Original Article

Multiple free-radical scavenging (MULTIS) capacity in cattle serum

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Multiple free-radical scavenging (MULTIS) activity in cattle and human sera was evaluated with electron spin resonance spectroscopy. Scavenging rates against six active species, namely hydroxyl radical, superoxide anion, alkoxyl radical, alkylperoxyl radical, methyl radical, and singlet oxygen were quantified. The difference in the electron spin resonance signal intensity in the presence and absence of the serum was converted into the scavenging rates. Comparative MULTIS measurements were made in sera from eight beef cattle, three fetal calves and fifteen healthy human volunteers. Further, we determined the MULTIS value of albumin, the most abundant component in serum. MULTIS values in cattle sera indicated higher scavenging activity against most free radical species tested than human sera. In particular, cattle serum scavenging activities against superoxide and methyl radical were higher than human serum by 2.6 and 3.7 fold, respectively. In cattle serum, albumin appears to play a dominant role in MULTIS activity, but in human serum that is not the case. Previous data indicated that the abundance of uric acid in bovine blood is nearly 80% less than humans; however, this difference does not explain the deviation in MULTIS profile.

***Key Words*: cattle serum, multiple radical scavenging capacity, MULTIS, antioxidants, spin trapping**

Antioxidant defense capacity in mammals may have species as well as age dependence. A clear indication of such notion is that some mammals, including humans and monkeys, lack ability to biosynthesize ascorbic acid (vitamin C), a major endogenous antioxidant.[1] Genetically altered mice that did not have ascorbate biosynthesis ability showed elevated lipid peroxidation in the liver tissue as compared with wild type.[2,3] Therefore, it is reasonable to speculate that free radical scavenging capacity in the body fluids of mammals could have species and age dependence. The present study was motivated by these notions.

Usually, antioxidant components in the blood show scavenging abilities against multiple free radical species that are found in biological systems.[4] The scavenging rates depend on the kind of target free radical species, while a majority of previous studies determined the scavenging rate against a single free radical species.[5–8] We have developed a new method to measure the scavenging ability against multiple free radical species (MULTIS method). This method has made it possible to readily determine the scavenging rates against multiple free radical species.[4] In addition, in this report serum scavenging ability (MULTIS value) was evaluated using the sum of each serum component's scavenging-rate, i.e., rate-constant multiplied by its concentration.

Previously, using the MULTIS method, free radical scavenging capacity against multiple free radical species, i.e., hydroxyl radical, superoxide anion, alkoxyl radical, alkylperoxyl radical, methyl radical, and singlet oxygen, was measured in human serum.[4] The report showed that the MULTIS capacity in the serum obtained from chronic kidney disease (CKD) patients was significantly modified as compared with healthy control group. Specifically in the CKD group, serum superoxide scavenging capacity increased by nearly 60%, while that for methyl radical decreased by 74%. Also, there was a moderate decrease in scavenging capacities for hydroxyl radical and singlet oxygen in CKD group.[4] Although there is a possibility that these changes are disease specific, whether these changes are related to the pathological states are not understood at present. We were hinted by this finding and have initiated a project to determine the variation in serum MULTIS profile among various mammals and age.

Materials and Methods

Six free radical species (FRS) include hydroxyl radical ($HO^{\bullet}$),[9] superoxide anion ($O_2^{\bullet-}$),[10] alkoxyl radical ($RO^{\bullet}$),[11] alkylperoxyl radical ($ROO^{\bullet}$),[12] methyl radical ($H_3C^{\bullet}$),[13] and singlet oxygen ($^1O_2$).[14] The essence of scavenging capacity (rate) evaluation with the MULTIS method is to compare free radical concentration in the presence and absence of the antioxidant. For the determination of free radical concentration, electron spin resonance (ESR) spin trapping method was employed.

Materials and equipment. Spin trapping compounds, DMPO (5,5-dimethyl-pyrroline *N*-oxide), CYPMPO [5-(2,2-dimethyl-1,3-propoxy cyclophosphoranyl)-5-methyl-1-pyrroline *N*-oxide], and 4-HO-TEMP (4-hydroxy-2,2,6,6-tetramethylpiperidine) were obtained from Funakoshi (Tokyo, Japan), Radical Research Inc. (Hino, Japan), and Tokyo Chemical Ind. (Tokyo, Japan), respectively. DMPO was used for the detection of $H_3C^{\bullet}$, $RO^{\bullet}$ and $HO^{\bullet}$, because it provides the stable spin adducts and simple ESR signal patterns. CYPMPO was used for $O_2^{\bullet-}$ and *tert*-$BuOO^{\bullet}$ because it has better trapping capability against these free radicals than DMPO.[15] Oxidation reaction of 4-HO-TEMP was utilized to quantify $^1O_2$. The experimental conditions when free radical precursors and photosensitizers were used to produce six FRS are listed in Table 1. These chemicals were purchased from Nacalai Tesque (Kyoto, Japan) and dissolved in 100 mM phosphate buffer (PB) and subjected to *in situ* photolysis. These FRS were produced with the UV/Vis light irradiation (RUVF-203S, Radical Research Inc.) in the presence or absence of serum.

