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Variability of Oxygen Radical Absorbance Capacity (ORAC) in Different Animal Species

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The oxygen radical absorbance capacity (ORAC) was measured both in whole (ORAC-T) and deproteinized (ORAC-AS) plasma samples of human, pig, cow, rabbit, dog, cat, sheep, horse, dolphin, turkey, guinea-hen and chicken. In the 12 species, ORAC-T data, expressed as micromoles of peroxyl radicals trapped by 1 l of sample, were found scattered between 8,600 and 23,000 μmol/l. The species with the highest ORAC-T values were cat among mammals and chicken among avies. ORAC-AS values ranged between 600 and 2000 μmol/l, with the highest values found in dolphin and sheep among mammals, while chicken was first among avies. In the 12 species, the relative contribution of ORAC-AS in relation to ORAC-T ranged from 5% to 20%. Protein SH-groups and uric acid were measured in plasma of all species, but no significant correlation was found between thiols and ORAC-T values or between uric acid and ORAC-AS values. Our results show that: (1) the ORAC method is reproducible and sensitive enough to be used in the comparison of the peroxyl-radical absorbance capacity of protein and non-protein plasma components in different animal species; (2) both in mammals and in avies, there is a deep intra-class heterogeneity of ORAC-T and ORAC-AS values; (3) by considering most species, plasma proteins and lipoproteins account for about 85–90% of the overall peroxyl-radical trapping capacity. In the dolphin only, the protein contribution decreases to 80%; (4) uric acid accounts for about one-half of the ORAC-AS value in human, guinea-hen and for about one-third in chicken, while it provides a very limited contribution in other species. We conclude that species with the highest ORAC-T, like cat and chicken, or with the highest ORAC-AS, like dolphin, are interesting models to study the reasons of such a marked antioxidant defense in the plasma.

Keywords: Peroxyl-radicals, plasma, serum, antioxidants, uric acid, animal species

Abbreviations: ORAC-T, oxygen radical absorbance capacity of whole plasma; ORAC-AS, oxygen radical absorbance capacity of deproteinized plasma; GSH, reduced glutathione; AAPH, Azobis(2-amidinopropane) dihydrochloride, B-PE, beta-phycoerythrin; ROS, reactive oxygen species; AUC, area under the decay curve of B-PE; LP, lag phase method; $T_{1/2}$, half-life time method

INTRODUCTION

Blood plasma is extensively and repeatedly exposed to oxygen radical species (ROS) formed by activated phagocytes, by reactions of oxygen with various metabolites or compounds derived from dietary intake and xenobiotics.[1] Plasma is endowed with several protein and non-protein

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components able to cope with various radical species,[2] but in certain situations these antioxidant defenses are overwhelmed and an oxidative stress occurs.[3] Under these conditions the oxidative products of lipids and proteins gradually appear in the blood stream.[4]

It has been shown that oxidative stress is involved in certain human diseases[4,5] and increasing endogenous antioxidant defenses by means of vitamins and oligoelements has received growing attention within the last two decades.[6] Many studies have indicated that vegetables, fruits and grains contain substances that appear to reduce the risk of cancer and vascular disease. Several molecules with striking antioxidant activity have been isolated from vegetables and used to enrich the diet of humans and domestic animals.[7–11] Important results came also from the animal kingdom. Comparative studies among animal species have suggested a relationship between antioxidant protection and longevity.[12] Moreover some molecules that give a genetic advantage against ROS have been discovered and studied in detail. For example, it was discovered that β-carnosine, a dipeptide with antioxidant properties, is present at higher level in the skeletal muscle of animals that are good sprinters, such as horses and greyhounds.[13] This dipepetide is actually used to supplement the diet of athletes and elderly subjects, who are particularly susceptible to oxidant stress.[14–16]

The positive effects of food supplementation with antioxidant molecules can be detected by measuring the increase of the concentration of specific antioxidant agents[9,10,17] or through the assay of the total plasma antioxidant protection.[18–21] Concerning the latter approach, a number of methods, labelled with the acronyms TRAP, FRAP, ORAC, TOSC, have been published.

