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Oxidative stress-inducible antioxidant adaptive response during prostaglandin $F_{2\alpha}$-induced luteal cell death *in vivo*

CATHERINE GARREL[1], IRENE CEBALLOS-PICOT[2], GUY GERMAIN[3], & KAÏS H AL-GUBORY[3]

[1]*Laboratoire de Biologie du Stress Oxydant, Département de Biologie Intégrée, Centre Hospitalier Universitaire de Grenoble, 38043 Grenoble cedex 9, France,* [2]*Laboratoire de Biochimie Médicale, Institut National de la Santé et de la Recherche Médicale, Centre Hospitalier Universitaire de Necker, 75743 Paris cedex 15, France, and* [3]*Unité de Biologie du Développement et de la Reproduction, Département de Physiologie Animale, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas cedex, France*

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Abstract

Oxidative stress-induced antioxidant adaptive response would be particularly important to cells in high reactive oxygen species (ROS) environments. We aimed to determine the dynamic adaptive response of antioxidant enzymatic systems in sheep corpus luteum (CL) during $PGF_{2\alpha}$-induced luteal cell death. Activities of superoxide dismutase (SOD1 and SOD2), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GSR), and *in situ* DNA fragmentation were determined in CL at day 10 of the estrous cycle (0 h) and at 12, 24 or 48 h after $PGF_{2\alpha}$ injection. A decrease in plasma progesterone concentration was first observed at 6 h after treatment ($P < 0.05$). Apoptotic cells were rarely observed in the CL at 0 h (less than 0.7%), and their incidence increased ($P < 0.01$) by 12 h post-$PGF_{2\alpha}$ (11.7%) and remained thereafter elevated through 48 h. Activities of SOD1, SOD2, GPX and GSR were not changed at any time points after $PGF_{2\alpha}$ treatment. CAT activity increased at 12 h ($P < 0.01$) and at 24 h ($P < 0.05$) after $PGF_{2\alpha}$ treatment as compared to that at 0 h. These findings demonstrate that $PGF_{2\alpha}$ induce luteal cell death without depressing the activity of antioxidant enzymes. It is suggested that transient increase in CAT activity is an adaptive response of the CL to oxidative stress induced by $PGF_{2\alpha}$.

Keywords: *Antioxidant enzymes, oxidative stress, luteal cell death, adaptive response*

Introduction

During the reduction of molecular oxygen to water, reactive oxygen species (ROS) are produced in low quantities as by-products of normal aerobic metabolism. An increase in the generation of ROS beyond the ability of intracellular antioxidant defenses is called oxidative stress, also defined as an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to cellular damage [1]. ROS include superoxide anion radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2). Under physiological and pathophysiological conditions, the generation of ROS in biological systems is known to damage lipids, proteins and nucleic acids [2] and ultimately lead to cell death. To prevent cell death caused by ROS, aerobic cells are endowed with enzymatic antioxidant defense systems. Superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH)-dependent enzymes are the key and complimentary antioxidant enzymatic systems that protect cells against toxic and damaging effects of ROS [3].

Correspondance: K. H. Al-Gubory, UMR Biologie du Développement et de la Reproduction, Institut National de la Recherche Agronomique (INRA), 78352 Jouy-en-Josas cedex, France. Tel: 33 1 34652362. Fax: 33 1 34652364. E-mail: kais.algubory@jouy.inra.fr

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Copper–zinc SOD (SOD1), which is located in the cytoplasm, and manganese SOD (SOD2), which is located in the mitochondria, both belong to the first enzymatic step that plays a vital protective role by catalyzing the conversion of $O_2^{\cdot-}$ into H_2O_2 [4,5]. Unlike $O_2^{\cdot-}$ radical, H_2O_2 is relatively stable and has a higher oxidant potential. In the presence of iron, H_2O_2 and $O_2^{\cdot-}$ can interact in a Haber–Weiss reaction to generate $OH^{\cdot}$ which is thought to be a powerful initiator of lipid peroxidation [2]. CAT, found primarily within peroxisomes and glutathione peroxidase (GPX), present in significant amounts in the cytoplasm, both belong to the secondary defense mechanism against ROS by catalyzing the conversion of H_2O_2 to H_2O [6]. GPX detoxifies H_2O_2 to H_2O through the oxidation of reduced GSH and metabolizes lipid hydro peroxides to less reactive hydroxy fatty acids [6]. The ability of GPX to reduce H_2O_2 or other hydro peroxides is dependent on the activity of glutathione reductase (GSR). This enzyme catalyses the reduction of the oxidized form of glutathione (GSSG) to GSH with NADPH as the reducing agent [6]. Therefore GSR is an important component of the cellular anti-oxidant defense mechanism as it maintains adequate levels of reduced-GSH. Glutathione-dependent enzymes represent therefore a coordinately regulated defense against ROS-induced oxidative stress [7].

Oxidative stress has been implicated in a large number of human diseases including atherosclerosis, pulmonary fibrosis, cancer, neurodegenerative diseases and aging [8]. ROS-induced oxidative stress play an important role in apoptosis [9,10]. ROS also function as double-edged swords: while excessive ROS production damages DNA, low levels of ROS affect cell signaling in physiological processes [11]. Over the last two decades, a great deal of evidence has been published suggesting that ROS affect multiple physiological processes such as sperm development, oocyte maturation, ovulation, follicular and luteal steroidogenesis, implantation and early embryo development [12,13]. There is also evidence that ROS cause pathological processes affecting female reproduction, such as infertility, endometriosis, pre-eclampsia, fetal embryopathies, preterm labor and abortions [14]. Yet the relationship between ROS-induced oxidative stress and the pathophysiology of these diseases is not clear, largely due to a lack of fundamental insights regarding cellular adaptive responses to oxidant environment under both physiological and disease states.

