Antioxidant adaptive responses of extraembryonic tissues from cloned and non-cloned bovine conceptuses to oxidative stress during early pregnancy

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Abstract

Placental oxidative stress has been suggested as a key factor in early pregnancy failure. Abnormal placental development limits success in pregnancies obtained by somatic cell nuclear transfer (SCNT). Malondialdehyde (MDA) content, an index of oxidative stress, and superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities were determined in bovine extraembryonic tissues of SCNT or artificial insemination (AI) conceptuses. Chorionic tissues of SCNT and AI conceptuses show no difference in MDA content at day 32 of pregnancy. MDA content in chorionic tissues of SCNT and AI conceptuses decreased from day 32 to 62 of pregnancy. MDA content was lower in chorionic tissues of SCNT conceptuses than that in chorionic tissues of AI conceptuses at day 62 of pregnancy. SOD1, SOD2 and GPX activities in chorionic tissues of SCNT conceptuses were not different from those in chorionic tissues of AI conceptuses at both gestational ages. CAT activity in chorionic tissues of SCNT conceptuses was lower at day 32, and it was higher at day 62 of pregnancy than that in chorionic tissues of AI conceptuses. CAT and GPX activities increased in chorionic tissues of SCNT conceptuses with gestational age. SOD1 activity decreased while that of SOD2 and GPX increased in chorionic tissues of AI conceptuses with gestational age. At day 62 of pregnancy, MDA content and enzyme activities in cotyledonary tissues were not different between AI and SCNT conceptuses. Different antioxidant mechanisms may operate within the chorion of AI and SCNT conceptuses. Further experiments are required to elucidate this point.

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Introduction

In domestic farm animals, such as cattle, somatic cell nuclear transfer (SCNT) is associated with pathological pregnancies (Hill *et al.* 1999) and important foetal losses during the first trimester of pregnancy (Hill *et al.* 2000) as well as in the late foetal and perinatal periods (Heyman *et al.* 2002). Abnormal early placental development has frequently been described in pregnancies derived from IVF and cloning procedures by SCNT in cattle (Stice *et al.* 1996, Hill *et al.* 2000, Hashizume *et al.* 2002, Chavatte-Palmer *et al.* 2006, Constant *et al.* 2006, Farin *et al.* 2006). Poor viability of cloned fetuses during days 35–60 of pregnancy is associated with rudimentary chorioallantoic development (Hill *et al.* 2000), fewer numbers of chorionic villi (Hashizume *et al.* 2002) and altered peri-implantation trophoblast differentiation (Arnold *et al.* 2006). Available evidence shows that

aberrant expression of antioxidant enzyme proteins and associated oxidative stress in the extraembryonic tissue derived from 26-day-old SCNT conceptuses is a major factor contributing to low birth rate in pigs (Chae *et al.* 2006).

Oxidative stress during early placental development is associated with pregnancy-related disorders in humans, such as embryonic resorption, abortion, intra-uterine growth restriction and foetal death (Wang & Walsh 2001, Hempstock *et al.* 2003, Agarwal & Allamaneni 2004, Gupta *et al.* 2007). Furthermore, placental oxidative stress (Myatt & Cui 2004, Poston & Raijmakers 2004) may be a general underlying mechanism that links altered placental function to foetal programming (Myatt 2006). The depletion of placental antioxidant systems has been suggested as a key factor in early human pregnancy failure (Liu *et al.* 2006). Adequate placental antioxidant status early in pregnancy could prevent those

disorders induced by oxidative stress that lead to impairment of placental function and development, foetal growth and pregnancy outcomes. Indeed, the effectiveness of antioxidant enzymatic defences against oxidative stress varies with the stage of placental development in humans (Sekiba & Yoshioka 1979, Takehara *et al.* 1990, Qanungo *et al.* 1999, Jauniaux *et al.* 2000, Qanungo & Mukherjee 2000) and sheep (Garrel *et al.* 2010). These previous data suggest that enhanced activities of key antioxidant enzymes with gestational ages may act as protective mechanism against oxidative stress during early human and sheep placental development and growth.