In this paper, we applied the ORAC method, developed by Cao *et al.*[22] and based on the work by De Lange and Glazer,[23] in an attempt to compare the peroxyl-radical absorbance capacity of plasma from mammalian and avian species. Our goal was to detect intrinsic differences among species or classes of animals and to find the species with the highest anti-peroxyl radical protection. These informations may open a new line of research on the presence of peculiar physiological mechanisms that help some species to cope with increased oxidative stress. We reported the ORAC values of the total and deproteinized plasma in such a way as to provide the anti-peroxyl radical contribution of both protein and non-protein components. The obtained values are discussed on the basis of the concentrations of protein thiols, vitamins, uric acid and other antioxidants.

MATERIALS AND METHODS

Chemicals

Porphiridium cruentum B-phycoerythrin (B-PE), uric acid test-combination Kit was obtained from Boehringer Mannhein (Germany). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Polyscience (Warrington, PA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other analytical grade chemicals were from Sigma (Milan).

Blood Samples

The present study was performed on heparinized blood from human (*Homo sapiens*), pig (*Sus scrofa*), cow (*Bos taurus*), rabbit (*Orictolagus cuniculus*), dog (*Canis familiaris*), cat (*Felix domesticus*), sheep (*Ovis aries*), horse (*Equus caballus*), dolphin (*Tursiups truncatus*), turkey (*Meleagris gallopavo*), chicken (*Gallus domesticus*) and guinea-hen (*Numida meleagris*). Free roaming animals, living under normal field conditions on farms, where the diet was based on cereals, as much natural as possible, were used in this study. In preliminary experiments, rat plasma was also used to assess reproducibility and sensitivity of the method, but

the results were not included in the comparative tables since this animal did not fit in the characteristic of non-caged animal fed with natural food. Dolphin blood was taken during the spring; only fresh fish and no particular diet supplementation was given to the cetacea. Four different animals for each species were sampled. Human blood was collected into heparin from normal male blood donors between aged 25 and 45.

Blood samples were immediately centrifuged at 1200 g for 10 min at 4°C. Plasma was removed and placed on ice. An aliquot was precipitated of proteins as follows: 0.05 ml of plasma was diluted to 0.1 ml with 75 mM Na,K-phosphate buffer pH 7.0; then 0.4 ml of 100% saturated ammonium sulfate were added, mixed and placed on ice for 30 min, samples were then centrifuged at 20,000 rpm for 10 min at 4°C in a Beckman (Fullerton, CA) ultracentrifuge, the supernatants were carefully removed and stored in ice, for immediate use or frozen at −80°C and utilized within 20 days.

ORAC Assay

It was essentially that of Cao *et al.*[22] with slight modifications. The final reaction mixture for the assay was as follows:

Reagents	Blank (μl)	Trolox (μl)	Sample (μl)
Phosphate buffer, 0.75 M	200	100	—
Trolox, 100 μM	—	100	—
Plasma (1 : 100 in phosphate buffer)	—	—	200
B-PE, 0.04 μM	1600	1600	1600
AAPH, 40 mM	200	200	200

A final volume of 2 ml was used in 10 mm wide cuvettes. Reached the stability of the fluorescence, the reaction was started with the addition of the AAPH. The final concentration of the Trolox was 5 μM and the plasma dilution was 1 : 1000. Fluorescence was measured every 5 min at the emission of 565 nm and excitation of 540 nm using a Perkin-Elmer (Norwalk, CT) LS-5 fluorescence spectrophotometer until zero fluorescence occurred.

Calculations

The peroxyl radical absorbing ability of a fluid is given by the number of micromoles of peroxyl radical trapped by 1 litre of fluid. The ORAC value refers to the net protection area under the curve (AUC) of B-PE in the presence of plasma or trolox, minus a blank performed with phosphate buffer or saturated ammonium sulfate. Having established that the trolox stoichiometric factor is 2, i.e. that 1 μmol of trolox is able to trap 2 μmol of peroxyl radicals, the results were calculated with the following equation:

$$\text{ORAC value (μmol/l)} = 2k \times (\text{S sample} - \text{S blank}) / [(\text{S Trolox} - \text{S blank})/5],$$

where k is dilution factor; 2 is the trolox stoichiometric factor; S is the area under the curve (AUC) of B-PE in the presence of the sample or trolox or the buffer, that was integrated by a computer program; 5 is the final concentration of trolox.

In each experiment, the starting fluorescence of B-PE was normalized to 100% arbitrary units to make up for the decay of the stock solution of B-PE or variations in the sensitivity of the fluorimeter.