The prostaglandins (PG's) are a group of oxygenated fatty acids that have been found in all mammalian tissues and they are important bioregulators as well as participants in pathological states [15]. Regression of the corpus luteum (CL) or luteal regression is an example of physiological degeneration of cellular structure, which is a necessary event of the mammalian reproductive cycle. Luteal regression is characterized by two associated events: loss of the capacity of the CL to synthesize and secrete progesterone (functional luteal regression) and death of luteal cells (structural luteal regression). In many mammalian species, $PGF_{2\alpha}$ is implicated in the process of functional and structural luteal regression [16]. A role of ROS in regulating ovarian steroidogenesis was first proposed based on the presence of $O_2^{\cdot-}$ in the rat ovarian tissue and its cyclic changes during the estrous cycle [17]. Then, most investigations reported that both natural and $PGF_{2\alpha}$-induced functional luteal regression in the rat are associated with a loss of progesterone production and an increase in the production of ROS [18,19]. ROS are also produced during enzyme reaction, particularly by the cytochrome P-450 family implicated in steroidogenesis [6,20] and by the arachidonic acid cascade during PG's synthesis [21], and thus ROS are considered as by-products of normal steroid and PGs synthesis by the CL.

The oxidative stress-induced adaptive response as a defense mechanism would be particularly important to cells in high ROS environments. Luteal cell's highly oxidizing environment and constant exposure to ROS due to their high capacity to synthesize and secrete steroid hormones make the CL an excellent *in vivo* model for studying the antioxidant adaptive response of these cells to ROS-induced oxidative stress and luteal cell death. The dynamic and rapid changes in antioxidant enzymatic activities may indicate how a cell or organ might respond to ROS oxidizing environment. The antioxidant enzymatic defences against ROS act synergistically to protect cells against ROS-induced oxidative stress and DNA damage [3,7] and should not be considered separately. The present study was therefore designed to determine the activities of key intracellular antioxidant enzymes, namely SOD1, SOD2, CAT, GPX and GSR, and the time course of *in situ* DNA fragmentation, characteristic of apoptotic cell death, in functionally active and healthy mature CL of the estrous cycle and in regressed CL obtained from ewes 12, 24 or 48 h after treatment with $PGF_{2\alpha}$.

Materials and methods

Animals and tissue collection

All procedures relating to care and use of animals were approved by the French Ministry of Agriculture according to the French regulation for animal experimentation (authorization no 78–34). The study involved 16 cyclic ewes of the Préalpes-du-Sud breed. All the ewes were treated for 14 days with intravaginal sponges containing 40 mg fluorogestone acetate (Intervet, Angers, France) to synchronize estrus as described [22]. Each ewe received immediately after removal of the sponges an intramuscular

injection of 500 IU of equine chorionic gonadotropin (eCG, Intervet). Twelve ewes were injected (i.m.) with 10 mg of $PGF_{2\alpha}$ (Dinolytic®, Pharmacia, Guyancourt, France) at day 10 of the estrous cycle and the CL were collected 12, 24 and 48 h ($n = 4$ ewes per group) after $PGF_{2\alpha}$-injection. Control CL (CL of ewes not given $PGF_{2\alpha}$) were obtained at day 10 of the estrous cycle (0 h, $n = 4$ ewes). In all the ewes, ovaries were obtained by surgery under general anesthesia and the CL were immediately dissected from the surrounding ovarian tissue and weighed. For the determination of luteal cell death, a piece of each CL was fixed overnight in 4% para-formaldehyde in phosphate-buffered saline (PBS), pH 7.4, washed in PBS, dehydrated through a series of increasing concentrations of ethanol, cleared in xylene, and embedded in paraffin wax. The remaining luteal tissue was snap-frozen in liquid nitrogen and stored at −80°C until processed for the antioxidant enzyme activities.

In situ *detection of luteal cell death*

Luteal cell death was identified with the *In Situ* Cell Death Detection Fluorescein Kit (Roche Diagnostics, Mannheim, Germany). This assay detects nuclear DNA fragmentation in apoptotic cells by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL). Briefly, tissue sections 6 μm thick were prepared, mounted on silane-coated slides (3-amino-propyltriethoxysilane-coated slides; Sigma-Aldrich Chimie Sarl, Saint Quentin Fallavier, France), dehydrated through a series of decreasing concentrations of ethanol (100–70%) to water. They were then treated with 100 μg/ml Proteinase K (Sigma-Aldrich Chimie Sarl) in 10 mM Tris, pH 8.0, for 30 min at 37°C and washed with PBS, pH 7.4. The slides were submerged in 0.1 M sodium citrate for 30 min at 70°C and then washed with PBS. Sections were incubated 60 min at 37°C with the TUNEL mixture, washed with PBS and counterstained with an aqueous solution of propidium iodide (DNA-PREP stain, Beckman Coulter Co. Fl, Miami, USA). Tissue sections were analyzed under a fluorescent microscope. Negative control sections were processed identically except that the labeling enzyme (terminal deoxynucleotidyl transferase) was omitted. Four different optical fields (magnification X250) were selected in a random manner for each CL tissue section and used to calculate the percentage of apoptotic nuclei. Tissue sections were also processed for routine histology and stained with haematoxylin and eosin for morphological analysis.

Antioxidant enzyme activity assays

Frozen unthawed luteal tissues obtained at 0, 12, 24 or 48 h after $PGF_{2\alpha}$ injection from each ewe 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 and measurement of enzymatic activities. Protein concentrations were determined by Lowry's method [23].Total SOD activity was measured using the pyrogallol assay as previously described [24], based on the competition between pyrogallol oxidation by superoxide radicals and superoxide dismutation by SOD. To measure SOD2 activity, sodium cyanide was included to inhibit SOD1 activity. 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 measured by a simple and rapid method as previously described [25]. In this method, the rate of hydrogen peroxide decomposition by CAT was followed at 240 nm. One unit was defined as the decomposition of 1 mmol hydrogen peroxide per minute per milligram protein. GPX activity was measured using the glutathione reductase-NADPH method [26]. Enzyme 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 being recorded at 340 nm. GPX activity was expressed in terms of nM of NADPH oxidized per minute per milligram of protein. GSR activity was assayed by the standard method of NADPH oxidation [27]. In this assay, oxidized glutathione is reduced to glutathione by GSR which oxidizes NADPH to $NADP^+$. NADPH consumption was determined at 340 nm. Enzyme activity was expressed in terms of nM of NADPH oxidized per minute per milligram of protein.

