellular reactive oxygen species (ROS) and the molecular systems involved in cell proliferation.

horse / sheep / dog / reactive oxygen species / species-specific cellular signalling

Résumé – **Systèmes anti-oxydants et prolifération lymphocytaire chez le cheval, le mouton et le chien.** Afin de définir l'influence de l'activité oxydo-réductrice intracellulaire sur le système immunitaire de différentes espèces animales, nous avons évalué l'activité de la glutathione peroxydase (GSHPx) lymphocytaire, de la glutathione (GSH) plasmatique et l'effet du peroxyde d'hydrogène (H_2O_2) sur la réponse des lymphocytes aux stimulations prolifératives. Parmi les trois espèces considérées, les moutons présentaient les plus faibles concentrations mineures en GSH plasmatique et l'activité du GSHPx lymphocytaire la plus élevée. Par contre, les chiens montraient un profil inverse

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(faible activité du GSHPx et concentration de GSH élevée). Les chevaux présentaient des valeurs moyennes pour les deux paramètres évalués. Les effets du H_2O_2 sur la capacité proliférative des lymphocytes ont été les mêmes dans les trois espèces étudiées, en particulier une dose de 200 µM s'est révélée fortement inhibitrice. Cependant, chaque espèce a montré des taux différents d'inhibition ; les moutons ont présenté une sensibilité maximale aux effets antiprolifératifs du H_2O_2. Nos résultats confirment que les concentrations élevées de H_2O_2 sont nocives pour la fonction cellulaire de tous les animaux. Par ailleurs, cet effet dépend, dans le contexte de la spécificité d'espèce, de la relation existant entre la concentration des dérivés réactifs à l'oxygène (ROS) intracellulaire et les systèmes moléculaires mis en jeu lors de la prolifération cellulaire.

cheval / mouton / chien / dérivé réactif à l'oxygène / signalement intracellulaire espèce-spécifique

1. INTRODUCTION

Reactive oxygen species (ROS) are normally produced by oxidative metabolism. During evolution, however, aerobic organisms have learned to counteract the effects of these dangerous radicals. The hydroxyl radical, superoxide anion and hydrogen peroxide can react with proteins, lipids and nucleic acids to predispose the cell to destruction [7, 10, 15]. In order to avoid or minimise oxidation damage, aerobic cells have developed molecular systems which can ensure their survival and protect their physiological functions. Immune system cells, on the contrary, have learned to use ROS as chemical weapons against pathogenic microorganisms (respiratory burst) [6].

Moreover, oxygen-free radicals have been shown to be involved in intracellular trafficking, although the role of ROS as signalling molecules is far from completely explained [18]. In this context, the antioxidant enzymes (Superoxide dismutase, Catalase, Glutathione peroxidase (GSHPx), Glutathione-S-transferase and Glutathione reductase) [20, 22, 25] and organic scavengers, such as glutathione (GSH), thiols, etc., also play a complex role which is still not completely understood. These antioxidants are not only involved in the defensive mechanisms against free radicals, but they also carry out specific functions and influence each other to maintain an appropriate intracellular oxidant-antioxidant balance [17, 27]. Because signal transduction is also related to the intracellular redox status, the antioxidants must be considered as mediators of intracellular signalling in a wide variety of cell types [2, 3, 16, 30].

To this regard, lymphocytes show an increase in intracellular reactive oxygen species when stimulated by different exogenous factors [11]. Reactive oxygen species (ROS) then act as second messengers, and specific pathways may be activated [1, 2, 24, 34]. However, the final targets of ROS could be reached using a preferential modulatory strategy specific to the animal species studied.

We previously reported that erythrocyte antioxidant systems and response capacity to oxidative injury vary significantly in different animal species [4]. In this report, we hypothesise that there may be similar differences in lymphocytes. Given that the intracellular redox status directly regulates the proliferation of immune cells [11], these differences may have fundamental implications for the regulation of diverse cellular events such as the responsiveness of lymphocytes to proliferative stimuli.