In some cases, values were also given with the lag phase (LP) or with the $T_{1/2}$ method. The former way is based on the period of complete protection of B-PE exherted by the plasma or trolox. As suggested by Ghiselli *et al.*,[24] this value was calculated by extrapolating the slope of maximal B-PE decay to intersect with the slope of plasma or trolox protection. The quantification of the antioxidant protection (TRAP) was given by the equation:

$$\text{TRAP (μmol/l)} = (\text{Plasma LP} : \text{Trolox LP}) \times 2\ (\text{Trolox conc.}) \times (\text{Dilution factor}),$$

where Plasma LP and Trolox LP are the lag phase times of plasma and trolox respectively and 2 is the stoichiometric factor of trolox.

The $T_{1/2}$ method was based on the determination of the time at which B-PE has reached one-half of the initial fluorescence. The antioxidant capacity of plasma was obtained with the following equation:

$$\text{ORAC } T_{1/2}(\mu\text{mol/l}) = (\text{Plasma } T_{1/2} : \text{Trolox } T_{1/2}) \times 2(\text{Trolox conc}) \times (\text{Dilution factor}),$$

where Plasma $T_{1/2}$ and Trolox $T_{1/2}$ are the half-life times of B-PE fluorescence in the presence of plasma and trolox respectively and 2 is the stoichiometric factor of trolox.

Statistical Analysis

The statistical significance of differences between animal and human plasma was determined with two-tailed Student's *t*-test or ANOVA. The statistical significance level was set at $p < 0.01$ with respect to the reference value.

Other Assays

Uric acid was assayed by the Boehringer test-combination kit. Protein thiols were assayed as reported by Motchnik *et al.*[17] and protein content by the Bradford's method.[25]

RESULTS

Figure 1 shows the plot of fluorescence loss of β-phycoerythrin (B-PE) versus time with rabbit plasma samples at different dilutions. The blank shows an initial acceleration of fluorescence loss followed by a slower decay at 50 min which goes asymptotically to zero. The trolox gives a shoulder of lag phase before the onset of the rapid decay, while the plasma samples show a decay, which is proportional to the dilutions used.

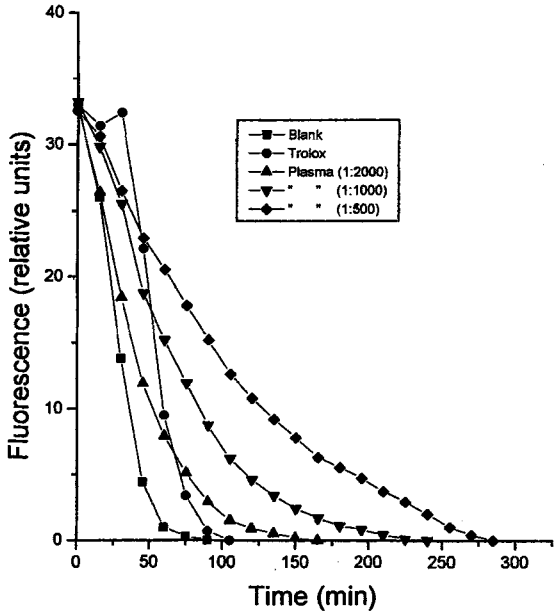

FIGURE 1 Time dependent fluorescence decay of β-phycoerythrin followed in the presence of: (■) none; (●) 5 μM Trolox; 200 μl of: (▲) 1:200, (▼) 1:100, (◆) 1:50 diluted rabbit plasma. The dilutions were done with 0.75 M Na,K-phosphate buffer pH 7.0.

Table I shows the antioxidant capacity of human, rabbit and rat plasma at different dilutions, calculated by three different parameters: the area under the curve (AUC), the lag phase time (LP) and the half-life time ($T_{1/2}$). The data are different in their absolute value; however values found with the AUC-method have a variability coefficient (CV) in the range 10–11%, while the $T_{1/2}$ and the LP method have CV not consistent with a good reproducibility. In this respect we preferred to detect ORAC values by using the AUC-technique, since the results were more sensible and reproduceable in the choosen dilution range.