In the presence of spin trap, hydroxyl and alkoxyl radicals were generated with UV irradiation of H_2O_2 and AAPH, respectively.

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Table 1. Precursors/sensitizers and experimental conditions for the formation of multiple free radicals in the MULTIS method

Free radical	Experimental concentrations of precursor/sensitizer and serum in 100 mM PBS	Filter	Irradiation time
$HO^{\bullet}$	H_2O_2 (5 mM), DETAPAC (1 mM), DMPO (5 mM), serum (3%)	None	5 s
$O_2^{\bullet-}$	Riboflavin (60 μM), EDTA (10 mM), CYPMPO (10 mM), serum (3%)	Band-path	30 s
$RO^{\bullet}$[a]	2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) (5 mM), DMPO (5 mM), serum (3%)	None	1 s
tert-$BuOO^{\bullet}$	*t*-Butylhydroperoxide (20 mM), EDTA (5 mM), CYPMPO (5 mM), serum (3%)	None	15 s
$^{\bullet}CH_3$	Dimethyl sulfoxide (100 mM), H_2O_2 (20 mM), DETAPAC (1 mM), DMPO (5 mM), serum (3%)	None	15 s
$^1O_2$	Rose Bengal (50 μM), 4-HO-TEMP (40 mM), serum (1%)	Band-path	1 s

[a]Alkoxyl radical derived from AAPH.

Table 2. Free radical scavenging rates[a] relative to the spin trap in cattle and human serum

Subjects	M/F	Age	$HO^{\bullet}$ (DMPO)[b]	$O_2^{\bullet-}$ (CYPMPO)	$RO^{\bullet}$ (DMPO)	*tert*-$BuOO^{\bullet}$ (CYPMPO)	$^{\bullet}CH_3$ (DMPO)	$^1O_2$ (4-OH-TEMP)	Attribute
Cattle 1	M		152 ± 22[c]	253 ± 18	75.0 ± 4.5	54.2 ± 4.3	160 ± 30	5,580 ± 540	castrated
Cattle 2	M		103 ± 10	232 ± 30	65.1 ± 2.9	46.2 ± 3.9	153 ± 27	5,260 ± 590	castrated
Cattle 3	M		152 ± 20	239 ± 19	74.6 ± 4.7	50.6 ± 7.6	172 ± 39	5,710 ± 640	castrated
Cattle 4	F		102 ± 19	263 ± 18	57.2 ± 2.9	52.4 ± 9.5	121 ± 19	4,180 ± 390	antepartum
Cattle 5	F		99.6 ± 10.1	218 ± 29	67.4 ± 4.3	53.8 ± 3.9	126 ± 15	4,960 ± 460	antepartum
Cattle 6	F		113 ± 21	146 ± 16	70.8 ± 4.0	68.6 ± 8.7	164 ± 32	5,260 ± 660	antepartum
Cattle 7	F		116 ± 16	208 ± 23	64.6 ± 2.7	56.0 ± 3.6	140 ± 31	4,080 ± 390	antepartum
Cattle 8	F		120 ± 17	143 ± 18	50.5 ± 3.2	47.8 ± 3.0	114 ± 17	4,120 ± 600	antepartum
Cattle Av. (*n* = 8)	3M/5F	—	120 ± 20	213 ± 42	65.7 ± 7.9	53.7 ± 6.4	144 ± 20	4,890 ± 630	—
Fetal bovine Av. (*n* = 8)	—	—	89.8 ± 8.4	256 ± 37	81.8 ± 4.8	65.1 ± 7.1	10.4 ± 4.1	4,520 ± 279	—
Bovine serum albumin (*n* = 1)	—	—	83.1 ± 4.6	61.2 ± 8.3	42.9 ± 6.1	45.2 ± 7.2	23.2 ± 1.9	4,880 ± 90	—
Human Av. (*n* = 15)	10M/5F	35 ± 15	90.8 ± 29.3	83.0 ± 26.6	111 ± 20	38.8 ± 13.6	39.3 ± 8.1	6,100 ± 950	—
Human serum albumin (*n* = 1)	—	—	58.2 ± 1.2	60.4 ± 1.1	17.0 ± 0.4	47.0 ± 0.8	~0	2,080 ± 80	—

