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Full Length Research Paper

Implication of hydrogen peroxide in biochemical and morphological alterations of cultured adventitial fibroblasts of *Psammomys obesus*

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Oxidative stress causes hypertrophy and apoptosis, which are both implicated in various pathogenesis, particularly in cardiovascular diseases. Within this framework, we chose to analyze certain physiopathological aspects (lipid peroxidation, proliferation, extracellular matrix remodeling, hypertrophy and oncosis conducting the cell to apoptosis) induced by hydrogen peroxide on adventitial fibroblasts in culture of *Psammomys obesus.* In secondary culture, the fibroblasts were exposed to 1.2 mM of H_2O_2 during 24 h. The cellular proliferation was evaluated by counting. A morphological and morphometric study was carried out after a coloring of the cells with orange acridine and May Grunwald Giemsa. The quantification of total malonaldehyde, total proteins and total collagens was carried out in the extra and intracellular compartments. The proportioning of NO was carried out by the determination of the concentration of total nitrites. The main results obtained indicated a reduction in cellular proliferation, an oncosis, a compensatory hypertrophy of the fibroblasts, a hypercondensation of chromatin, which is typical of apoptotic cells, an increase in malonaldehyde and nitrite production. Alterations such as a decrease in the production of proteins and total collagens were observed in the extracellular compartment of the cultured fibroblasts. Oxidative stress caused by high amounts of H_2O_2 induces biochemical deteriorations marked by an overproduction of NO, of malondialdéhyde as well as a reduction in proteins and total collagens. These biochemical disturbances are at the origin of the morphological deteriorations which are responsible for the loss of cellular viability leading to apoptosis.

Key words: Adventitial fibroblasts, oxidative stress, cellular culture, H_2O_2, apoptosis.

INTRODUCTION

Free radicals and their precursors are members of a reactive chemical family named reactive oxygen species (ROS). Free radical stress results from disequilibrium in the prooxidant/antioxidant balance. In mammalian cells, potential enzymatic sources of ROS include xanthine oxidase, nitric oxide synthase (NOS) and peroxydases (Favier, 2003). These systems primarily catalyse one electron reduction of molecular oxygen to form the superoxide anion ($O_2•^-$) which rapidly reacts with the nitric oxide (NO•) to form peroxynitrite (Favier, 2003). Under ambient conditions, some $O_2•^-$ is dismutated to H_2O_2 sponta-neously or catalyzed by superoxide dismutase (SOD). Some enzymes, such as xanthine oxidase and glucose oxidase, can directly produce H_2O_2 by donating two electrons to oxygen. In the presence of heavy metals, H_2O_2 undergoes Fenton reaction to form highly reactive hydroxyl radical (•OH). When bound to peroxidases such

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as catalase, H_2O_2 forms compound which oxidizes NO• to nitrogen dioxide anion (NO_2-) (Favier, 2003, Cai, 2005).

The oxidative stress is implicated in the several pathologies like hyperlipidemia, diabetes, consumption of tobacco and hormonal disorders such as hypothyroidism which accelerate the development of pathological processes such as cardiovascular diseases, and constitute a real public health problem (Pincemail et al., 1998).

Atherosclerosis is a major component of cardiovascular diseases and the main clinical manifestations are heart attacks of ischemic origin (tonsillitis) and myocardial infarctions (Pincemail et al., 1998). Many authors have shown the implication of oxidative stress in the development of the atherosclerotic plaque (Stocker and Keaney, 2004). At some physiological levels, the pro-oxidizing toxic molecules produced are neutralized by antioxydants (vitamins, oligoelements, and enzymes); when there is an imbalance in favor of pro-oxidant products, oxidative stress settles in. The latter is responsible for many structural (lipids, proteins, DNA) as well as physiological deteriorations which affect the cell and lead it to its death (Favier, 2003).

Free radicals are necessary species to life and fulfill many functions; they are involved in several ways such as functioning of some enzymes, immune defense against pathogenic agents, destruction of tumoral cells by apoptosis, cell cycle, regulation of capillary dilatation, operation of certain neurons, gene regulation and in the redox control of genes They constitute a system of signal transmission and can be regarded as intra- and extra-cellular messages (Favier, 2003). However, they can provoke cellular damages, inducing DNA mutation, inactivating proteins or inducing lipidic peroxidation processes of the lipoproteins or the cellular membrane polyinsaturated fatty-acids, leading the cells to apoptosis (Pincemail et al., 2001).

The apoptotic process can be divided into three successive chronological phases (Thornberry and Lazebnik, 1998):

1. The initiation phase which is started by varied stimuli like oncogenes, growth factors, cytokines, ROS and ceramides (Thornberry and Lazebnik, 1998).
2. The effector phase during which the cell undergoes biochemical changes, a result from the systematic activation of the catabolic hydrolases (proteases and nucleases), facilitating the morphological changes of the cells,
3. The degradation phase which is marked by the cleavage of the proteins, DNA, and the lipids (Thornberry and Lazebnik, 1998).