Blood sampling and progesterone assay

Blood samples were taken from each ewe by jugular venipuncture beginning 30 min before $PGF_{2\alpha}$ treatment and then continued every 6 h until the surgical removal of ovaries. After centrifugation (3000*g*, 4°C) for 30 min, plasma was stored at −20°C until assayed. Plasma was analyzed for concentrations of progesterone as a means to monitor the timing of functional luteal regression, using a validated radioimmunoassay in unextracted plasma as described [28], except that charcoal–dextran solution was used instead of polyethylene glycol for the separation of bound and free radioactivity. Tritiated progesterone

([1,2,6,7−^{3}H]−progesterone, sp.act. 88 Ci mmol^{-1}) was obtained from Amersham International (Amersham, Bucks, UK) and a specific antiprogesterone antibody was obtained from the Institut Pasteur (Paris, France). All plasma samples were run in duplicate in a single assay. The minimum detectable concentration of progesterone was 0.1 ng/ml, and the intra-assay coefficient of variation was less than 10%.

Statistical analysis

Data were expressed as mean ± SEM. The number of positively stained apoptotic cells was counted in 4 random fields/section/CL and results are expressed as the percentage of apoptotic cells of the total number of cells counted at each time after $PGF_{2\alpha}$ treatment. The data were analyzed by one-way ANOVA followed by a Turkey multiple comparison test (PRISM Graph Pad version 2; Graph Pad Software, San Diego, CA, USA). The acceptable level of significance was set at $P < 0.05$.

Results

$PGF_{2\alpha}$ injection induces functional luteal regression

Plasma progesterone concentrations (ng/ml) before and after $PGF_{2\alpha}$ treatment are shown in Figure 1. A significant decrease ($P < 0.05$) in plasma progesterone concentration was first observed at 6 h after $PGF_{2\alpha}$ treatment as compared with the level at 0 h. Then plasma progesterone further decreased ($P < 0.001$) at and after 12 h post-$PGF_{2\alpha}$ treatment.

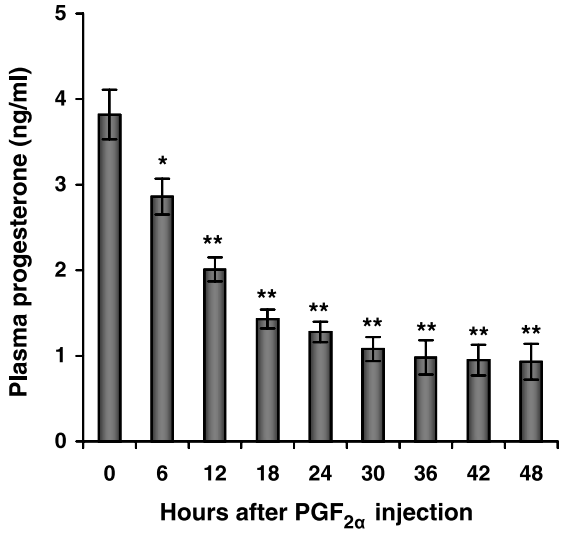

Figure 1. Mean (± SEM) plasma progesterone concentration before (0 h) and at different time points after $PGF_{2\alpha}$ treatment. Ewes were treated (i.m.) with 10 mg of $PGF_{2\alpha}$ at day 10 of the estrous cycle. *$P < 0.05$, **$P < 0.001$ compared with value at 0 h.

$PGF_{2\alpha}$ injection induces luteal cell disorganization

Representative histological features of fully healthy CL collected at day 10 of the estrous cycle (0 h) from non-treated ewe and regressed CL obtained at different time points (12, 24 and 48 h) from $PGF_{2\alpha}$-treated ewes are shown in Figure 2. In the healthy CL, the predominating cell type is the large polyhedral luteal cells which were relatively uniform in shape and had abundant cytoplasm containing central rounded nuclei. At 12 h after treatment with $PGF_{2\alpha}$, the large luteal cells still appeared morphologically similar to that seen in the healthy CL but are irregular in outline. Marked morphological changes characteristic of luteal regression occur 24 h after $PGF_{2\alpha}$ treatment, particularly cellular disorganization and apparent nuclear chromatin condensation. Widespread cellular disorganization and prominent shrinking of luteal cells occur 48 h post-$PGF_{2\alpha}$ treatment. At this stage of $PGF_{2\alpha}$-induced luteal regression, nuclear chromatin condensation, densely staining pyknotic bodies and vacuoles became a predominant morphological feature of the CL.

$PGF_{2\alpha}$ injection induces luteal cell death and structural luteal regression

We used *in situ* nick-end labeling (TUNEL) method to determine the onset and the time course of nuclear DNA fragmentation within luteal tissue during $PGF_{2\alpha}$-induced luteal regression. The fluorescent nuclear DNA fragments were rarely detected in luteal tissues of the healthy functional CL, whereas they were extensively present in the regressed CL (Figure 2). Irrespective of time after $PGF_{2\alpha}$ treatment, the distribution of DNA fragments was heterogeneous within the region of the CL examined, and DNA fragments were easily detected. The weight of CL recovered at 0 h was different ($P < 0.01$) from that recovered 24 and 48 h following $PGF_{2\alpha}$ treatment (Figure 3). The percentage of apoptotic nuclei of luteal cells increased ($P < 0.01$) at 12, 24 and 48 h post-$PGF_{2\alpha}$ treatment compared to that at 0 h (Figure 3). There was no difference between apoptotic indices in CL collected at different time points after $PGF_{2\alpha}$ treatment.