Table II shows the ORAC activity in the plasma of some mammalian and avian species. The ORAC-T column refers to the whole plasma and shows a wide variability of protection in the range of 8,600–23,000 μmol/l both for mammals and avies. The cat has the highest value of ORAC-T among mammals and chicken among

TABLE I Values of antioxidant capacity of human, rabbit and rat plasma at different dilutions obtained by three different methods

Animal	Plasma dilution	Net AUC	ORAC (μmol/l)	Lag phase (min)	ORAC lag phase (μmol/l)	$T_{1/2}$ (min)	ORAC $T_{1/2}$ (μmol/l)
Human male	100	24031	10656	30′	1000	255′	9444
	250	12548	13911	10′	833	103′	9537
	500	6194	13733	10′	1667	47′	8703
	1000	2587	11472	5′	1667	25′	9259
Mean ± SD			12443		1291		9235
			SD = 1410		SD = 379		SD = 323
			CV = 11%		CV = 29%		CV = 3.5%
Rabbit	500	7216	14249	15′	2143	84′	7925
	1000	3803	15020	10′	2857	56′	10566
	2000	1443	11398	10′	5714	36′	13584
Mean ± SD			13555		3571		10691
			SD = 1557		SD = 1542		SD = 2311
			CV = 11%		CV = 43%		CV = 21%
Rat	250	13098	10047	20′	1428	100′	5555
	500	6748	10352	10′	1428	60′	6666
	1000	4113	12620	10′	2857	42′	9333
Mean ± SD			11006		1904		7184
			SD = 1147		SD = 673		SD = 1585
			CV = 10.4%		CV = 35%		CV = 22%

Orac values were calculated as reported under methods. The reference values for 5 μM Trolox were: AUC, 2620 ± 320; Lag phase, 35 min; $T_{1/2}$, 40 min. Plasma values were diluted with 0.75 M Na,K-phosphate buffer pH 7.0 at the indicated final dilutions. A blank was used in each assay. AUC is the area under the curve of phycoerythrin decay. Statistical analysis was performed with ANOVA. SD = standard deviation; CV = variability coefficient.

TABLE II ORAC values in whole and deproteinized plasma of mammalian and avian species

Order	Scientific name (Common name)	ORAC-T (μmol/l)	ORAC-AS (μmol/l)	ORAC-AS/ORAC-T (%)[a]
Primata	*Homo sapiens* (Caucasian male)	10931 ± 1054	1691 ± 155	15.5
Carnivora	*Canis familiaris* (Dog)	16483 ± 1645*	1362 ± 115*	8.3
	Felix domesticus (Cat)	22627 ± 1011*	1077 ± 66*	4.8
Artiodactila	*Sus scrofa* (Pig)	11374 ± 734	716 ± 63*	6.3
	Ovis aries (Sheep)	16521 ± 1140	1858 ± 123	11.2
	Bos taurus (Cow)	10913 ± 698	686 ± 40*	6.3
Perossidactila	*Equus caballus* (Horse)	9302 ± 699	1000 ± 71*	10.7
Lagomorpha	*Orictolagus cuniculus* (Rabbit)	12820 ± 998	667 ± 41*	5.2
Cetacea	*Tursiops truncatus* (Dolphin)	10714 ± 518	2160 ± 132*	20.2
Galliformes	*Meleagris gallopavo* (Turkey)	13318 ± 1295	862 ± 68*	6.5
	Numida meleagris (Guinea-hen)	8670 ± 713	1165 ± 81*	13.4
	Gallus domesticus (Chicken)	23320 ± 1527*	1717 ± 159	7.4

Values of ORAC total were determined on 200 μl of 1 : 100 diluted plasma, while ORAC-AS values were determined on 200 μl of 1 : 10 diluted ammonium sulphate (AS) deproteinized plasma. Values were mean ± SD of four different experiments. [a] These values represented the antioxidant contribution of non-protein plasma components with respect to the whole plasma. * The statistical significance level was set at $p < 0.01$ with respect to Homo Sapiens.

avies. The ORAC-AS column refers to deproteinized samples and shows that plasma non-protein components protect less than whole plasma. The relative contribution of ORAC-AS in relation to ORAC-T ranges from 4.8% to 20.2%. Relatively high values of ORAC-AS were found in dolphin and sheep among mammals, while chicken was first among avians.