Taking account these aspects, the aim of this study was to evaluate the adventitial fibroblasts induction to the oxidative stress *in vitro*. To do this, we analyzed the effects of hydrogen peroxide (1.2 mM/ 24 h) in the adventitial fibroblasts of *Psammomys obesus* in culture by an estimate of the rate of cellular proliferation, a morphological and morphometric study after an acridine orange and May Grunwald Giemsa staining (MGG), a dosing of the lipidic peroxidation products (malondialdehyde) and of nitric monoxyde and a quantification of the proteins and total collagens contained in the intra- and extra-cellular compartments.

MATERIALS AND METHODS

Biological material

We did our study on a series of 08 sand rats (*P. obesus*). Under captivity, the animals are maintained in conditions previously described by Aouichat et al. (2001); they are placed in individual cages and are fed halophilous plants at a rate of 50 g/day. These rats are desert rodents we meet in several areas of the Sahara, especially nearby Beni-Abbès (Wilaya of Bechar, 30° 7 Northern latitude and 2° 10 Western longitudes) in South-western Algeria. *P. obesus* is specie with diurnal activity, commonly called “Gerd” by the local populations of the various Saharan regions. It belongs to the Muridae family, a subfamily of Gerbillidae. It has a three year life expectancy and under the severe climatic conditions of the desert, it feeds on halophilous plants, which are rich in water and minerals (particularly sodium chloride) of the Chenopodiaceae family; especially *Tragnum nudatum*, *Salsola foetida* and *Sueda mollis* (Daly and Daly, 1973).

Analytical methods

A weekly weighing is carried out in order to monitor the body weight variations in the animals. The analysis of the biochemical parameters consists in a monthly blood sampling carried out by a puncture at the level of the retro-orbital plexus of the eye (Aouichat Bouguerra et al., 2001). This technique prevents using anesthetics, which could influence biochemical parameters. The blood is sampled in heparinized tubes and immediately centrifuged (3000 rpm). The plasmas were collected and preserved at -20°C, ready for the dosing of their biochemical parameters (glycemia, Triglyceridemia, cholesterolemia and proteinemia) which are carried out using Biosystem kits. The insulinemia is determined by radio immunological method (CIS Bio).

Culture of adventitial fibroblasts

The adventitial fibroblasts of *P. obesus* were put in culture using the explants technique (Aouichat et al., 2001; Daly and Daly, 1973). The animal was anaesthetized with urethan (0.4 ml/ 100 g). After opening the thorax, we took the aorta out and immediately plunged it into a box of Petri containing Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, USA), supplemented with 10% fetal calf serum (FCS) (Sigma,USA), 1% antibiotics (streptomycin 50 µg/ml, penicillin 50 IU/ml), 1.2% L-glutamine at 200 mM (Gibco, USA) and 5% of HEPES to preserve the pH. The aortic lumen was then emptied of its blood. The aorta was incubated for 20 min at 37°C in 0.1% collagenase to remove the endothelium and to facilitate the separation between the media and the adventitia, and was cut out into 1 mm explants. 8 to 10 explants were placed inside flasks and incubated in the presence of DMEM containing 10% FCS, 1.2% glutamine and 1% antibiotics and plunged into the incubator at 37°C under a humidified atmosphere with 95% air and 5% CO_2. The culture of explants corresponds to primo-culture. In secondary culture and at the 6th passage, the adventitial fibroblasts were suspended a second time by trypsinisation (0.08% of trypsin; Gibco, USA); they were sown in plaques of 6 wells at a rate of 8×10^5 cells per well, in 1.5 ml of DMEM supplemented with 10% of FCS, 1.2%

L-glutamine, 1% antibiotic. At confluence, the medium was eliminated and the cells were exposed to hydrogen peroxide at 1.2 mM, prepared in DMEM with 1.2% L glutamine and 1% antibiotics without FCS and incubated for 24 h. A plaque of control cells was subjected to the same conditions with no addition of H_2O_2.

Study of the proliferation

At the 7th passage and after exposure to H_2O_2, the cells were trypsinized (0.08% of trypsin); the rate of proliferation was carried out on the cellular suspension by counting using the Mallasez cells.