[a]Relative scavenging rate [$(I_0/I_{Serum}) - 1$] of 100% serum against 1 mM ST (ST = DMPO and CYPMPO). For singlet oxygen, relative rate of 100% serum against 1 μM 4-HO-TEMP. [b]Spin traps used in this study are shown in parentheses. [c]SD.

After UV irradiation, the ESR signal intensities of the radical adducts were measured. Superoxide and singlet oxygen were formed by using the photosensitizers riboflavin and rose bengal, respectively. The alkylperoxyl radical was generated according to the established reaction mechanism reported by Bors *et al.*[12] Methyl radical was produced from the photolysis of dimethyl sulfoxide (DMSO) plus hydrogen peroxide. In methyl radical scavenging, the hydroxyl radical adduct of DMPO was not present, suggesting that the reaction of hydroxyl radical with antioxidants in serum is negligible.

A JEOL FA200 X-band spectrometer (Akishima, Japan) was used to record ESR spectra of trapped free radicals (spin adducts). ESR spectrum was assigned to each spin adduct and ESR signal height was measured to obtain free radical concentration.

Serum collection. Cattle sera were collected by jugular vein venipuncture from 8 beef cattle of various age and sex (Table 2) that were maintained at Sumiyoshi livestock science station, the University of Miyazaki. The treatment of animals strictly complied with the "Guide to humane treatment of experimental animals" that was published by the University of Miyazaki (https://www.miyazaki-u.ac.jp/guide/files/kisoku_01.pdf). Fetal bovine serum [*n* = 3 (country of origin: USA, Canada, and Mexico)] were purchased from Life Technologies Company. Human serum was collected from 15 healthy volunteers (5 female, 10 male; average age 35) at Asao Clinic following the protocol approved by the human subject use committee in Asao Clinic.

Bovine serum albumin (bovine origin, Lot No. M3H125) and human serum albumin (human origin, Product No. SRP6182) were separated from blood by Nacalai Tesque (Kyoto, Japan) and Aldrich Chemical Company Inc. (Milwaukee, WI), respectively: These specimens are claimed to be fatty acid and globulin free, purity >98%. Based on the previous data determined by colorimetric and electrode method,[16–18] we prepared the PB solutions of bovine (3.9 g/dl) and human (4.3 g/dl) albumins.

Scavenging rate calculation. Formulations to calculate scavenging rate were described elsewhere:[4] It is important to point out that the free radical scavenging activity in serum is due to the activity by multiple but unknown number (*n*) of antioxidants. Therefore, in the presence of serum and the spin trap (ST), the free radical ($R^{\bullet}$) scavenging reaction should occur as follows:

$$\begin{aligned} &ST + R^{\bullet} \rightarrow \text{Spin adduct} && \text{rate constant: } k_{ST}. \\ &AOx(1) + R^{\bullet} \rightarrow P_1, && \text{rate constant: } k_1 \\ &AOx(2) + R^{\bullet} \rightarrow P_2, && \text{rate constant: } k_2 \\ &\quad\vdots \\ &AOx(n) + R^{\bullet} \rightarrow P_n, && \text{rate constant: } k_n \end{aligned}$$

where AOx(*i*) denotes antioxidant (*i*) that is present in serum. "Relative scavenging rate (v_{Serum}/v_{ST})" for the above reaction of antioxidants in serum is calculated according to the following equation[4]:

$$\frac{v_{Serum}}{v_{ST}} = \frac{I_0 - I_{Serum}}{I_{Serum}} = \frac{\sum_i^n k_i[AOx(i)]_0}{k_{ST}[ST]_0} = \frac{\sum_i^n k_i\alpha_i[AOx(i)\%]_0}{k_{ST}[ST]_0} \quad (1),$$

where I_{Serum} and I_0 are ESR signal heights in the presence and absence of serum, respectively. The $[\,]_0$ and [%] symbols express the initial concentration (M) and the concentration in volume %, respectively. α_i is constant.