Reactive oxygen species (ROS) are produced in low quantities as by-products of normal aerobic metabolism. An increase in the generation of ROS and/or a decrease in cellular antioxidant capacity can induce oxidative stress (Fridovich 1999). Mammalian cells have evolved a variety of antioxidant mechanisms to control ROS-induced oxidative stress (Michiels *et al.* 1994). Superoxide dismutases (SODs) are metalloenzymes that catalyze the dismutation of superoxide radical ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2). Thus, they constitute the first enzymatic step that plays a vital role in the control of cellular $O_2^{\cdot-}$ production. Two intracellular isoforms of SOD have been identified in mammals. Copper–zinc-containing SOD (Cu, Zn-SOD or SOD1) is a dimeric protein, essentially located in the cytoplasm. Manganese-containing SOD (Mn-SOD or SOD2) is a homotetrameric protein, located in the mitochondria. The control of H_2O_2 production, before their subsequent transformation to highly reactive hydroxyl radical ($OH^{\cdot}$), is the second enzymatic step that plays a vital role against ROS propagation and oxidative damage. Glutathione peroxidases (GPXs) are located within the mitochondrial matrix and the cytoplasm, and catalase (CAT) found primarily within peroxisomes, both belong to the secondary defence mechanism by catalyzing the conversion of H_2O_2 to H_2O . The major enzymes, SOD1, SOD2, CAT and GPX, are therefore specifically compartmentalized within the cell, and represent co-ordinately operating network of defences against oxidative stress (Fig. 1).

While the aerobic post-implantation period constitutes a critical stage of pregnancy due to high susceptibility of the developing conceptuses to oxidative stress, antioxidant defences can be altered during early placental development. Thus, the characterisation of antioxidant adaptive responses could improve the understanding of placental development, and may help in the design of a strategy for prevention and treatment of placental insufficiency, particularly in SCNT pregnancies. More than 80% of foetal deaths in the SCNT pregnancies occurred between days 30 and 60 (Hill *et al.* 2000). We aimed therefore to provide insights into the pro-oxidative and anti-oxidative status of bovine extra-embryonic tissues derived from SCNT and artificial insemination (AI) conceptuses sampled at days 32 and

Figure 1 Schematic representation of the pathways producing reactive oxygen species (ROS) and key cellular antioxidant enzymatic systems controlling ROS production. Molecular oxygen (O_2), superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxynitrite ($ONOO^-$), hydroxyl radical ($OH^{\cdot}$), copper/zinc-superoxide dismutase (SOD1), manganese-SOD (SOD2), catalase (CAT), glutathione peroxidase (GPX), reduced glutathione (GSH) and oxidized glutathione (GSSG).

62 of pregnancy. In this study, we determine the content of malondialdehyde (MDA), as an index of ROS-induced oxidative stress and lipid peroxidation, along with the activities of the key antioxidant enzymes, SOD1, SOD2, CAT and GPX, in chorionic and cotyledonary tissues derived from SCNT and AI conceptuses.

Results

MDA content in chorionic tissues at days 32 and 62 of pregnancy

The content of MDA in chorionic tissues obtained at days 32 and 62 of pregnancy from AI and SCNT conceptuses is shown in Fig. 2. MDA content in chorionic tissues of SCNT conceptuses was not different from that in chorionic tissues of AI conceptuses at day 32. MDA content in chorionic tissues of AI conceptuses tended to be lower ($P=0.056$) at day 62 of pregnancy than that at day 32 of pregnancy (Fig. 2). MDA content in chorionic tissues of SCNT conceptuses significantly decreased ($P<0.01$) from day 32 to 62 of pregnancy. Chorionic tissues of SCNT conceptuses demonstrated significantly lower ($P<0.01$) content of MDA at day 62 as compared to that in chorionic tissues of AI conceptuses.

SOD1, SOD2, CAT and GPX activities in chorionic tissues at days 32 and 62 of pregnancy

The activities of SOD1, SOD2, CAT and GPX in chorionic tissues obtained at days 32 and 62 of pregnancy from AI and SCNT conceptuses are shown in Fig. 3a–d. SOD1, SOD2 and GPX activities in chorionic tissues were not different between AI and SCNT conceptuses, neither at day 32 nor at day 62 of pregnancy (Fig. 3a, b and d). CAT activity in chorionic tissues of SCNT conceptuses was significantly lower

Figure 2 Malondialdehyde (MDA) content in chorionic tissues derived from AI (open bars) and SCNT (solid bars) conceptuses obtained at day 32 (four AI and four SCNT pregnant animals) and at day 62 (seven AI and seven SCNT pregnant animals) of pregnancy. Values are means $\pm$ S.E.M. for the number of animals used.

