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Measurement of malondialdehyde as oxidative stress biomarker in goat plasma by HPLC-DAD

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ABSTRACT

During the cattle breeding, the animals are exposed to stressful conditions (confinement, competition, extreme temperatures, pathogens); these have been associated to the increase of reactive oxygen species that attack cell membrane unsaturated lipids and other biomolecules and thus, inducing oxidation. This oxidative stress increases the incidence of degenerative diseases and decreases productivity. It has been proposed that the supplementation of the animal diet with antioxidants decreases the oxidative stress. Malondialdehyde (MDA) is an advanced oxidation product and a recognized oxidative stress biomarker. The aims of this work were to validate a methodology to determine MDA in goat plasma by HPLC-DAD and its assay in an animal feeding experiment. The MDA level was measured after condensation reaction with thiobarbituric acid (TBA). Free MDA (FMDA) was directly quantified after TBA condensation; while, Protein-bound MDA (PBMDA) needed to be released by an alkaline hydrolysis step and followed by TBA condensation. The MDA detection and quantitation limits were 0.034 μM and 0.086 μM, respectively. The linear range was 0.086–9.1 μM ($R^2 = 0.9998$). The accuracy was 100.5% to FMDA and 102.4% to PBMDA. The precision values intra and interday were 3.7 and 3.9%, respectively, for FMDA; and 3.8 and 4.1%, respectively, for PBMDA. Samples were analyzed in triplicate to obtain the figures of merit. The MDA level was quantified in plasma samples of 19 goats (*Capra hircus*) randomly distributed in three groups and fed with isocaloric and isoproteic diets: control, and supplemented with 12.5% d.b. of native woody species as polyphenol sources. The MDA plasma levels were statistically different among treatments in regard to the animal diet. A protector effect of the supplementation against oxidative stress was observed. The proposed methodology was selective, sensitive, reproducible, and suitable to evaluate MDA in goat plasma as oxidative stress biomarker.

1. Introduction

In animal production, oxidative stress has been identified as a cause of reproductive disorders and various diseases such as sepsis, mastitis, and pneumonia, among others [1]. The origin of this oxidative stress in the animals is related with their growing conditions during intensive and extensive breeding. The metabolic activity and the level of reactive oxygen species (ROS) increase as a consequence of animal exposition to these environmental stressors; some of the most relevant being confinement, competition with other animals, extreme temperatures in summer periods and possible infections with pathogens [2]. The excess of ROS, which cannot be eliminated by the endogenous antioxidant system, may attack the unsaturated lipids of the biological membranes and induces the lipid oxidation process where malondialdehyde is produced as an advanced oxidation product and it is recognized as an oxidative stress biomarker [3].

The main goals in cattle breeding are to obtain high productivity, healthy animals and getting quality products [4]; however, animal oxidative stress increases the incidence of degenerative diseases and decreases productivity [5]. To address this, the supplementation to the animal diet with antioxidants has been evaluated in ruminants. In this regard, sheep previously fed with a diet rich in antioxidant compounds maintained redox homeostasis when exposed to oxidative stress induced by high temperatures (28–40 °C) [6]. For this kind of studies, the measurement of oxidative stress biomarkers is a useful tool to evaluate the stress level in a biological system and to implement therapeutic actions designed to decrease it.

The scientific literature concerning the evaluation of oxidative stress level in human medicine is wide [7–12]. MDA quantitation as oxidative stress biomarker has been used in different human biological fluids, such as semen [13], plasma [14,15], serum [16], gingival fluid [17], among others. The main methodology used to determine MDA level is the traditional spectrophotometric thiobarbituric reactive specie

Abbreviations: ACN, acetonitrile; DAD, diode array detection; FMDA, free malondialdehyde; HPLC, high performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantitation; MDA, malondialdehyde; PBMDA, protein-bound malondialdehyde; ROS, reactive oxygen species; RSD, relative standard deviation; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive species; TCA, trichloroacetic acid; TEP, 1,1,3,3-tetraethoxypropane.