Activities of SODs, CAT and GSH-dependent enzymes during $PGF_{2\alpha}$-induced luteal regression

The activities of SOD1 and SOD2 in the CL collected from the non-treated ewes (0 h) were not different from those in the CL collected from ewes at 12, 24 and 48 h after $PGF_{2\alpha}$ treatment (Figure 4). The activity of CAT increased 12 h ($P < 0.01$) and 24 h ($P < 0.05$) after $PGF_{2\alpha}$ treatment compared to that at 0 h, but was not different in the CL collected 48 h following $PGF_{2\alpha}$ treatment (Figure 4). The activities of GPX

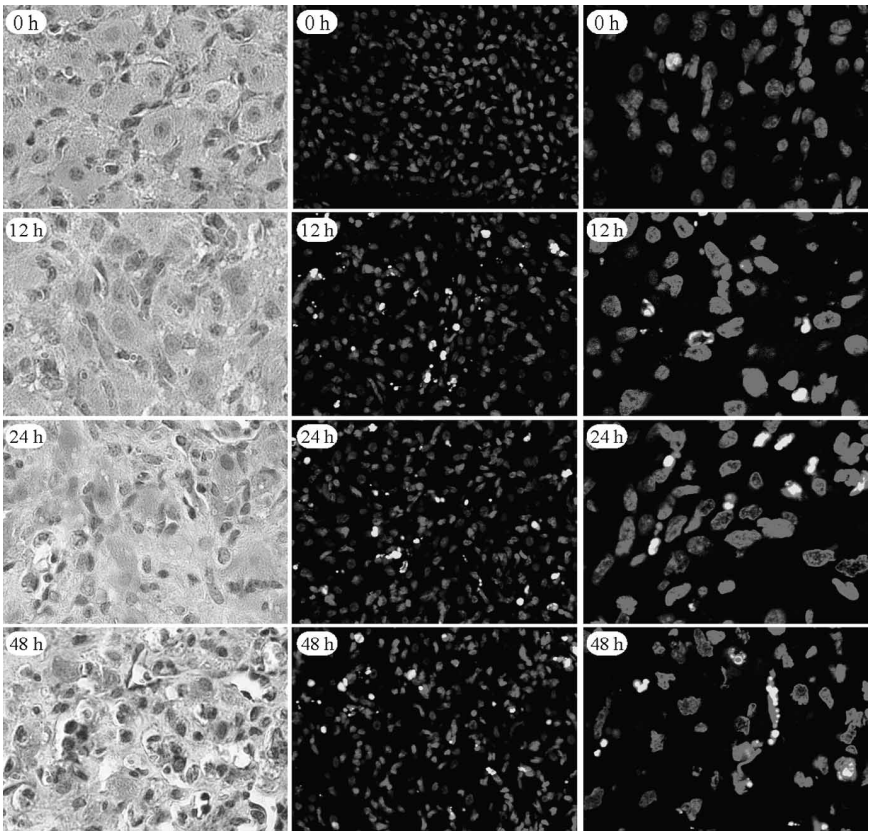

Figure 2. Histological feature representation of the luteal tissues and *in situ* identification of apoptotic nuclei by fluorescence labeling of nicked DNA in CL of vehicle-treated control ewes (0 h) and $PGF_{2\alpha}$-treated ewes at different time points after treatment. Sections (6 μm) were stained with hematoxylin and eosin or subjected to TUNEL analysis. In hematoxylin and eosin photographs (left panels, X400), note that luteal cells have normal size and appearance in CL of vehicle-treated ewes (0 h), whereas they were abnormal in the CL of ewes obtained 12 h post-$PGF_{2\alpha}$ treatment. Densely staining bodies are easily distinguishable in CL recovered 24 h after $PGF_{2\alpha}$ treatment and increased then after at 48 h post-injection. In TUNEL photographs (middle panels, X250 and right panels, X400), non-fragmented nuclei are stained red (propidium iodide) whereas apoptotic nuclei with fragmented DNA are stained red and yellow–green. Note that CL recovered 12, 24 and 48 h after $PGF_{2\alpha}$ treatment displayed a high number of TUNEL-positive apoptotic cells compared with CL of vehicle-treated control. Ewes were treated (i.m.) with vehicle or 10 mg of $PGF_{2\alpha}$ in vehicle at day 10 of the estrous cycle.

and GSR in the CL collected from the non-treated ewes (0 h) were not different from those in the CL collected from ewes at 12, 24 and 48 h after $PGF_{2\alpha}$ treatment (Figure 4).

Discussion

The inhibition of nuclear DNA cleavage in cultured isolated rabbit CL with SOD, CAT or a putative stimulator of endogenous GPX [29], the induction of apoptotic DNA fragmentation in bovine luteal cells by down-regulation of GPX [30] and the induction of apoptosis in cultured bovine luteal cells by simultaneous treatment with H_2O_2 and with a specific inhibitor of GPX [31] suggest a role for SOD, CAT and GPX in the maintenance of CL structure through inhibition of luteal cell death. In addition, depletion of non-enzymatic antioxidant ascorbic acid occurs following $PGF_{2\alpha}$ treatment of rats [32,33] and pigs [34] and during spontaneous luteal regression in rats [35], and has been suggested to be one of the possible intracellular mechanisms by which $PGF_{2\alpha}$ exercises its luteolytic action. Recently, it was proposed that $PGF_{2\alpha}$ by decreasing the expression of free radical scavenger proteins facilitates the accumulation of ROS, which in turn initiates a cascade of events that will finally cause luteal cell death in the mouse CL during luteal regression [36]. In the present study, we investigated the intracellular antioxidant response in sheep CL during $PGF_{2\alpha}$-induced functional luteal regression and cell death. The activities of SOD1, SOD2, GPX and GSR in the CL were not different between the different time points after $PGF_{2\alpha}$ treatment (12, 24 and 48 h) and from those in the control group (CL of ewes not given $PGF_{2\alpha}$). In addition, the activity of CAT significantly increased at 12 and 24 h after $PGF_{2\alpha}$ treatment as compared to that in the control group. These results demonstrate for the first time that oxidative DNA damage in luteal cells is not related to the enzymatic antioxidant status in these cells. The results of the present study also indicate that cleavage of DNA into oligonucleosome occurs in the sheep CL during $PGF_{2\alpha}$-induced functional and structural luteal regression *in vivo*