In an attempt to characterise the biochemical pattern involved in ROS mediated lymphocyte activation, we analysed both

the proliferative capacity of lymphocytes exposed to H_2O_2 and, at the same time, two biochemical parameters which show the antioxidant status of these immune cells: Glutathione peroxidase (H_2O_2 oxido-reductase, EC 1.11.1.9) activity in lymphocytes and glutathione in plasma. In particular, GSHPx catalyses the reduction of hydrogen peroxide (H_2O_2), organic hydro-peroxides and lipid peroxides by oxidating GSH [8]. Plasmatic GSH is used by various tissues and indicates a scavenger capacity throughout the body [13]. It has been reported that its values can be altered in the course of certain diseases [21], indicating its important role in supplying the intracellular GSH of different tissues. GSHPx activity in lymphocytes is an expression of cellular scavenger capacity and should be related to the effect of free radicals on immune activity. One must take into consideration that, in physiological conditions, both of these antioxidant defence mechanisms maintain a low steady-state concentration of free radicals in the cell, and that their activities are very precisely regulated. The determination of lymphocyte GSHPx activity has a different significance with respect to that of the same enzyme in red blood cells inasmuch as such activity considerably affects transcriptional nuclear movement [2, 8].

The aim of this study, therefore, was to analyse the antioxidant systems of lymphocytes in order to confirm the differences found in the erythrocytes from different animal species and to determine whether such differences are related to the proliferative capacity of lymphocytes.

2. MATERIALS AND METHODS

2.1. Animals

Three different animal species were included in this study: horses, 8 Maremmana stallions from the same breeding farm; sheep, 8 Comisana ewes from the same breeding farm and dogs, 8 subjects of different breeds (3 Mongrels, 3 German Short-haired Pointers, 2 English Setters). All of these animals, age-matched, were in normal health as indicated from clinical observations and were fed a calorically and nutritionally adequate diet.

Peripheral blood samples were collected at rest, from the jugular veins of each animal, directly into Vacutainer tubes containing lithium heparin as an anticoagulant.

2.2. Plasmatic glutathione assay

Literature values of GSH in plasma vary greatly as a consequence of minor hemolysis, oxidation or enzymatic cleavage [14]. To minimise errors in sample collection, storage and processing, the plasma was immediately separated from whole heparinised blood by centrifugation and anaerobically stored at –20 °C until analysis. The total GSH content (which included both reduced glutathione and glutathione disulfide) was measured using the Glutathione reductase – Ellman reagent recirculating assay according to the method of Tietze [32]. Briefly, 0.025 mL plasma was mixed with 0.825 mL of 0.1 M sodium phosphate/5 mM EDTA buffer (pH 7.5) plus 0.6 μmol 5,5'dithiobis(2-nitro-benzoic acid) and 1.5 U yeast glutathione reductase; 0.2 μmol of NADPH (dissolved in 0.1 mL buffer) was added and the absorbance was recorded at 412 nm. The rate of reaction at 25 °C was expressed as the change in absorbance for 6 min at 412 nm. The GSH content in the plasma was expressed as μM.

2.3. Cell separation

Mononuclear cells were separated from whole heparinised blood by density gradient centrifugation in Ficoll-Paque (Amersham Pharmacia Biotech, Milan, Italy) [12]. Briefly, the blood collected was mixed with RPMI 1640 (Gibco-BRL Life Technologies,

Milan, Italy) supplemented with 100 IU/mL penicillin and 100 μg/mL streptomycin. After mixing, the suspension was carefully layered on Ficoll-Paque (30 mL diluted blood: 10 mL Ficoll-Paque) and centrifuged at 400 $\times g$ for 20–30 min. The lymphocyte ring was removed and washed once in RPMI 1640 and once in RPMI 1640 containing 10% foetal calf serum (RPMI growth medium).

Erythrocyte contamination, which might interfere with the results of the lymphocyte GSHPx assay, checked by Giemsa staining, was less than 1%. More than 97% of the separated mononuclear cells were viable (trypan blue dying, see the Cell Viability section).