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(TBARS) assay [18]. This assay considers that MDA is the main advanced-lipid oxidation product able to react with thiobarbituric acid (TBA), and the condensation product MDA-TBA_2 can be measured by UV–VIS spectrophotometry. However, other oxidation products (saturated and unsaturated aldehydes) can also react with TBA under the same experimental conditions [18]. Therefore, other methodologies have been developed to overcome this lack of specificity. These analytical approaches are based on the resolution of the MDA-TBA_2 condensation product using high performance liquid chromatography (HPLC) coupled to UV–Vis [14,16,17] and mass spectrometry (MS) detection [15], as well as capillary electrophoresis (CE) with fluorescence detection [19]. The last methodology was applied to MDA quantification performed in rat organs [19].

In veterinary medicine, the development of methods concerning MDA quantification has not been so deeply and broadly explored; and the main methodology to evaluate the level of MDA is limited to TBARS [20]. For that, the standardization of new methods to study the oxidative stress in animal samples is still a subject to be explored [21]. In addition, opposite results in respect to MDA levels are found in the literature when different methodologies were applied. As an example, different levels of MDA in plasma samples have been informed in calving cows in days close to the parturition compared with non-pregnant ones assaying a HPLC methodology [22]; however, under similar conditions, the MDA levels were not significantly different when using the TBARS methodology [23]. Taking into account these results, it is unclear whether or not the level of oxidative stress during this period is responsible for the poor yield in terms of animal health and production.

In a previous work of our group, the MDA determination in cryopreserved bovine semen samples was carried out by high performance liquid chromatography with detection diode array (HPLC-DAD) and by TBARS in order to compare the determinative capacity of both techniques; several advantages being found in the chromatographic method as sensitivity and selectivity [24].

The main aim of this study was to evaluate the MDA level as oxidative stress biomarker in plasma obtained from goats fed with antioxidant supplemented diet during growth stage. Taking into account that the chemical composition of the matrix (interferences, protein presence, lipid composition and thus, oxidation susceptibility) determines the most convenient methodology to analyze the biomarker level in goat plasma samples, a new validation process was carried out based on HPLC-DAD. In addition, a comparison of the MDA levels in goat plasma samples between HPLC-DAD quantification and those obtained by the conventional TBARS methodology was undertaken.

2. Experimental

2.1. Chemicals and reagents

As a precursor of MDA, 1,1,3,3-tetraethoxypropane (TEP) was purchased from Sigma Aldrich (St. Louis, MO, USA). TBA was purchased from Merck (Darmstadt, Germany). Chromatography-grade acetic acid and acetonitrile were obtained from Sintorgan (Buenos Aires, Argentina). Milli-Q ultrapure water was used to prepare all the aqueous solutions.

A TEP stock solution in 50:50 (v/v) methanol–water was prepared to a concentration of 1.1 mM. Finally, TEP standard solutions for calibration purposes were prepared in a 1% (v/v) H_2SO_4 solution by subsequent incubation to hydrolyze TEP into MDA at 40 °C for 30 min. The final concentration of MDA in every standard solution was determined by measuring its absorbance at 245 nm ($\varepsilon = 13{,}700\ M^{-1}\ cm^{-1}$) [25].

2.2. Animal management

A total of 19 healthy creole goats (*Capra hircus*) of 3-month age, and with similar body weight (BW) were selected and fed with different diets for 50 days. The formulations of the diets were isoproteic and isocaloric and were developed according to the National Research Council Lamb Feeding Standard (Table 1). Goats were randomly divided into three groups. Two groups were fed with diets supplemented with leaves from native woody plants from the Argentinean as, *Acacia aroma* (known locally as tusca) and *Larrea divaricata* (known locally as jarilla) as sources of polyphenols, at 12.5% inclusion level (w/w) on dry basis; the base substrate was alfalfa. One group was fed with a control diet without supplementation.

Plasma samples from each goat group were collected after 50 day of animal feeding. Whole blood (2.0 mL) was collected by venipuncture into tubes containing 0.5 mL of 0.5 mM EDTA as anticoagulant. Plasma was obtained by centrifugation at 2500 × *g* for 10 min at 4 °C [26]; it was separated and stored at −20 °C until analysis. The MDA quantitation in plasma samples was performed within 15 days after sampling.

The validation of the HPLC-DAD methodology was carried out using a homogeneous goat plasma sample.