Table III shows the protein content and the net peroxyl radical trapping capacity of 1 mg of

proteins in the plasma of each species. This latter value was obtained from the ORAC-T deprived of the ORAC-AS contribution and expressed per mg of protein. Figure 2 gives a clearer comparative view of this data in an histogram plot. Chickens among avians and cats among mammals have the highest ORAC per mg protein. Table III also shows the content of thiol groups per liter (l) of plasma and the thiols per ORAC units. In both columns, humans have the highest value. An attempt to correlate the thiol values with the net ORAC per mg proteins by linear regression analysis, did not yield any significant correlation, either by considering all species or mammals only (data not shown).

Since uric acid is recognized as the most efficient antioxidant among non-protein plasma components,[26] we measured this metabolite in the plasma of all the species under study. Considering that the relative peroxyl radical absorbing capacity of 1 μM uric acid is 1.84,[22,28] we drew the corresponding ORAC-uric acid value of our samples. Table IV shows the uric acid concentration and ORAC-uric acid values. From these data, it emerges that among all the species, humans, chickens and guinea-hens receive from uric acid about one-third of ORAC-AS protection, while the other species received a lower

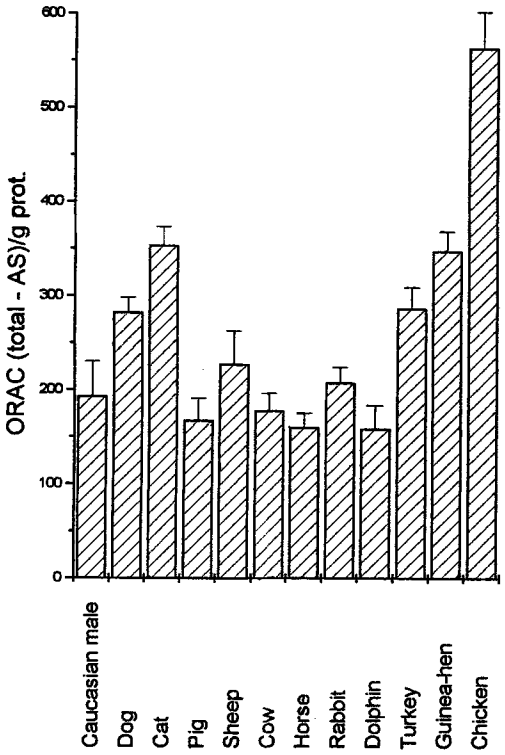

FIGURE 2 ORAC units per mg of plasma proteins in different animal species. The histogram was drawn with data of Table II, column 4. The net ORAC value was drawn by subtracting the ORAC-AS from the ORAC-T and dividing the obtained value for the protein content.

TABLE III ORAC due to protein and thiol contribution in plasma of mammalian and avian species

Animal	Plasma proteins (g/l)[a]	ORAC (Total-AS) (μmol/l)	ORAC (Total-AS)/g prot (μmol/g prot.)[b]	Thiols (μmol/l)[c]	Thiols/ORAC (%)[d]
Caucasian male	47.8 ± 5.3	9240 ± 844	193.3 ± 37	284 ± 23	3.07
Dog	53.6 ± 5	15121 ± 1544	282.1 ± 16*	166 ± 13*	1.09
Cat	61.0 ± 8.9	21550 ± 2202	353.3 ± 20*	244 ± 21	1.13
Pig	63.7 ± 2.1	10658 ± 1069	167.3 ± 24	31.8 ± 2.9*	0.30
Sheep	64.5 ± 1.3	14663 ± 1234	227.3 ± 35	238 ± 19	1.60
Cow	57.6 ± 2.3	10227 ± 1096	177.6 ± 19	224 ± 20*	2.19
Horse	52 ± 2.4	8302 ± 777	159.7 ± 15.5	135 ± 11*	1.62
Rabbit	58.6 ± 1.8	12153 ± 987	207.4 ± 16.5	199 ± 16*	1.63
Dolphin	53.9 ± 2.7	8557 ± 699	158.7 ± 25	11 ± 2*	0.12
Turkey	43.5 ± 3	12456 ± 918	286.3 ± 23*	52 ± 4*	0.42
Guinea-hen	21.6 ± 2.5	7505 ± 621	347.5 ± 21*	52 ± 4*	0.69
Chicken	38.2 ± 8.4	21603 ± 1612	565.5 ± 38.5*	50 ± 3*	0.23

[a,c] Values were mean ± SD from four different experiments.
[b] ORAC values were drawn from the data of Table I.
[d] This value represents the percentage of ORAC due to one micromole of thiols.
* Statistical significance level set at $p < 0.01$ with respect to Caucasian males.