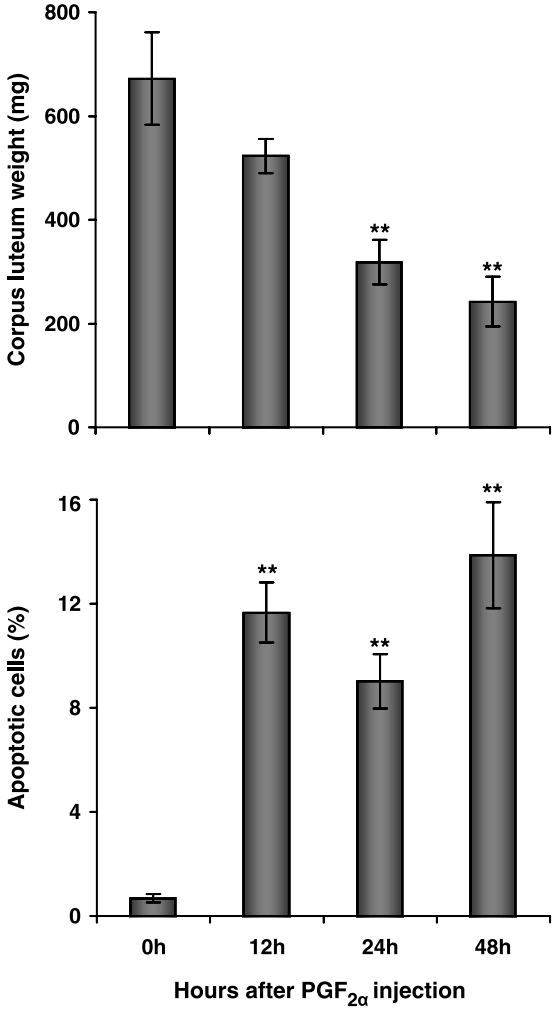

Figure 3. Mean (±SEM) CL weight and incidence of TUNEL-positive apoptotic nuclei of luteal cells in the vehicle-treated ewes and the $PGF_{2\alpha}$-treated ewes at different time points after treatment. The number of positively stained apoptotic cells were counted in 4 random fields per section per CL (a mean of 1680 cells were counted for each CL). Ewes were treated (i.m.) with vehicle or 10 mg of $PGF_{2\alpha}$ in vehicle at day 10 of the estrous cycle. Results represent the mean (±SEM, $n = 4$ ewes per group). $^{\star}P < 0.05$, $^{\star\star}P < 0.01$ compared with value at 0 h.

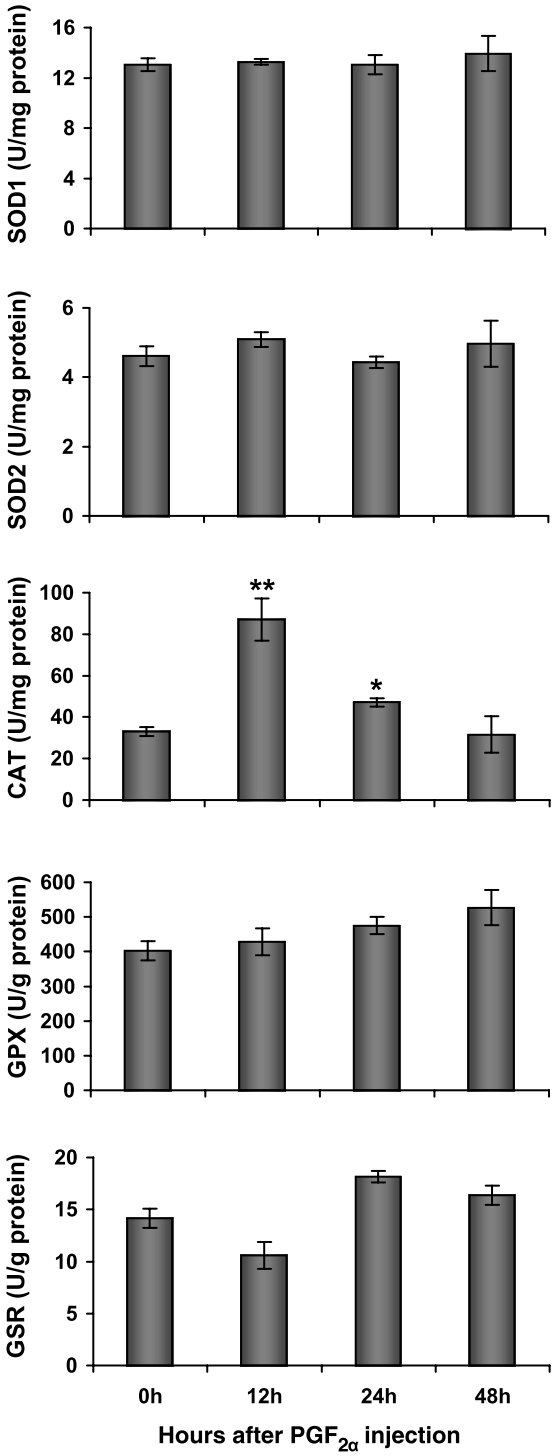

Figure 4. Changes in the activities of SOD1, SOD2, CAT, GPX and GSR in the sheep CL collected from ewes at day 10 of the estrous cycle (0 h) and at different time points after $PGF_{2\alpha}$ treatment. Ewes were treated (i.m.) with vehicle or 10 mg of $PGF_{2\alpha}$ in vehicle at day 10 of the estrous cycle. Results represent the mean (±SEM, $n = 4$ ewes per group). $^{\star}P < 0.05$, $^{\star\star}P < 0.01$ compared with value at 0 h.

without any depression in the activities of key antioxidant enzymes. They do not support the hypothesis that depletion of the activities of key intracellular antioxidant enzymes during luteal regression contributes to the initiation and/or the progression of luteal cell death in response to progesterone withdrawal induced by $PGF_{2\alpha}$.

Our results show that luteal cell death, although detectable at a very low level in functionally active CL, increased rapidly following $PGF_{2\alpha}$ treatment. The present study extend previous report [37]. In that study, sheep CL were not obtained until 16 h following treatment with 10 mg of $PGF_{2\alpha}$. In our study, we showed that extensive luteal cell death could be demonstrated as early as 12 h after $PGF_{2\alpha}$ treatment. Our current finding is in agreement with previous report [38] in which inter-nucleosomal DNA fragmentation (DNA laddering), as measured by DNA gel electrophoresis, was observed 12 h after $PGF_{2\alpha}$ treatment in sheep. Since fragmentation of

nuclear DNA constitutes the final cell event of apoptosis, rapid and relatively high incidence of DNA fragmentation mediated in the luteal cells 12 h after $PGF_{2\alpha}$ treatment (present study) must therefore involve early activation of intracellular apoptotic signaling cascade that precedes the DNA cleavage, at least in part via activation of key proteolytic enzyme, caspase-3. Indeed, DNA laddering in sheep CL removed 12 h after an i.m. injection of 7.5 mg of $PGF_{2\alpha}$ at day 10 of the oestrous cycle occurred concomitantly with an increase in caspase-3 activity [39]. All these findings support the hypothesis that $PGF_{2\alpha}$-induced luteal cell death is mediated, at least partly, by oxidative stress.