2.3. Sample pre-treatment

MDA levels in goat plasma samples were determined using a strategy that quantify free MDA (FMDA) and protein-bound MDA (PBMDA) separately. In these sense, to estimate MDA distribution in both fractions, Grotto et al. [14] and Roca et al. [27] procedures were combined with some modifications. Initially, different amounts of samples were assayed as well as different acid concentration levels were evaluated for deproteinization step. The optimized sample pre-treatment was as follows: 0.50 mL of goat plasma sample was mixed with 0.50 mL 20% (w/v) TCA in order to precipitate proteins. The sample was centrifuged at 10,000 rpm for 20 min at 4 °C to separate FMDA in the supernatant and PBMDA in the pellet. Free MDA was determined by condensation reaction with TBA. For this, TBA was dissolved at 0.5% (w/v) using an aqueous solution of TCA (20% w/v) as solvent. An aliquot of 2.0 mL of TBA solution in TCA was added to the supernatant and made up to 5.0 mL. The dilution was incubated at 100 °C for 15 min. Subsequently, the mixture was cooled in an ice bath for 10 min, and finally, the emulsion was centrifuged at 10,000 rpm at 4 °C for 20 min. The supernatant was then separated for spectrophotometric and HPLC analyses. PBMDA was recovered from the pellet. The precipitate was redissolved in 0.25 mL 3 N NaOH. This mixture was placed into a water bath at 60 °C for 45 min to hydrolyze MDA–protein bindings. Aliquots of 0.75 mL of 6% (v/v) H_3PO_4 aqueous solution and 2.0 mL of the TBA solution were added to this sample. This mixture was taken to 5.0 mL and kept at 100 °C for 15 min. After cooling, the sample was centrifuged and the supernatant was then separated for spectrophotometric and HPLC analyses.

2.4. Experimental conditions for TBARS analysis

Spectrophotometric analyses of TBARS were carried out in UNICAM UV2 equipment (UNICAM Limited, Thermo Optek, UK). Absorption

Table 1
Composition and nutrients of the diets offered to creole goats.

	Control	*L. divaricata* diet	*A. aroma* diet
Ingredient, %			
Alfalfa hay	51.0	45.0	40.0
Corn	27.0	22.5	29.5
Soybean expeller	22.0	20.0	18.0
L. divaricata dried leaves	–	12.5	–
A. aroma dried leaves	–	–	12.5
Chemical composition			
CP, %	16.7	16.7	16.4
ME, Mcal/kg DM	2.56	2.53	2.51
NDF, %	42.2	41.1	40.1
EE, %	3.2	3.2	3.2

CP: crude protein. ME: metabolizable energy. NDF: neutral detergent fiber. EE: ether extract. DM: dry matter.

spectra were scanned between 200 and 700 nm and the absorbance was measured at 532 nm.

2.5. Liquid chromatography conditions

The HPLC analyses were performed in the conditions previously optimized and reported [24]. The chromatographic system used was a Lab Alliance Series III-5 mL (LABALLIANCE Corporation, State College, PA, USA) equipped with two pumps and a column thermostating oven. A Grace Vydac Protein & Peptide C18 column (250 × 4.6 mm ID, 5 mm) was used and it was acquired from VYDAC (Hesperia, CA, USA). The injection volume was 20 μL. Mobile phase elution was carried out at 1 mL min^{-1} using a binary system, solvent A being a 95: 5 (v/v) mixture of 0.57 M (pH 2.5) acetic acid solution and ACN and solvent B being pure ACN. The gradient started with 100% solvent A for 2 min, increased to 20% solvent B at 3.0 min, held for 8.0 min and then returned to the initial conditions for 4.0 min. Thus, the total chromatographic run time was 15 min. The column was thermostatized at 40 °C. The detection wavelength was fixed at 532 nm using a Shimadzu (SHIMADZU Corporation, Japan) photodiode array detector.

2.6. Assay validation

The methodology to evaluate MDA level in goat plasma by HPLC-DAD was validated according to International Conference Harmonisation guidelines. [28] Sensitivity, linearity, accuracy and precision analytical parameters were determined. Detection and quantitation limits (LOD and LOQ) were calculated taking into account a ratio of 3.3 and 10 times between the standard deviation of the blank and the slope of the calibration curve [28]. Linearity was evaluated using at least seven MDA concentration levels within the working range. The standard solutions were prepared in triplicate. Precision was estimated as the relative standard deviation (RSD) of the measurements considering intraday precision or repetitibility and interday precision or reproducibility. Accuracy was expressed as recovery percent and was determined after adding MDA standard in quantities perfectly known to a homogenous goat plasma sample. The MDA addition levels were: 0.34, 0.90, and 2.7 μM. These spiked samples were prepared with three replicas for each level and were processed during three different days.