($P < 0.05$) at day 32 of pregnancy, and it was significantly higher ($P < 0.05$) at day 62 of pregnancy as compared with that in chorionic tissues of AI conceptuses (Fig. 3c). SOD1 activity in chorionic tissues of AI conceptuses significantly decreased ($P < 0.05$) from day 32 to 62 of pregnancy (Fig. 3a). SOD1 activity in chorionic tissue of SCNT conceptuses was not different between days 32 and 62 of pregnancy (Fig. 3a). SOD2 activity in AI chorionic tissues significantly increased ($P < 0.01$) from day 32 to 62 of pregnancy (Fig. 3b). SOD2 activity in chorionic tissue of SCNT conceptuses was not different between days 32 and 62 of pregnancy (Fig. 3b). CAT activity in chorionic tissue of AI conceptuses was not different between days 32 and 62 of pregnancy (Fig. 3c). CAT activity in chorionic tissues of SCNT conceptuses significantly increased ($P < 0.01$) from day 32 to 62 of pregnancy (Fig. 3c). GPX activity significantly increased in chorionic tissues of AI ($P < 0.01$) and SCNT ($P < 0.05$) conceptuses with gestational ages (Fig. 3d).

MDA content and SOD1, SOD2, CAT and GPX activities in chorionic and cotyledonary tissues at day 62 of pregnancy

Cotyledonary tissues of AI and SCNT conceptuses had high activities of SOD1 ($P < 0.01$), CAT ($P < 0.001$ and $P < 0.05$ respectively for AI and SCNT conceptuses) and GPX ($P < 0.001$ and $P < 0.05$ respectively for AI and SCNT conceptuses), and low activity of SOD2 ($P < 0.01$ and $P < 0.05$ respectively for AI and SCNT conceptuses) as compared with the activities of these enzymes in chorionic tissues (Fig. 4a–d). It is interesting to note that neither MDA content (result not shown) nor SOD1, SOD2, CAT and GPX activities (Fig. 4a–d) in the cotyledonary tissues were different between AI and SCNT conceptuses.

Discussion

To our knowledge, this is the first report of changes in antioxidant enzymatic pathways in extraembryonic tissues derived from cloned and non-cloned conceptuses at early stages of placental development and growth. In the present study, the extent of lipid peroxidation and the activities of the key antioxidant enzymes, namely SOD1, SOD2, CAT and GPX, were determined in chorionic and cotyledonary tissues of viable bovine conceptuses obtained by SCNT biotechnology or conventional AI. Lipid peroxidation is one of the most important expressions of oxidative stress induced by ROS. MDA, an end product of lipid peroxidation processes (Mukai & Goldstein 1976), is the most frequently used biomarkers providing an indication of

Figure 3 Changes in the enzymatic activities of (a) SOD1, (b) SOD2, (c) CAT and (d) GPX in chorionic tissues derived from AI (open bars) and SCNT (solid bars) conceptuses obtained at day 32 (four AI and four SCNT pregnant animals) and at day 62 (seven AI and seven SCNT pregnant animals) of pregnancy. Values are means $\pm$ S.E.M. for the number of animals used.

Figure 4 Enzymatic activities of (a) SOD1, (b) SOD2, (c) CAT and (d) GPX in chorionic and cotyledonary tissues derived from AI (open bars) and SCNT (solid bars) conceptuses obtained at day 62 of pregnancy (seven AI and seven SCNT pregnant animals). Values are means $\pm$ S.E.M. for the number of animals used.

the extent of ROS-induced oxidative stress. MDA is found to be produced by the human placenta during normal or complicated pregnancy (Walsh & Wang 1993, Poranen *et al.* 1996). Overall, the extent of lipid peroxidation in chorionic tissues derived from SCNT or AI conceptuses decreased as gestation proceeded (present study). Importantly, the extent of lipid peroxidation was lower in chorionic tissues derived from SCNT conceptuses than that in chorionic tissues derived from AI conceptuses at day 62 of pregnancy. This difference could be explained, at least in part, by developmental changes in the antioxidative status of chorionic tissues derived from cloned and non-cloned conceptuses with gestational age.