The procedure to use Eq (1) was as follows: first, prepare a phosphate buffer containing the spin trap and the free radical source and UV light was illuminated, then the height (I_0) of the selected ESR line was recorded. In the separate experiment, the

same procedure was repeated after the addition of a finite volume (typically 3% of total volume) of the serum and the ESR height (I) was recorded. Using I_0, I, $[Serum]_0$, and $[ST]_0$ according to Eq (1), the relative scavenging rates (v_{Serum}/v_{ST}) of 100% serum against 1 mM ST can be readily calculated. Using Eq (1), the relative antioxidant ability of serums (1) and (2) can be expressed as follows:

$$\frac{v_{Serum(2)}}{v_{Serum(1)}} = \frac{(I_0 / I_{Serum(2)}) - 1}{(I_0 / I_{Serum(1)}) - 1} = \frac{\sum_i^n k_i \alpha_i [AOx(i)(2)\%]}{\sum_i^n k_i \alpha_i [AOx(i)(1)\%]} = \frac{\sum_i^n k'_i [AOx(i)(2)\%]}{\sum_i^n k'_i [AOx(i)(1)\%]} \quad (2),$$

where $k'_i = k_i \alpha_i$.

The above equation indicates that no knowledge is necessary about the antioxidant component that is responsible for the FRS scavenging activity (MULTIS capacity) when the relative MULTIS values are calculated.

Results

Fig. 1 demonstrates the decrease of ESR peak intensity of DMPO's HO• adduct by the addition of human serum. Using the ESR peak height I_0 before the addition of serum and the peak height I_{Serum} after the addition, the scavenging rate against HO• radical was calculated with Eq (1) as $(I_0 - I_{Serum})/I_{Serum}$. Because this calculation uses the intensity ratio but not the absolute intensity, the results did not depend on the kind of spin trap used. This procedure was repeated for other five free radical species. The measurement was repeated for 5 times for the same serum and the error was shown as SD. The reproducibility for the active species scavenging rates in various dilution of serum ([Serum %] = 1, 3, and 5) was within 8% (SD).

Table 2 shows relative scavenging rates (MULTIS values) for bovine serum albumin, individual beef cattle, fetal bovine serums, and averaged human values are listed. In Fig. 2, MULTIS results are illustrated in bar graph that represents the relative scavenging abilities of cattle serum against fetal bovine serum, together with those of bovine serum albumin. Fig. 3 shows the difference in the active species scavenging abilities of human and cattle serum.

Discussion

For many years, veterinarians recognized that vitamin rich feeds for daily cows increase milk production and promote overall health. In peripartum cows, vitamin E and zinc supplementation have shown to increase energy metabolites and milk production, and decrease lipid peroxidation.[19,20] Such effects are believed to be due to direct or indirect free-radical scavenging activity of these agents. *In vivo*, various endogenous antioxidants in body fluids may play the same role.

This investigation took advantage of the MULTIS method and determined MULTIS capacity of beef cattle serum for the first time. Although beef cattle from which blood was collected did not

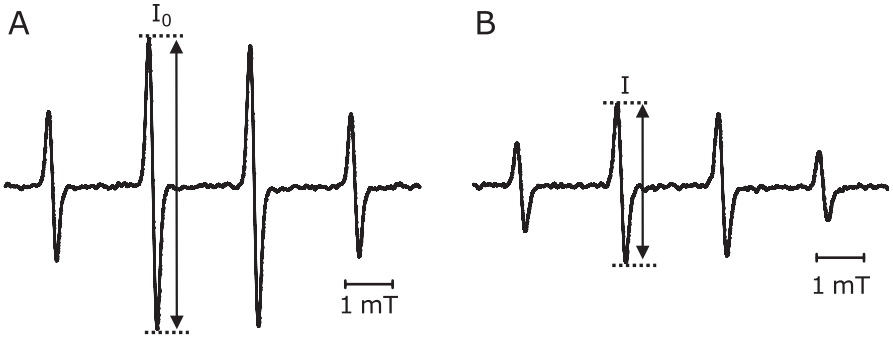

Fig. 1. ESR spectra of HO• adduct produced after photolysis of hydrogen peroxide (5 mM) solution containing DMPO (5 mM), and DETAPAC (1 mM) in the absence (A) and the presence (B) of 3% human serum. Peak heights used for the calculation of the scavenging rate are shown in the spectra.