2.7. Statistical analyses

Analysis of variance (ANOVA) was used to determine significant differences among data. Each statistical analysis was done using the software program INFOSTAT version 2012 (Universidad Nacional de Cordoba). Fisher LSD-test was used to compare means when the effects were found to be significant ($P < 0.05$).

3. Results and discussion

3.1. Analytical characterization

Several preliminary assays were carried out to define the most appropriate sample pre-treatment. The results showed that the MDA determination, taking into account both fractions FMDA and PBMDA, resulted the most convenient (data not shown). Under these conditions, the elution of MDA-TBA_2 condensation product from the samples at 6.2 min as retention time was satisfactory. Typical chromatograms corresponding to both FMDA and PBMDA fractions are shown in Fig. 1. These chromatograms correspond to goat plasma samples with and without addition of MDA standard.

The methodology to quantify MDA in goat plasma by HPLC-DAD (including the sample pretreatment selected) was validated using MDA released quantitatively from TEP as validation standard.

Sensitivity was evaluated in terms of LOD and LOQ; these values were 0.034 and 0.086 μM, respectively.

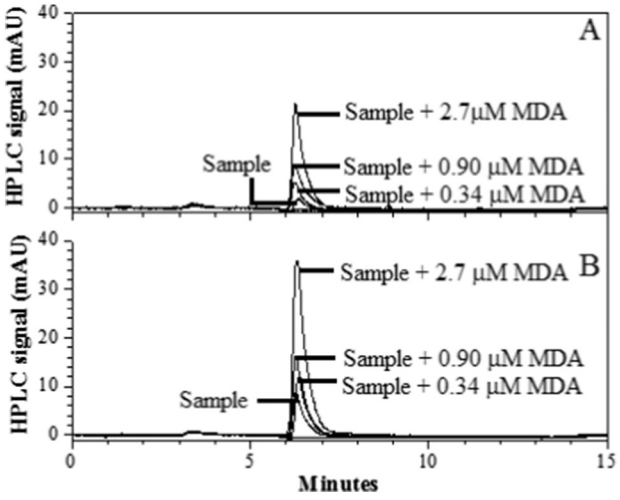

Fig. 1. HPLC-DAD chromatograms of MDA-TBA_2 condensation product in goat plasma samples. A: FMDA fraction. B: PBMDA fraction.

In respect to the linear range, an excellent linearity between peak area and analyte concentration was obtained in a range of at least two orders of magnitude (Fig. 2).

The accuracy was determined taking into account the MDA recovery from a homogenous goat plasma sample. This recovery value was calculated as the difference between mean value and the true value. MDA basal level was quantified in the homogenous sample before the standard addition. Concordances between MDA added and detected were found. The recovery percent was 100.5% to FMDA and 102.4% to PBMDA (Table 2). These recovery values are not different from 100% ($P = 0.6953$ and $P = 0.1536$) and they were in agreement with the values informed by Hong et al. [29] in human plasma.

Precision is defined as the degree of concordance between individual assays using the same sample and it was evaluated through the analysis by triplicate to the samples used in the recovery studies. Repetitibility and reproducibility were determined in the same day and in three consecutive days, respectively. The RSD values obtained are shown in Table 2 and they were within the range normally informed in biological fluids that is 5–10% [30].

Taking into account the matrix complexity, the reported values for the figures of merits corresponding to the proposed HPLC methodology in goat plasma can be considered highly satisfactory.