Chorionic tissues of AI conceptuses respond to oxidative stress and related lipid peroxidation MDA product early in pregnancy by increasing SOD2 and GPX activities as pregnancy progresses. This is likely to be an important cellular adaptive antioxidant

mechanism because mitochondria are the major sites of endogenous ROS production (Wallace 2005). The mitochondria are therefore the prime target for ROS-induced oxidative damage. Under physiologically relevant conditions of bovine placental development between days 32 and 62 of pregnancy (present study), it may be assumed that increased activity of SOD2 and GPX in chorionic tissues of AI conceptuses would, in theory, counteract the production of ROS and the propagation of highly reactive radicals in the mitochondria and cytoplasm. The adaptive protective mechanisms that operate in chorionic tissues of SCNT conceptuses as gestation proceeds are different from those observed in chorionic tissues of AI conceptuses. The decrease in chorionic MDA content from day 32 to 62 of pregnancy is accompanied by an increase in CAT and GPX activities. Oxidative stress and the associated increase in the activity of H_2O_2 detoxification enzymes, CAT and GPX, have also been reported in human placental tissues of early pregnancy failure, suggesting that such an antioxidative mechanism might be developed and operate against possible oxidative damage in patients with a susceptibility for miscarriage (Biri *et al.* 2006). The marked increase in the activities of CAT and GPX and a decrease in MDA content from day 32 to 62 of pregnancy in bovine chorionic tissues derived from SCNT conceptuses (present study) are probably an important protective mechanism against the oxidative stress and lipid peroxidation associated with early placental development. Overall, these different operating antioxidative mechanisms within the chorion of non-cloned and cloned conceptuses obtained by AI and SCNT respectively could be a part of developmental adaptation to control oxidative stress and lipid peroxidation early in pregnancy.

Unlike $O_2^{\cdot-}$, H_2O_2 is more stable, and can diffuse out of the mitochondria into the cytoplasm and the nucleus where it can lead to cell damage (Wallace 2005). In the presence of transition metals, usually iron, H_2O_2 and $O_2^{\cdot-}$ can interact in a Haber-Weiss reaction (Kehrer 2000) to generate highly toxic $OH^{\cdot}$, which is thought to be directly responsible of lipid peroxidation and oxidative damage (Halliwell 1978, Halliwell & Gutteridge 1989). As an unstable molecule, nitric oxide (NO) may also react with $O_2^{\cdot-}$ in a reaction controlled by the rate of diffusion of both radicals to form peroxynitrite ($ONOO^-$), which is a powerful oxidant and a potent inducer of lipid peroxidation (Radi *et al.* 1991). In addition, $ONOO^-$ can react through several different mechanisms, including the formation of an intermediate through a reaction with $^{\cdot}OH$ (Koppenol *et al.* 1992). Therefore, tightly controlled H_2O_2 production by CAT protects cells against lipid peroxidation by limiting the generation and propagation of highly reactive ROS. Our observation of the depression of CAT activity in chorionic tissues derived from 32-day-old bovine SCNT conceptuses as compared with that in chorionic

tissues derived from AI conceptuses is in agreement with previous findings showing decreased CAT protein expression in the porcine extraembryonic tissue derived from 26-day-old SCNT conceptuses (Chae *et al.* 2006). Taken together, these previous data in pigs and our present findings in cattle suggest that aberrant expression of CAT protein and the depression of CAT enzymatic activity in chorionic tissues derived early in pregnancy from cloned conceptuses are therefore likely to decrease placental defences against deleterious H_2O_2 reactions, and could contribute to the increased lipid peroxidation during early placental development. It is important to note that the activity of CAT was increased from day 32 to 62 of pregnancy in chorionic tissues derived from SCNT conceptuses (present study). This specific adaptive response operates only in cloned conceptuses early in pregnancy and are likely to reinforce the cellular antioxidant defence systems, thereby enhancing cell tolerance against the forthcoming lipid peroxidation during placental development.