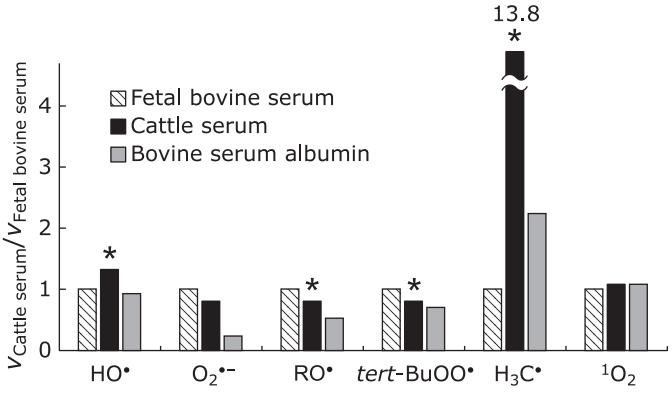

Fig. 2. A bar graph that represents relative scavenging rates in fetal bovine serum, cattle serum, and bovine serum albumin. The symbol of * denotes that statistical difference is significant (p<0.05 vs fetal bovine serum).

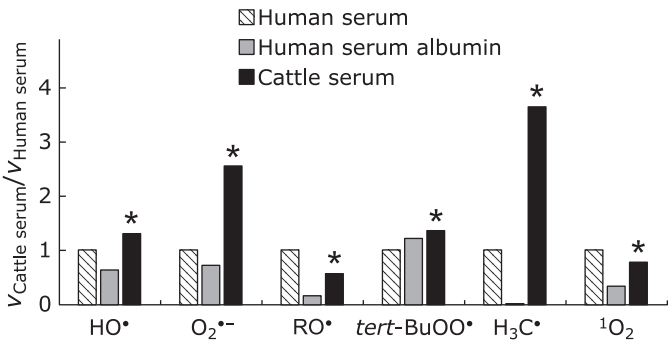

Fig. 3. A bar graph that represents relative scavenging rates in human serum, human albumin, and cattle serum. The symbol of * denotes the presence of significant difference (p<0.05 vs human serum).

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Table 3. Abundance of blood component in cattle, fetal bovine, and human blood

Blood component		Cattle[a] (whole blood)	Fetal bovine[c]	Human[d] (plasma)
TP	Total protein (g/dl)	6.99 ± 0.50	5.0	6.0–8.3
Alb	Albumin (g/dl)	3.87 ± 0.36	1.6	3.5–5.2
BUN	Blood urea nitrogen (mg/dl)	12.4 ± 3.0	15.9	9–21
Glu	Glucose (mg/dl)	75.1 ± 7.0	31.8	70–105
T-cho	Total cholesterol (mg/dl)	105.5 ± 19.6	75.0	100–220
TG	Triglyceride (mg/dl)	29.9 ± 6.1		25–300
Pi	Inorganic phosphorus (mg/dl)	6.67 ± 1.16		2.3–4.5
Ca	Calcium (mg/dl)	9.73 ± 0.68	1.39	8.4–11.5
Cre	Creatinine (mg/dl)	0.77 ± 0.14[b]	2.4	0.8–1.5
UA	Uric acid (mg/dl)	0.71 ± 0.43[b]	4.8	2.6–6.0

[a]19–24 months, *n* = 94 ref (16). [b]various age, *n* = 12 ref (17). [c]Data base of Tokyo University of Marine Science and Technology (http://www2.kaiyodai.ac.jp/~hasobe/2014-Kit-Level2/Exp%20Data/Set1-TF-Brows.html). [d]Cited from ref (18).

have specific disease, they have diverse health history such as pregnancy or castration. Because the total number of cattle were relatively small, it was not possible to conclude that the discrepancy in MULTIS values was caused by the difference in physical conditions. Therefore, serum MULTIS value for each FRS was averaged and deemed as that of healthy cattle.

Fig. 2 shows the difference in the relative free radical scavenging abilities in adult cattle serum of Miyazaki against fetal bovine serum. The scavenging capacities against superoxide, alkoxyl radical, and alkylperoxyl radical are slightly lower than those of fetal bovine serum. Conversely, the scavenging abilities of hydroxyl and methyl radicals is higher compared to those of fetal bovine. It is noted that healthy cattle group showed a remarkably high scavenging activity against methyl radical.