A decline in the activities of key antioxidant enzymes in the regressing CL may result in increased intra-luteal concentrations of ROS which induce functional and structural CL regression. Yet $PGF_{2\alpha}$ did not deplete the activities of SOD1, SOD2, GPX and GSR in sheep CL (present study). Furthermore, the observations that treatment of rats with luteolytic doses of $PGF_{2\alpha}$ induces a rapid transient increase in ROS formation within minutes of treatment and before decreases of progesterone secretion suggests that an early event in luteal regression may involve stimulation of ROS production leading to rapid irreversible inactivation of progesterone synthesis [40,41]. In the present study, the choice of 12, 24 and 48 h time point following $PGF_{2\alpha}$ treatment to study adaptive response of key antioxidant enzymatic systems and luteal cell death *in vivo* is in some way not informative of change in ROS levels in our experimental model. Therefore, it will be of interest to investigate intra-luteal ROS production in the ovine CL during the processes of luteal formation, development and regression.

Glutathione in its reduce form (GSH) can either act as a substrate in the cytosolic GSH redox cycle or is able to directly inactivate ROS [42]. Little is known about the level of GSH in the tissue of the reproductive system under different physiological states. In addition, the regulation of GSH components in the female reproductive tissues by PGs under natural physiological environment of the cell in living animals is currently unknown. The enzyme GSR, by generation of GSH, indirectly participates in the protection of cells against deleterious effects of ROS and is involved in the maintenance of the redox status of cells. In the present study, GSR activity in the CL collected from the non-treated ewes was not different from that in the CL collected from ewes at different time point after $PGF_{2\alpha}$ treatment. Under these experimental conditions, intraluteal GSH levels would be maintained by GSR. Interestingly, it has been found that luteal regression was associated with a transient increase in ovarian GSH in rats [33]. Furthermore, it has been shown from *in vitro* experiments that J_2 series of the PGs (PGJ_2) at low sublethal concentrations induces an adaptive protection response through augmentation of cellular GSH synthesis [43–45]. Although our current work did not examine GSH, is it possible that $PGF_{2\alpha}$ also altered the luteal GSH components.

Luteal cell death occurs in the CL of several mammals including sheep [37] and human [46] only after the drop in luteal progesterone production during luteal regression. In the present study we showed that peripheral levels of progesterone fell rapidly within 6 h and then further decreased at and after 12 h post-$PGF_{2\alpha}$-treatment. Consequently following rapid progesterone withdrawal, CL exhibited extensive luteal cell death (12 h post-$PGF_{2\alpha}$ injection). However, it is unclear whether luteal cell death occurs because of rapid ROS generation by $PGF_{2\alpha}$ treatment or whether progesterone withdrawal by $PGF_{2\alpha}$ treatment promotes ROS generation and subsequent luteal cell death. Although it is difficult to conclude that withdrawal of progesterone within hours of $PGF_{2\alpha}$ treatment is the cause or the consequence of $PGF_{2\alpha}$-induced extensive luteal cell death as evaluated by studying *in situ* nuclei undergoing DNA fragmentation in the CL of sheep (present study), there is increasing evidence that progesterone promotes survival of luteal cells. This includes: 1) progesterone antagonist induces apoptotic cell death in bovine luteal cells [47] and in late pregnant rat CL [48]. In addition, *in vivo* administration of progesterone delayed the occurrence of DNA fragmentation in postpartum rat CL [49]. From all these findings, one can speculate that the rapid decline in progesterone production (present study) within hours after $PGF_{2\alpha}$ treatment contributes to the initiation and progression of luteal cell death in the sheep luteal cells. In support with this conclusion are the recent results showing that intraluteal progesterone suppresses apoptosis in bovine luteal cells through the inhibition of Fas and caspase-3 mRNA expression and inhibition of caspase-3 activation [50].

Adaptive response to ROS-induced oxidative stress may function as an important component of a cell's defense system and contributes to the maintenance of cellular integrity in high ROS environments. Adaptive response to oxidative stress has been observed in microorganisms and mammalian cells [51,52]. Mammalian cells actively respond to oxidative stress by setting up many different reactions that increase cell defense or lead to adaptation to oxidant environments [52]. The adaptive response of eukaryote cells to oxidative stress induced by ROS has been reported *in vitro*. Exposure of cultured hamster tracheal epithelial cells [53], rat neonatal cardiac myocytes [54] and Chinese hamster lung fibroblasts [55] to H_2O_2 selectively up regulates CAT, but not GPX. The antioxidant defence systems, CAT and GPX, were enhanced in neural PC12 cells by the their pretreatment with low doses of H_2O_2, suggesting that this

adaptive response enriches the cellular antioxidant defence systems, thereby enhancing cell tolerance against the forthcoming oxidative insults induced by H_2O_2 and related hydroxyl radicals [56].To our knowledge, the selective *in vivo* increase of CAT activity reported in the sheep CL in response to $PGF_{2\alpha}$-induced cell death (present study) has not previously been highlighted in mammalian CL and in other steroidogenic tissues and organs. In the present study, luteal cells exhibited several morphological changes characteristic of structural CL regression and the percentage of apoptotic nuclei of luteal cells significantly increased at 12 h after treatment with $PGF_{2\alpha}$. At that time, the activity of CAT was maximal. From these findings, one can speculate that the rapid decline in progesterone production (6 h post-$PGF_{2\alpha}$ treatment) contributes to the initiation of luteal cell death, and that the CL can no longer be rescued even in the presence of high activity of CAT, because irreversible structural luteal regression has occurred. Nevertheless, only 11.7% of total luteal cells exhibited nuclear DNA fragmentation. This raises question on specific sites of changes in activities of antioxidant enzymes. Indeed, enzyme activities were determined in whole luteal tissue and thus it is possible that CAT may be overexpressed only in surviving cells. It is therefore tempting to suggest that adaptive response of the CL to $PGF_{2\alpha}$-induced oxidative stress through up-regulation of CAT activity may function as an important component of luteal cell's defense against ROS formation and thus contributes to the maintenance of luteal cellular integrity.