To prevent the production and propagation of highly reactive and toxic ROS, H_2O_2 generated after a SOD catalyzed reaction is quickly converted into H_2O by GPX, which has selenocysteine within its active site, and therefore, it is a selenium dependent antioxidant activity. The retention of the placenta in selenium-deficient cattle suggests a role for selenium–GPX in pregnancy outcomes and foetal delivery (Eger *et al.* 1985). The high efficiency of GPX in controlling and limiting H_2O_2 production and the propagation of highly reactive ROS may be attributable to the fact that it is located in both the cytoplasm and the mitochondrial matrix, and that it can utilize both H_2O_2 and lipid peroxides as substrates. The increased GPX activity in chorionic tissues derived from AI and SCNT conceptuses with early gestational ages (present study), also reported during early placental development in humans (Jauniaux *et al.* 2000), and sheep (Garrel *et al.* 2010), is probably an important adaptive mechanism protecting the early placenta against ROS-mediated lipid peroxidation and cell damage. Possibly chorionic cells induce enhancement of GPX to counteract the generation of H_2O_2 and the subsequent propagation of highly toxic ROS known to induce lipid peroxidation.

The present study for the first time provides insight into the enzymatic antioxidant status of the extraembryonic tissues derived from cloned and non-cloned bovine conceptuses. We have demonstrated that the activities of the key antioxidant enzymes show major changes in chorionic tissues of SCNT and AI conceptuses during early placental development. Enhanced activities of H_2O_2 detoxification enzymes, namely CAT and GPX, with gestational age observed in the chorion derived from SCNT conceptuses may represent a protective or adaptive mechanism to prevent damage from oxidative insult early in pregnancy.

Materials and Methods

Bovine somatic cell nuclear transfer

Methods of somatic clone embryo production were as previously described (Vignon *et al.* 1998). Briefly, bovine ovaries were collected from the abattoir and transported in sterile PBS. Cumulus–oocyte complexes (COCs) were aspirated from follicles 2 to 7 mm in diameter, washed and selected morphologically for *in vitro* maturation. Batches of 30–40 COCs were matured in TCM-199 containing 10% (v/v) FCS (Life Technologies, Paisleys, Scotland, UK), 10 μ g FSH/ml and 1 μ g LH/ml for 22 h at 39 °C in a humidified atmosphere of 5% CO_2 in air. Skin cells from three different adult females of Holstein bred were cultured over several passages before being used for nuclear transfer (NT). Cells were trypsinated, pelleted at 1200 *g* for 5 min and resuspended in fresh medium prior to NT. Each isolated cell was inserted under the zona pellucida of the recipient cytoplasm and fused by electrostimulation. Reconstituted embryos were co-cultured in microdrops of 50 μ l B2 medium (CCD, Paris, France) with 2.5% FCS and vero cells. After 7 days of *in vitro* culture at 39 °C and under 5% CO_2 , expanding or hatching blastocysts (grade 1) were removed from the culture drops and were transferred into the uterus of synchronized recipient Holstein heifers.

Embryo transfer

Recipient heifers, 15–18 months of age, were treated for oestrus synchronisation with progestagen implants for 9 days (Crestar, Intervet, Angers, France). Two days before removal of implant, each heifer was treated with an analogue of prostaglandin $F_{2\alpha}$ (0.5 mg Estrumate/2 ml per day, Schering-Plough Vétérinaire, Levallois-Perret, France). One week after induced oestrus, heifers with a palpable corpus luteum (CL) were used. SCNT pregnancies were obtained after non-surgical transfer of a single NT blastocyst into the uterine horn ipsilateral to the CL of each recipient animal using the miniaturized embryo transfer syringe and sanitary sheath (IMV, L'Aigle, France) under epidural anaesthesia. Control pregnancies were obtained after AI of Holstein heifers using frozen semen from a single Holstein bull. Control AI pregnant heifers were bred and managed in the same facilities as SCNT pregnant heifers (INRA, Unité Commune d'Expérimentation Animale, Leudeville, France). Pregnancy was made by transrectal ultrasonography at days 29–30 and then repeated at days 45 and 60 of gestation.

Tissue collection

Pregnant animals bearing SCNT or AI viable conceptuses were randomly allocated for slaughter at days 32 and 62 of gestation. The animals were humanely put down at the experimental slaughterhouse of INRA in accordance with protocols approved by the institutional ethical committee. The reproductive tracts were collected and immediately transported to the laboratory adjacent to the slaughterhouse for tissue dissection. Chorionic tissues from cloned and non-cloned conceptuses were sampled at day 32 (four SCNT and four AI pregnant animals) and at day 62 (seven SCNT and seven AI pregnant animals) of pregnancy, and cotyledonary tissues were collected at day 62 of pregnancy (seven SCNT and

seven AI pregnant animals). Immediately after dissection, chorionic and cotyledonary tissues were snap-frozen in liquid nitrogen, and then stored at -80°C until processed for MDA content and SOD1, SOD2, CAT and GPX enzymatic activities.