TABLE IV ORAC due to uric acid and ORAC due to other non-protein plasma components

Animal	Uric acid (μmol/l)	ORAC-Uric acid (μmol/l)[a]	ORAC-Undef. (μmol/l)[b]
Caucasian male	297.7 ± 28	547.8 ± 51.6	1143.2 ± 103
Dog	10.7 ± 3.3	19.6 ± 6*	1342.3 ± 70*
Cat	28.8 ± 2.4	52.9 ± 4.4*	1024 ± 87
Pig	7.4 ± 0.32	13.6 ± 1.4*	702.4 ± 40*
Sheep	40.5 ± 13.6	74.5 ± 32.4*	1783.5 ± 134*
Cow	n.d.	n.d.	n.d.
Horse	9.0 ± 1.6	16.6 ± 3.0*	983.4 ± 48
Rabbit	10.2 ± 0.93	18.8 ± 1.6*	648.2 ± 35*
Dolphin	41.8 ± 4.6	76.9 ± 8.4*	2083.1 ± 129*
Turkey	69.8 ± 23	128.4 ± 42*	733.6 ± 46*
Guinea-hen	206 ± 38	379 ± 68*	786 ± 54*
Chicken	238 ± 71	437.9 ± 130.6	1279 ± 72

Values are mean ± SD of the different experiments; n.d. = not detectable.
[a] ORAC uric acid is the antioxidant contribution of uric acid, obtained by considering a protection of 1.84 units per mole, as reported by Cao *et al.*[22] and confirmed by us.
[b] ORAC-Undef. is the antioxidant contribution of non-protein components different from uric acid, which was calculated by subtracting ORAC-Uric acid from ORAC-AS values.
* Statistical significance level set at $p < 0.01$ with respect to Caucasian male.

protection. The difference between ORAC-AS and ORAC-uric acid, reported in column 4 of Table IV, was referred as to ORAC-undefined, a value that expresses the anti-peroxyl radical capacity of plasma non-protein components different from uric acid. Data in this column show that sheep and dolphin have a marked protection among mammals and chicken among avians.

DISCUSSION

Several methods[19–24,27–31] have been developed to assess the total antioxidant capacities in serum and plasma. These methods have been needed for two reasons: one is the difficulty in measuring each antioxidant component separately; another is the potential interactions among different antioxidant components in complex biological samples. Almost all of these methods are inhibition methods: a free radical reaction is involved and its inhibition by an added antioxidant sample is quantitated as its antioxidant capacity. Two elements need to be considered in measuring the inhibition of the reaction by an antioxidant sample: one is the length of time that the inhibition lasts and the other is the degree of inhibition displayed at different times. The ORAC assay is, to date, the only method that takes a free radical reaction to completion and uses an area under the curve technique for quantitation, thus combining both inhibition time and inhibition degree of the free radical action by antioxidants into a single quantity.[22,28] Other methods[24,27,29–31] relate either the inhibition time at a specific inhibition percentage, i.e. a lag phase, or the inhibition percentage at a specific time point, i.e. ($T_{1/2}$), to the antioxidant capacity of a sample. This represents the main disadvantage of these methods, since some antioxidants, such as melatonin,[32] GSH,[33] and albumin[34] do not even show a lag phase in inhibiting free radical actions and two compounds having the same inhibition percentage at one time point, may exhibit different inhibition percentages at another time point.[33,34] One of the advantages of the ORAC assay is that different reactive species can be used in the assay.[28,33] However, peroxyl radicals are the most important physiologically and pathologically. With AAPH as a peroxyl radical generator, the ORAC assay measures all non-enzymatic antioxidants, whether they are water- or lipid-soluble. Examples of these antioxidants include ascorbic acid, α-tocopherol,

β-carotene, GSH, methionine, uric acid, bilirubin, phenolic acids, flavanols, flavonols, flavones, isoflavones, flavanones and anthocyanins.[22,28,32,33,35–38]

Among the available methods, we preferred to compare the peroxyl radical absorbance capacity of plasma from mammalian and avian species by the ORAC method of Cao *et al.*[22,28] In our experience, the evaluation by a computer program of the AUC provides a precise and reproducible ORAC-T values, which take into account not only the SH-groups but also other protein residues that work as radical scavengers during the propagating reactions within the protein structure.[5] By the ammonium sulfate (AS) precipitation, we were able to separate the proteins from the serum and we provided the ORAC-AS values, i.e. the anti-peroxyl radical capacity of non-protein components.