The washed cells were resuspended at 2×10^6 cells/mL in RPMI 1640 containing 10% foetal calf serum, placed in plastic dishes and then incubated for 1 h at 37 °C in a 5% CO_2 air humidified atmosphere to remove monocytes. The non-adhering cells (lymphocytes) were collected, washed and counted in a Neubaur counting chamber by trypan blue exclusion.

2.4. Glutathione peroxidase assay

The separated lymphocytes were lysed and the resulting lysate was analysed for GSH-Px activity according to the method of Paglia and Valentine [26]. The assay of enzyme activity is based on the measurement of GSSG production by coupling to the reaction catalysed by glutathione reductase.

The enzyme unit of the GSH-Px activity was defined as the number of micromoles of NADPH oxidised per minute per mg of total protein and was calculated on the basis of the linear decrease in NADPH absorption at 340 nm.

Proteins of lymphocyte lysates were determined by the method of Lowry [19].

2.5. Cell proliferation

The proliferative capacity of mononuclear cells was assessed monitoring the incorporation of [^{3}H]thymidine (μCi/5×10^5 cells) into newly synthesised DNA after Concanavalin A (ConA) (SIGMA-Aldrich srl, Milan, Italy) stimulation.

Although each animal species has its own optimum concentration of mitogen to elicit a proliferative response [31], preliminary experiments were performed with a wide range of ConA concentration (0.5 to 10 μg/mL) in order to establish the optimum dose for each species. It was found that 2.5 μg/mL is the best concentration for all three species examined, and this dose was then used in all subsequent experiments.

Lymphocytes were plated in quadruplicate samples in flat bottom, 96-well culture plates at a density of 0.5×10^6 cells/well in RPMI growth medium. After 48 hr of incubation at 37 °C in a 5% CO_2 air humidified atmosphere in the presence of ConA, 1 μCi/well of [^{3}H]thymidine (SIGMA-Aldrich srl, Milan, Italy) was added. Eighteen hours after [^{3}H]thymidine addition, the cells were harvested using an automatic cell harvester, and radioactivity was measured (DPM) with a liquid scintillation counter (Packard, USA).

2.6. Cellular response to hydrogen peroxide

Mononuclear cells, cultured in an RPMI growth medium for 48 hr at 37 °C in a 5% CO_2 air humidified atmosphere and stimulated to proliferate with 2.5 μg/mL of Concanavalin A were treated with different concentrations of hydrogen peroxide (H_2O_2) (10, 100 and 200 μM) for 18 hr immediately following the addition of [^{3}H]thymidine. Cell proliferation was determined as reported above.

2.7. Cell viability

To exclude a toxic effect due to hydrogen peroxide, we tested cell viability in cells exposed to the same conditions used in the cell proliferation tests which excluded [^{3}H]thymidine addition. The cells were harvested from microwell plates and diluted in PBS before being counted in a Neubaur counting chamber by trypan blue exclusion. Viable cells, with intact cell membranes, did not take up the dye. Non-viable cells appeared blue.

2.8. Statistical analyses

Values are represented as mean ± SEM. Significant differences were computed by the analysis of variance (one way ANOVA) with Fisher post hoc tests (significant at 95%). The StatView program (Abacus Concepts, Inc, Berkeley, CA, 1996) was used for all statistical analyses.

3. RESULTS

3.1. GSH and GSHPx

We found significant differences among the three species relative to GSH concentration in the plasma ($p < 0.001$) (Fig. 1a). As far as GSHPx activity is concerned, we found significant differences in lymphocytes between sheep and dogs and horses, but no significant difference between dogs and horses ($p < 0.001$) (Fig. 1b).

Among the three species, sheep presented the lowest plasmatic GSH and the highest lymphocyte GSHPx activity. Dogs showed an inverted pattern (low GSHPx activity-high plasmatic GSH concentration). The horses displayed intermediate values for both parameters analysed.