Antioxidant enzyme activity assays

Chorionic and cotyledonary tissues were homogenized separately in cold phosphate buffer (50 mM, pH 7.4), and then, the homogenates were centrifuged at 15 000 *g* for 30 min at 4°C . The resulting supernatant was used for determination of protein concentration, MDA content and measurement of enzymatic activities. Protein concentrations were determined by Lowry's method (Lowry *et al.* 1951). Enzyme activities of SOD1, SOD2, CAT and GPX in the supernatant were determined as described previously in detail (Al-Gubory *et al.* 2005, Garrel *et al.* 2007). Total SOD activity was measured using the pyrogallol assay based on the competition between pyrogallol oxidation by superoxide radicals and superoxide dismutation by SOD (Marklund & Marklund 1974). SOD2 is encoded in the nuclear chromatin, synthesized as a precursor in the cytoplasm, and imported posttranslationally into the mitochondrial matrix in its mature form (Wispe *et al.* 1989). The freeze-thaw approach results in mitochondrial release of large amounts of SOD2 into the cytosol (Jin *et al.* 2005). Enzymatic activity of SOD2 was determined by assaying for SOD activity in the presence of sodium cyanide, which selectively inhibits SOD1 but not SOD2 (Jin *et al.* 2005). SOD1 activity was calculated by subtracting SOD2 activity from total SOD activity. The rate of auto-oxidation is taken from the increase in the absorbance at 420 nm. One unit of SOD activity is defined as the amount of the enzyme required to inhibit the rate of pyrogallol auto-oxidation by 50%. CAT activity was determined as described previously (Nzengue *et al.* 2008). Activity was assayed by determining the rate of decomposition of H_2O_2 by CAT in 10 mM of potassium phosphate buffer (pH 7). The reaction rate was related to the amount of CAT present in the mixture. The rate of H_2O_2 decomposition by CAT was followed at 240 nm. One unit was defined as the decomposition of 1 mmol H_2O_2 per min per mg protein. GPX activity was measured using the glutathione reductase–NADPH method. Activity was determined by a coupled assay system, in which oxidation of glutathione was coupled to NADPH oxidation catalyzed by glutathione reductase. The rate of glutathione oxidized by tertiary butyl hydroperoxide was evaluated by the decrease of NADPH in the presence of EDTA, excess reduced glutathione and glutathione reductase. The rate of decrease in concentration of NADPH was recorded at 340 nm. GPX activity was expressed in terms of nM of NADPH oxidized per min per mg protein.

MDA measurement

The most widely used method for the determination of MDA in biological materials is based on its reaction with thiobarbituric acid (TBA). Reversed-phase HPLC technique in which the MDA–TBA adducts are separated from interfering substances (Londero & Lo Greco 1996) was used for determining MDA in chorionic and cotyledonary tissue

homogenates. The breakdown product of 1,1,3,3-tetraethoxypropane (TEP) was used as standard. TEP undergoes hydrolysis to liberate stoichiometric amounts of MDA. Stock standard solution (480 μl of TEP in 100 ml ethanol) was prepared, and this primary solution was diluted to the concentrations of 0, 1, 2, 3, 4, 5 and 6 μM . Tissue extract aliquots or standards (100 μl) were mixed with 750 μl of 0.8% TBA. The tubes were placed in a water bath at 95°C for 1 h, and then, they were cooled. Samples were neutralized with methanol–NaOH mixture (pH 6). After centrifugation, 50 μl of protein-free supernatant were chromatographed in the HPLC system. The column used for the separation was Adsorbosphere C18 (5- μm particle diameter, 250×4.6 mm ID). The MDA–TBA adduct is eluted from the column with potassium dihydrogen phosphate buffer (10 mM, pH 6.0)–acetonitrile (17%). The quantification of MDA derivative was established by comparing the absorption to the standard curve of MDA equivalents generated by acid-catalyzed hydrolysis of TEP as μmol s per gram tissue protein.

Statistical analysis

The MDA content and the enzymatic activities were analyzed by one-way ANOVA and the Newman–Keuls multiple comparison test (PRISM Graph Pad version 2; Graph Pad Software, San Diego, CA, USA). The acceptable level of significance was set at $P < 0.05$. Data are presented as the mean $\pm$ S.E.M.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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