With human plasma, our results were similar to those found by Cao *et al.*[28] who obtained 4267 ± 414 μmol of trolox equivalents per liter of sample. Since 1 μmol of trolox is able to trap 2 μmol of peroxyl radicals, the micromoles of peroxyl radical trapped by 1 l of sample must be obtained by multiplying their value for a factor 2, which provides results very similar to ours.

The present results show a deep intra-class heterogeneity both in mammals and in avians for ORAC-T and ORAC-AS values. Significant differences were also present inside to the same order, like in the case of galliformes, where chicken ORAC-T value was 2.6 fold higher than that of guinea-hen.

Concerning the relative contributions of ORAC-AS versus ORAC-T values, we can infer that the antioxidant contribution of non-protein plasma components was about 15% of the total. In all mammals, except dolphin, the ORAC-AS contribution was within the range 6–15% with respect to ORAC-T. In cats, the mammal with the highest ORAC-T value, we found the lowest contribution of the ORAC-AS (4.8%).

Since in most animal species, the protein components account for about 90% of the total plasma peroxyl radical absorbance capacity, we inferred that the plasma antioxidant protection is mainly of genetic origin and depends on the type and concentration of plasma proteins. Among these, the most important is albumin, which represents from 40% to 70% of plasma proteins[39] and can react directly with peroxyl radicals mainly by means of its thiol-groups. It must be noted that protein sulfhydryl groups are "sacrificial" antioxidants, which are not easily regenerable when oxidized.[2,40] However albumin is characterized *in vivo* by a rapid turnover that means a rapid renewal of oxidized groups with reconstitution of antioxidant efficiency. Another interesting insight into the antioxidant role of albumin comes from the recent discovery,[41] that serum albumin possesses a glutathione-linked thiol-peroxidase activity. This can explain why species with the highest thiol concentration do not show the highest ORAC/g protein. The reason of this lack of correlation between the two parameters, shown in Table III, probably lies in the different contribution of the thiol-peroxidase activity of albumin of the animal species. Moreover, some species may take advantage of the fact that the thiol-peroxidase activity of albumin may be sustained by a fast regeneration of glutathione and other reductants by means of plasmatic glutathione reductase activity,[42] which is another enzyme presenting wide differences in vertebrates.

It is unlikely that the explanation of the very high antioxidant contribute of protein versus non-protein components may be traced to the content of plasma vitamin E, which is included in chylomicrons with lipoproteins.[6] Plasma vitamin E concentration shows consistent differences among species[39] but values are in any case too low for representing a sensitive contribution to the ORAC-T values.

Another interesting result regards the dolphin, which has ORAC-T value similar to humans, but shows the highest ORAC-AS value. This means that this mammal is endowed of active non-protein antioxidant molecules, which remain to be determined.

Among the non-protein radical scavengers, the most important plasma component is uric acid, which is able to scavenge the peroxyl radicals directly.[2] The contribution of uric acid to the ORAC-AS value was very low in dolphin and in other species, while it was 25% in chicken and about 32% in human and guinea-hen. The contribution to ORAC-AS of molecules different from uric acid was grouped in a numerical value, called ORAC-undefined.

In conclusion, plasma of different animal species is a complex mixture of antioxidant and pro-oxidant molecules, so that the individual evaluation of single components is very difficult to be performed. The evaluation of the whole antioxidant capacity of freshly drawn plasma and serum is thus an obligate step in the study of the oxidant stress that takes place *in vivo*. The ORAC method is reproducible and sensitive enough to be used in the comparison of the peroxyl radical absorbance capacity of protein and non-protein plasma components in different animal species. In all species, the protein antioxidant contribution ranges from 85% to 90% of the total plasma antioxidant capacity, but this cannot be ascribed only to the thiols but likely to some plasma enzymes which remain to be determined. Species, such as cat and chicken, which have the highest ORAC-T values and like dolphin, which has the highest ORAC-AS, are interesting models to study the physiological mechanisms that guarantee such a high and efficient protection against peroxyl radicals.

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