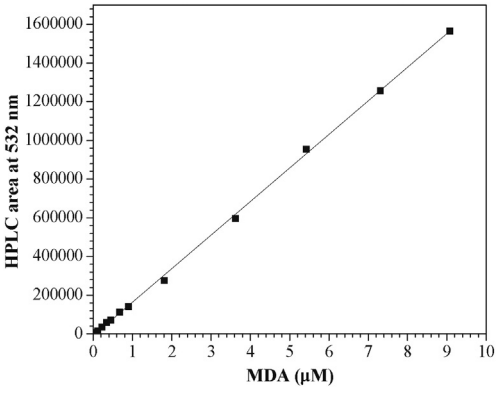

Fig. 2. Typical MDA calibration curve in the range measured with linear regression equation: $Y = 1.73 \times 10^5 X - 9.36 \times 10^4$ and $R^2 = 0.9998$.

Table 2
Analytical parameters in the quantitation of the FMDA and BPMDA fraction in goat plasma samples by HPLC-DAD.

MDA fraction	MDA added	MDA found (μM)			Precision (RSD %)			Interday	Accuracy (% recovery)		
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3		Day 1	Day 2	Day 3
Free	0.00	0.38 ± 0.02	0.35 ± 0.01	0.37 ± 0.02	4.8	3.7	4.6	4.4	–	–	–
	0.34	0.77 ± 0.03	0.69 ± 0.03	0.76 ± 0.03	4.3	4.9	4.6	4.6	101	100	102
	0.90	1.37 ± 0.04	1.19 ± 0.06	1.34 ± 0.03	3.0	5.0	2.2	3.6	103	95	102
	2.70	3.30 ± 0.09	2.90 ± 0.14	3.07 ± 0.06	2.8	4.9	1.8	3.2	105	95	99
Protein-bound	0.00	1.19 ± 0.06	1.18 ± 0.05	1.32 ± 0.06	4.7	4.9	4.8	4.8	–	–	–
	0.34	1.69 ± 0.08	1.67 ± 0.04	1.76 ± 0.09	4.7	2.5	5.1	4.1	110	109	105
	0.90	2.09 ± 0.09	2.13 ± 0.10	2.22 ± 0.08	4.4	4.9	3.8	4.4	99	102	99
	2.70	3.81 ± 0.12	3.89 ± 0.11	4.04 ± 0.13	3.1	2.8	3.2	3.0	98	100	100

Values: mean ± standard deviation.

3.2. Application assay

The methodology based en HPLC-DAD was used at the first time to evaluate the MDA concentration in goat plasma samples. As mentioned before, the goat plasma samples were obtained from animals randomized into three groups. Two groups of animals were fed with diets supplemented with *A. aroma* and *L. divaricata* as antioxidants source, mainly polyphenols.

The results of the MDA quantitation in both fractions, FMDA and PBMDA, are shown in Table 3. Statistically significant differences ($P < 0.0001$) at the MDA level were found among the three different diets. Taking into account the oxidative deterioration reactions advance, the best results were obtained in plasma of animals whose diet was supplemented, especially with *A. aroma*.

These results are very useful for the animal production area of knowledge because they represent a direct study concerning the oxidative stress remediation in different clinical situations. In this work, an antioxidant therapy was evaluated using directly in animal blood samples and with a simple and reliable methodology.

Some possible nutritional interventions have been discussed before; Se and vitamin E being the antioxidants most commonly investigated [31–33]. Both, vitamin E and Se reduced the respiration rate of the animals that grow under stress conditions such as high temperatures and as a consequence, their intake attenuates the negative effects of the oxidative stress. By contrast, a new source of antioxidants was evaluated in the present study using regional woody plants.

3.3. Comparison with MDA levels by TBARS method

A comparison between the mean total MDA levels detected in goat plasma samples using the separative methodology and the mean of total MDA levels determined by the traditional TBARS assay, was performed (Table 3). In this sense, in the TBARS assay only PBMDA fraction could be quantified because the detection limit of the spectrophotometric technique did not allow a reliable quantitation of FMDA fraction. Thus, total MDA levels evaluated with the TBARS methodology was equivalent to PBMDA. By contrast, both fractions, FMDA and PBMDA can also be quantified with the HPLC-DAD methodology, and so, the total MDA value was equivalent to a sum of these values.

The results using the TBARS assay showed an overestimation in MDA levels of goat plasma samples. The MDA concentrations determined by TBARS assay were approximately two times higher in the mentioned samples than the MDA concentrations quantified with the separative method validated in this work [22,29].