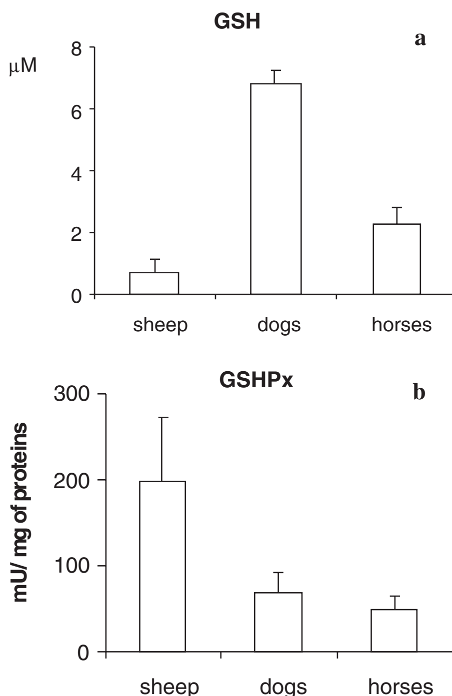

Figure 1. Differences among the three species studied regarding GSH content in the plasma (a) and GSHPx activity in the lymphocytes (b). Each value is the mean ± SEM of eight determinations.

3.2. Lymphocyte proliferation

Although the blastogenic activity of 2.5 µg/mL ConA induced the best blastogenic response in each species, we ascertained different response rates among the species. Indeed, [^{3}H]thymidine incorporation after mitogenic stimulation was high in sheep (activated lymphocyte DPM/ resting lymphocyte DPM = 68.2 ± 28.6), intermediate in horses (41.1 ± 27.3) and low in dogs (13.9 ± 7.8) ($p < 0.05$).

The proliferation of lymphocytes was unaffected by 10 µM H_2O_2, while the 200 µM dose was strongly inhibiting. This effect was the same for all three species. However, each species showed different

rates of inhibition which seemed directly related to their ConA induced proliferative capacity. In particular, the lymphocytes obtained from sheep blood (Fig. 2b) were more sensitive to hydrogen peroxide 200 μM than were those obtained from horses (Fig. 2c) and dogs (Fig. 2a). The administered doses of H_2O_2 caused no significant observable cell death with respect to the viability of those cells cultured without H_2O_2. Indeed, cell viability was only slightly reduced in the 200 μM H_2O_2 treated lymphocytes in the three groups of animals.

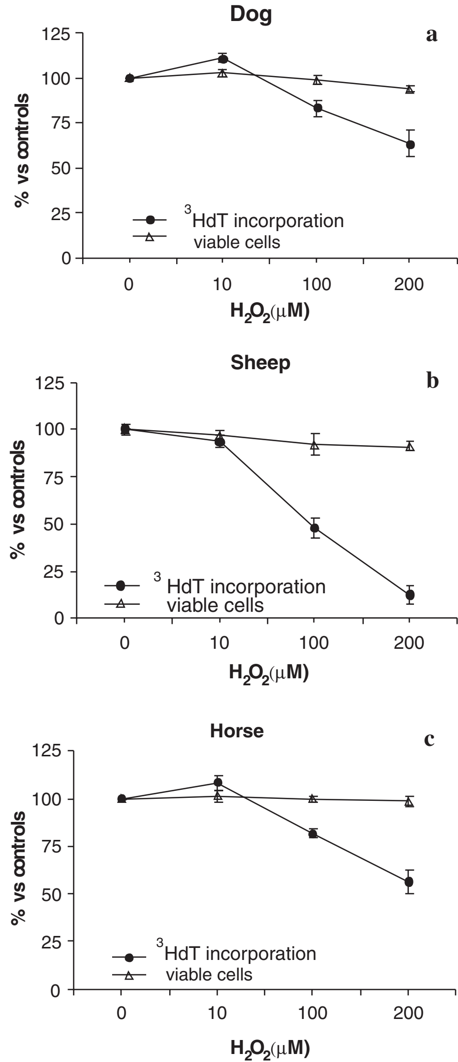

Figure 2. Effect of different H_2O_2 concentrations on the proliferative capacity and viability of lymphocytes from (a) dog, (b) sheep, (c) horse.
Viability (–△–) is expressed as the percentage of viable cells after H_2O_2 incubation vs. viable non treated cells (control). Lymphocyte proliferation (–●–) is expressed as the percentage of [^{3}H]thymidine incorporation (H_2O_2 treated cells vs. non treated cells). Each point is the mean ± SEM of eight determinations.