In conclusion, these *in vivo* findings demonstrate that $PGF_{2\alpha}$ induces CL progesterone withdrawal and luteal cell death in sheep CL without depressing the activity of key intracellular anti-oxidant enzymes involved in the defense mechanism; i.e. SOD1, SOD2, CAT, GPX and GSR. It is suggested that transient increase in CAT activity is a specific adaptive response of the CL to oxidative stress induced by $PGF_{2\alpha}$.

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References

[1] Sies H. Oxidative stress: From basic research to clinical application. Am J Med 1991;91:31S–38S.

[2] Halliwell B, Gutteridge JMC. Protection against oxidants in biological systems: The superoxide theory of oxygen toxicity. In: Halliwell B, Gutteridge JMC, editors. *Free Radical in Biology and Medicine*. Oxford: Clarendon Press; 1989. p 86–123.

[3] Michiels C, Raes M, Toussaint O, Remacle J. Importance of Se-glutathione peroxidase, catalase, and Cu/Zn-SOD for cell survival against oxidative stress. Free Radic Biol Med 1994;17:235–248.

[4] McCord JM, Fridovich I. Superoxide dismutase. An enzymatic function for erythrocuprein. J Biol Chem 1969;244: 6049–6055.

[5] Weisiger RA, Fridovich I. Mitochondrial superoxide dismutase. Site of synthesis and intramitochondrial localization. J Biol Chem 1973;248:4791–4793.

[6] Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. Physiol Rev 1979;59:527–605.

[7] Hayes JD, McLellan LI. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free Radic Res 1999;31: 273–300.

[8] Halliwell B, Gutteridge JM, Cross CE. Free radicals, antioxidants, and human disease: Where are we now? J Lab Clin Med 1992;119:598–620.

[9] Chandra J, Samali A, Orrentius S. Triggering and modulation of apoptosis by oxidative stress. Free Radic Biol Med 2000;29: 323–333.

[10] Simon HU, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis 2000; 5:415–418.

[11] Martin KR, Barrett JC. Reactive oxygen species as double-edged swords in cellular processes: Low-dose cell signaling versus high-dose toxicity. Hum Exp Toxicol 2002;21:71–75.

[12] Riley JCM, Behrman HR. Oxygen radicals and reactive oxygen species in reproduction. Proc Soc Exp Biol Med 1991; 198:781–791.

[13] Fujii J, Iuchi Y, Okada F. Fundamental roles of reactive oxygen species and protective mechanisms in the female reproductive system. Reprod Biol Endocrinol 2005;3:43–52.

[14] Agarwal A, Allamaneni SSR. Role of free radicals in female reproductive diseases and assisted reproduction. Reprod Biomed Online 2004;9:338–347.

[15] Kuehl FA, Jr, Egan RW. Prostaglandins, arachidonic acid, and inflammation. Science 1980;210:978–984.

[16] Arosh JA, Banu SK, Chapdelaine P, Madore E, Sirois J, Fortier MA. Prostaglandin biosynthesis, transport, and signaling in corpus luteum: A basis for autoregulation of luteal function. Endocrinology 2004;145:2551–2560.

[17] Laloraya M, Kumar GP, Laloraya MM. Changes in the levels of superoxide anion radical and superoxide dismutase during the estrous cycle of rattus norvegicus and induction of superoxide dismutase in rat ovary by lutropin. Biochem Biophys Res Commun 1988;157:146–153.

[18] Sawada M, Carlson JC. Superoxide radical production in plasma membrane samples from regressing rat corpora lutea. Can J Physiol Pharmacol 1989;67:465–471.

[19] Behrman HR, Kodaman PH, Preston SL, Gao S. Oxidative stress and the ovary. J Soc Gynecol Investig 2001;8:S40–S42.

[20] Cross AR, Jones OT. Enzymic mechanisms of superoxide production. Biochim Biophys Acta 1991;1057:281–298.

[21] Cadenas E. Biochemistry of oxygen toxicity. Annu Rev Biochem 1989;58:79–110.

[22] Menegatos J, Chadio S, Kalogiannis T, Kouskoura T, Kouimtzis S. Endocrine events during the periestrous period and the subsequent estrous cycle in ewes after estrus synchronization. Theriogenology 2003;59:1533–1543.

[23] Lowry OH, Rosebrough NJ, Farr AL, Randall RF. Protein measurement with folin phenol reagent. J Biol Chem 1951; 193:265–275.

[24] Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient

assay for superoxide dismutase. Eur J Biochem 1974;47: 469–474.

[25] Beers B, Sizer WA. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 1952;195:133–139.

[26] Flohé L, Günzler WA. Assays of glutathione peroxidase. Methods Enzymol 1984;105:114–121.

[27] Carlberg I, Mannervik B. Glutathione reductase. Methods Enzymol 1985;113:484–490.

[28] Schanbacher BD. Radioimmunoassay of ovine and bovine serum progesterone without extraction and chromatography. Endocr Res Commun 1979;6:265–277.

[29] Dharmarajan AM, Hisheh S, Singh B, Parkinson S, Tilly KI, Tilly JL. Antioxidans mimic the ability of chorionic gonadotropin to suppress apoptosis in the rabbit corpus luteum *in vitro*: A novel role for superoxide dismutase in regulating bax expression. Endocrinology 1999;140:2555–2561.

[30] Nakamura T, Ishigami T, Makino N, Sakamoto K. The down-regulation of glutathione peroxidase causes bovine luteal cell apoptosis during structural luteolysis. J Biochem 2001; 129:937–942.

[31] Nakamura T, Sakamoto K. Reactive oxygen species up-regulates cyclooxygenase-2, p53 and Bax mRNA expression in bovine luteal cells. Biochem Biophys Res Commun 2001;284: 203–210.

[32] Sato T, Iesaka T, Jyujo T, Taya K, Ishikawa J. Prostaglandin-induced ovarian ascorbic acid depletion. Endocrinology 1974; 95:417–420.