The true differences among MDA levels corresponding to the treatments evaluated were not found by TBARS. In addition, wrong conclusions could be obtained. In this sense, the conclusion at the present work could be that the antioxidant therapy not was effective taking into account only the TBARS results. On the opposite, the results obtained with the MDA determination using the separative method showed a protector effect since the MDA levels with the antioxidant diet were lower than the control diet.

The origin of this overestimation has been ascribed to interferences from other compounds that also react with TBA, and whose complex absorb in the same region of the spectrum that the condensation product MDA-TBA_2 [34].

In the Fig. 3 can be noted that the absorption band at 532 nm in the case of goat plasma samples is wider than in the case of standards. This situation can be explained taking into account the complexity of the samples and the interference of other substances [34]. Thus, it has been reported that other compounds such as bilirubin, biliverdin, pyrimidine and 2-aminopyrimidine, can also react with TBA and absorb in the 530–535 nm range [35]. Other interfering compounds are unsaturated aldehydes such as 2-butenal and 2,4-decadienal, which also react with TBA and their complexes absorb approximately at 445 nm, 500 nm and 532–535 nm. The absorption of these compounds at the 532–535 nm range is higher in the presence of sugars such as sucrose, glucose and fructose [34].

Table 3
MDA levels as oxidative stress biomarker in plasma samples of goats fed with different diets.

Analytical technique	Treatment	MDA concentration (μM)		
		Total MDA	BPMDA	FMDA
HPLC-DAD	Control	$(1.61 \pm 0.08)^a$	$(1.54 \pm 0.01)^a$	$(0.13 \pm 0.01)^a$
	L. divaricata	$(1.22 \pm 0.06)^b$	$(1.120 \pm 0.007)^b$	$(0.100 \pm 0.005)^b$
	A. aroma	$(1.02 \pm 0.08)^c$	$(0.930 \pm 0.005)^c$	$(0.090 \pm 0.003)^c$
TBARS	Control	$(2.5 \pm 0.1)^a$	$(2.5 \pm 0.1)^a$	ND (<0.034)
	L. divaricata	$(2.4 \pm 0.2)^a$	$(2.4 \pm 0.2)^a$	ND (<0.034)
	A. aroma	$(2.3 \pm 0.2)^a$	$(2.3 \pm 0.2)^a$	ND (<0.034)

Superscripts with different letters at columns correspond to means statistically different ($p < 0.05$). $n = 6$ for each treatment. ND: no detectable.

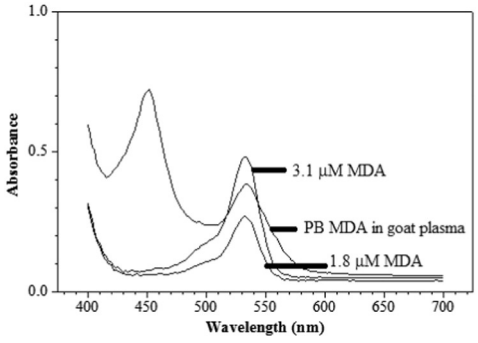

Fig. 3. Absorption spectra of a goat plasma sample and MDA standard solutions in the concentration range.

4. Conclusion

MDA levels in goat plasma samples were quantify by HPLC-DAD. The methodology was validated and the analytical figures of merit were determined. This methodology was satisfactorily assayed at the first time, to monitor the concentration of the mentioned oxidative stress biomarker in a feeding experiment in small ruminants. Total MDA, FMDA and PBMDA levels were significantly lower in the case of diets rich in antioxidants in respect to a control diet.

In addition, a comparison between MDA levels in goat plasma quantified by the proposed separative method and the traditional TBARS approach was undertaken. Overestimation of the MDA concentration levels was observed when the TBARS methodology was used, which could lead to unrealistic conclusions regarding the animal oxidative stress status and about the efficacy of the antioxidant diet. The interferences in the MDA determination were eliminated using the HPLC-DAD methodology instead of the TBARS method. The advantage of the separative method is that statistical differences could be found among MDA levels corresponding to the different diets. Thus, the MDA quantitation as biomarker of the oxidation advance using the HPLC-DAD methodology constitutes a viable tool to evaluate the oxidative status in animals exposed to different conditions and real knowledge in clinical studies that links animal health with oxidative stress can be obtained.

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