4. DISCUSSION

Our findings with respect to the species-specific differences in plasmatic GSH seem to indicate that scavenger capacities differ among dogs, sheep and horses. Indeed, the abundance of GSH found in the tissues is evidence of its ubiquitous function in direct and mediated scavenger pathways. GSH levels in the plasma also reflect a concentration of this important ubiquitous cellular antioxidant in other, less accessible, compartments. Moreover, GSH plasma levels under normal conditions are not readily altered, as in other tissues, by acute or transient perturbations such as dietary changes or toxicants [23, 28].

In light of the above findings, it may be confirmed that there are substantial scavenger capacity differences among species. These findings are not particularly surprising given the different molecular structure compositions found in the different species. It is in fact well-known that fatty acids in the tissues of ruminant animals are more saturated due to ruminal bio-hydrogenation. Consequently, it is possible to hypothesise that ruminant tissues are less susceptible to lipoperoxidation, and thus to oxidative damage, than are those of dogs and horses.

The supposed differences among the antioxidant systems in the three species studied were confirmed by our findings regarding GSHPx activity in the lymphocytes.

Because the role of GSHPx in lymphocytes concerns both the counteraction to free radicals and the red-ox modulation of intracellular signalling, the above differences found among dogs, sheep and horses may be interpreted in the light of the different role of this enzyme in cellular trafficking. We believe that GSHPx may have analogous effects in cellular trafficking, although through different, species-specific mechanisms. In other words, during its evolution the species may have developed preferential pathways from those available. For this reason, plasmatic glutathione and lymphocyte GSHPx do not necessarily have the same functional role even though both are antioxidants.

Such a hypothesis could, on the one hand, explain the differences observed among species regarding plasmatic GSH and lymphocyte GSHPx, and is, on the other hand, consistent with our findings concerning the lymphocyte proliferative response to mitogenic stimuli. Indeed, we observed both a different range of blastogenic responses to ConA and different inhibition rates by H_2O_2 during lymphocyte proliferation (Fig. 2). In fact, horse and dog lymphocytes show similar patterns in response to both ConA and H_2O_2. Sheep exhibit the greatest sensitivity to the antiproliferative effects of H_2O_2, as well as the highest proliferative capacity in response to ConA.

This interpretation also enables us to explain the apparent contradictions relative to our previously cited findings [4], which evidenced no significant differences between GSHPx activity in the erythrocytes of different animal species. Indeed, the erythrocyte continuously risks oxidative damage as a result of its high concentrations and close proximity to oxygen and heme [29, 33]. Red blood cells independently of the species from which they are derived, need high levels of both enzymatic and non-enzymatic antioxidants. Only non-genetic factors, nutritional intake of antioxidants or chronic exposure to external stressors may predispose erythrocytes to modify their scavenger systems' efficiency, such as their GSHPx [5].

Our findings may also indicate that 200 μM H_2O_2 is a very dangerous concentration for lymphocyte response capacity to antigenic stimuli (ConA) in all the animal species tested. This, in turn, could affect the main immune function of these cells, namely their blastogenic capacity. On the contrary, it appears that the 10 μM H_2O_2 treatment proved capable of eliciting a proliferative response in the horse and dog, but not in sheep. This last observation concerning horses and dogs is in agreement with evidence that in vitro exposure to low concentrations of H_2O_2 (lower than those used by us) can stimulate growth in some cell types [9].

In conclusion, while high H_2O_2 concentrations are dangerous for the cellular functions of all animals, our findings indicate that it is impossible to predict with certainty, without taking into consideration the animal species studied, the value below which H_2O_2 functions in mediating signalling events.

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