[33] Aten RF, Duarte KM, Behrman HR. Regulation of ovarian antioxidant vitamins, reduced glutathione, and lipid peroxidation by luteinizing hormone and prostaglandin F2 alpha. Biol Reprod 1992;46:401–407.

[34] Petroff BK, Ciereszko RE, Dabrowski K, Ottobre AC, Pope WF, Ottobre JS. Depletion of vitamin C from pig corpora lutea by prostaglandin F2 alpha-induced secretion of the vitamin. J Reprod Fertil 1998;112:243–247.

[35] Endo T, Aten RF, Wang F, Behrman HR. Coordinate induction and activation of metalloproteinase and ascorbate depletion in structural luteolysis. Endocrinology 1993;133: 690–698.

[36] Foyouzi N, Cai Z, Sugimoto Y, Stocco C. Changes in the expression of steroidogenic and antioxidant genes in the mouse corpus luteum during luteolysis. Biol Reprod 2005;72: 1134–1141.

[37] Murdoch WJ. Temporal relationships between stress protein induction, progesterone withdrawal, and apoptosis in corpora lutea of ewes treated with prostaglandin $F_{2\alpha}$. J Anim Sci 1995; 73:1789–1792.

[38] Rueda B, Wegner JA, Marion SL, Wahlen DD, Hoyer PB, Internucleosomal DNA. fragmentation in ovine luteal tissue associated with luteolysis: *In vivo* and *in vitro* analysis. Biol Reprod 1995;52:305–312.

[39] Rueda BR, Hendry IR, Tilly JL, Hamernik DL. Accumulation of caspase-3 messenger ribonucleic acid and induction of caspase activity in the ovine corpus luteum following prostaglandin F2alpha treatment *in vivo*. Biol Reprod 1999;60:1087–1092.

[40] Sawada M, Carlson JC. Rapid plasma membrane changes in superoxide radical formation, fluidity, and phospholipase A_2 activity in the corpus luteum of the rat during induction of luteolysis. Endocrinology 1991;128:2992–2998.

[41] Minegishi K, Tanaka M, Nishimura O, Tanigaki S, Miyakoshi K, Ishimoto H, Yoshimura Y. Reactive oxygen species mediate leukocyte–endothelium interactions in prostaglandin $F_{2\alpha}$-induced luteolysis in rats. Am J Physiol Endocrinol Metab 2002;283:E1308–E1315.

[42] Knapen MF, Zusterzeel PL, Peters WH, Steegers EA. Glutathione and glutathione-related enzymes in reproduction. A review. Eur J Obstet Gynecol Reprod Biol 1999;82:171–184.

[43] Levonen AL, Dickinson DA, Moellering DR, Mulcahy RT, Forman HJ, Darley-Usmar VM. Biphasic effects of 15-deoxy-12,14-prostaglandin J_2 on glutathione induction and apoptosis in human endothelial cells. Arterioscler Thromb Vasc Biol 2001;21:1846–1851.

[44] Lim SY, Jang JH, Na HK, Lu SC, Rahman I, Surh YJ. 15-deoxy-12,14-prostaglandin J_2 protects against nitrosative PC12 cells death through up-regulation of intracellular glutathione synthesis. J Biol Chem 2004;279:46263–46270.

[45] Chen ZH, Yoshida Y, Saito Y, Sekine A, Noguchi N, Niki E. Induction of adaptive response and enhancement of PC12 cell tolerance by 7-hydroxycholesterol and 15-deoxy-12,14-prostaglandin J_2 through up-regulation of cellular glutathione via different mechanisms. J Biol Chem 2006;281:14440–14445.

[46] Shikone T, Yamoto M, Kokawa K, Yamashita K, Nishimori K, Nakano R. Apoptosis of human corpora lutea during cyclic luteal regression and early pregnancy. J Clin Endocrinol Metab 1996;81:2376–2380.

[47] Rueda BR, Hendry IR, Hendry WJ, Stormshak F, Slayden OD, Davis JS. Decreased progesterone levels and progesterone receptor antagonist promote apoptotic cell death in bovine luteal cells. Biol Reprod 2000;62:269–276.

[48] Telleria CM, Goyeneche AA, Cavicchia JC, Stati AO, Deis RP. Apoptosis induced by antigestagen RU486 in rat corpus luteum of pregnancy. Endocrine 2001;15:147–155.

[49] Goyeneche AA, Deis RP, Gibori G, Telleria CM. Progesterone promotes survival of the rat corpus luteum in the absence of cognate receptors. Biol Reprod 2003;68:151–158.

[50] Okuda K, Korzekwa A, Shibaya M, Murakami S, Nishimura R, Tsubouchi M, Woclawek-Potocka I, Skarzynski DJ. Progesterone is a suppressor of apoptosis in bovine luteal cells. Biol Reprod 2004;71:2065–2071.

[51] Crawford DR, Davies KJ. Adaptive response and oxidative stress. Environ Health Perspect 1994;102(Suppl 10):25–28.

[52] Wiese AG, Pacifici RE, Davies KJA. Transient adaptation of oxidative stress in mammalian cells. Arch Biochem Biophys 1995;318:231–240.

[53] Shull S, Heintz NH, Periasamy M, Manohar M, Janssen YM, Marsh JP, Mossman BT. Differential regulation of antioxidant enzymes in response to oxidants. J Biol Chem 1991;266:24398–24403.

[54] Lai CC, Peng M, Huang L, Huang WH, Chiu TH. Chronic exposure of neonatal cardiac myocytes to hydrogen peroxide enhances the expression of catalase. J Mol Cell Cardiol 1996; 28:1157–1163.

[55] Sen P, Mukheriee S, Bhaumik G, Das P, Ganguly S, Choudhury N, Raha S. Enhancement of catalase activity by repetitive low-grade H2O2 exposures protects fibroblasts from subsequent stress-induced apoptosis. Mutat Res 2003;529:87–94.

[56] Chen ZH, Yoshida Y, Saito Y, Niki E. Adaptation to hydrogen peroxide enhances PC12 cell tolerance against oxidative damage. Neurosci Lett 2005;383:256–259.