The molecular weight of the albumin in humans is comparable with that of bovine. Human and bovine albumins contain 16% nitrogen and are often used as standards in protein calibration studies. As shown in Table 3, cattle and human serums contain a large amount of albumin. Therefore, MULTIS measurement of bovine serum albumin may be informative to elucidate the active species that is responsible for MULTIS activity. Fig. 2 and Table 3 show that the scavenging abilities of the bovine serum albumin against alkylperoxyl radical and singlet oxygen are comparable to those of the cattle serum, suggesting that albumin is the dominant antioxidant component in cattle serum for scavenging of alkylperoxyl radical and singlet oxygen. In the case of human serum, albumin's contribution was significant only in alkylperoxyl radical scavenging (Fig. 3). Because albumin content in human and bovine serum is close (Table 3), the difference in MULTIS profile may be originated from other components than albumin. Although the components that are available for comparison between cattle and humans are limited, the difference in uric acid abundance is outstanding: i.e., 0.3–1.1 mg/dl for cattle and 2.6–6.0 mg/dl for human. Independent from the present study, MULTIS capacity of pure uric acid has been published,[21] where uric acid showed scavenging capacity against superoxide nearly 5 times higher than the standard antioxidant trolox. It is obvious that the present higher superoxide scavenging capacity in bovine serum is not explainable with the difference in uric acid content. Rather, the tendency is opposite. Also, uric acid shows no scavenging capacity against methyl radical.[21] We believe that the differences in dietary and metabolism (the production of volatile fatty acids in the case of cattle) participate in the MULTIS values of cattle and human. Alkyl radical including methyl radical is known to be highly hydrophobic[22] and the serum of beef cattle is rich in hydrophobic components such as lipids. Taken together, we speculate that free radical scavengers in lipid portion could be effective in raising methyl radical MULTIS capacity in beef cattle serum.

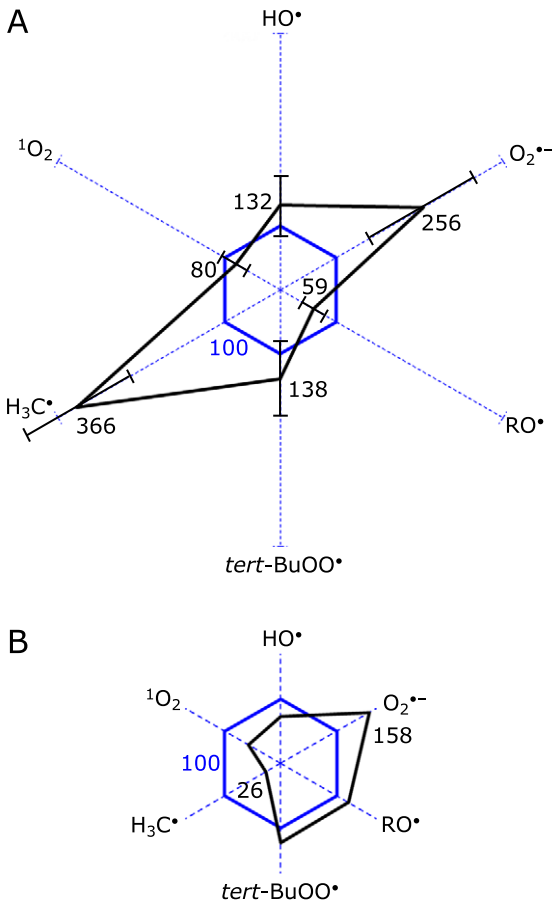

Fig. 4. (A) Radar chart illustration of relative MULTIS values of human and cattle serum. MULTIS values in human serum are set to 100, thus its radar chart is a normal hexagon. (B) The previously published MULTIS radar chart for the healthy human and CKD patients group.[4]

In human serum, relative changes in MULTIS values by disease conditions provided meaningful results;[4] likewise, we focus this investigation to the comparison of MULTIS values between cattle and human. For the purpose of visible comparison, the difference is illustrated using radar chart method (Fig. 4A). In Fig. 4B, the previously published MULTIS radar chart for the healthy human subjects (normal hexagon) and CKD patients group is shown.[4] As indicated in Table 2 and Fig. 3, notable differences in MULTIS

values were obtained in the scavenging capacities against superoxide radical (2.6 fold increase in cattle) and methyl radical (3.7 fold increase in cattle). It is of interest to point out that in human serum, scavenging capacities against superoxide anion and methyl radical are the two of the most affected by the presence of disease conditions.[4]

In conclusion, using the MULTIS method we determined serum scavenging capacities against six free radical species in healthy cattle and compared them with humans. Beef cattle serum showed the high scavenging abilities against superoxide anion and methyl radical as compared with human serum. We plan to expand this study to other mammals such as rodents, so that species dependence of antioxidant defense could be revealed.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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