Morphometric and morphological study

After 24 h of incubation in the presence of 1.2 mM hydrogen peroxide, the mediums were eliminated, the cells were washed with a phosphate-buffered saline (PBS, 1X) and then fixed to the aqueous Bouin and colored with a May Grunwald-Giemsa (MGG; V/V, 1/1) solution diluted into 1/3 in distilled water and 100 mg/ml orange acridine. The observation was done with an inverted microscope (Zeiss) for the MGG coloring and a fluorescence microscope (Zeiss, blue filter) for the coloring with acridine orange. In order to analyze the state of the adventitial fibroblasts of *P. obesus* subjected to the oxidizing agent (H_2O_2), we took the measurements of the cellular and nuclear main axis using a micrometer and we numbered the nucleoli on cells colored with MGG. Each parameter was measured on 100 cells, in various fields and on several wells.

Proportioning of malondialdehyde (MDA)

The MDA was determined both for extra and intracellular compartments of the fibroblasts after reaction with thiobarbituric acid (TBA) (Heath and Packer, 1968). These compartments were buffered (Na_2HPO_4/NaH_2PO_4 – 0.2 M, pH 6.5) and centrifuged at 10000 g during 20 min at 4ºC. The MDA contained in supernatant, in the presence of trichloracetic acid (TCA); 10% reacted with the TBA and caused the formation of a complex readable at 532 nm.

Proportioning of NO

The formation of NO is classically evaluated in an indirect manner by determining the concentrations of nitrites and nitrates which constitute the oxidative breakdown products of NO. The extra and intracellular compartments of the pilot cells and subjected to H_2O2 are deproteinized by centrifugation at 10000 g for 10 min at -20°C. The determination of nitrites and nitrates is directly carried out from the obtained supernatant. The Griess reaction only allows the measurement of nitrites. Therefore, to be quantified the nitrates were reduced into nitrites. The concentration thus measured represents the sum of nitrites and nitrates. The transformation of nitrates into nitrites is based on a reaction of reduction by cadmium and regenerated using a 5 min contact with a buffer solution of $CuSO_4$ at 5 mM in a glycine-NaOH. The nitrites contained in the deproteinized and regenerated extra and intracellular compartments of the pilot cells and subjected to the H_2O_2 at 1.2 mM for 24 h are quantified after adding the Griess reagent [0.1% N-(1 naphthyl) ethylenediamine dihydrochloride, 1% sulphanilamide, and 5% phosphoric acid]. The optic density (OD) reading was carried out at 543 nm.

Proportioning of total proteins and collagens

The samples of the extra (ECC) and intracellular (ICC) compartments of the cultured fibroblasts were recovered and submitted to 2 successive dialysis (Serva, 8/32). The latter undergo two successive dialyzes of 24 h, respectively against running water and acetic acid at 0.5 M at 4°C, under soft agitation. After dialysis, an aliquot was collected to quantify the total amount of proteins; the remainder underwent a 3rd dialysis in the presence of pepsin (Merck) with 200 mg/ml, against the acetic acid at 0.5 M for 24 h at 4°C. The total proteins and pepsino-resistant proteins (collagenous) contained in the ECC and the ICC reacted with the Bradford reagent, resulting in the formation of a colored complex readable at a 595 nm wave length of Bradford (1976).

Statistical analysis

Our results correspond to the affected averages of the standard deviation which are analyzed by the test of student.

RESULTS

In vivo study

The average body weight of the animals is of 94 g. Glycemia, cholesterolemia, triglyceridemia, proteinemia and insulinemia deferred on Table 1 are the parameters carried out on the animal in captivity after two weeks of adaptation. Under our experimental conditions, the animal is subjected to a hypocaloric diet rich in water and minerals and corresponds to the control group.

In vitro study

Study of the proliferation

Our results show that the proliferation rate increases by 9.3% in pilot cells and decreases by 32.3% in cells exposed to H_2O_2 ($p \leq 0.01$) (Figure 1). The reduction in proliferation testifies to the toxicity caused by hydrogen peroxide on fibroblasts in culture.

Morphometric and morphological study

The measurement of the nuclear and cellular main axis, carried out on 100 cells in various fields reveals respectively, after addition of hydrogen peroxide, an increase of 65.2 and 23% ($p < 0.001$) compared to the corresponding reference (Figure 2). This increase in the cellular and nuclear sizes marks compensatory hypertrophy. In addition, we noted an increase in the number of nucleoli in the fibroblasts subjected to H_2O_2 (Figure 3). Observation under the inverted microscope enabled us to note that the cells subjected to H_2O_2 for 24 h are characterized by a vacuolization of the cytoplasm, a sign of oncosis (Figure 4). A morphological analysis of the apoptotic cells is carried out by coloring with orange acridine. The observation of the cells under a fluorescence microscope shows that the intensity of

Table 1. Evaluation of body weight (g), glycemia, cholesterolemia, triglyceridemia, total protein (mg/dl) and insulinemia (µUI/ml) of *P. obesus* witnesses after their adjustment period in captivity and subjected to natural diet consisting of halophyl plants.

Body weight (g)	Glycemia (mg/dl)	Cholesterolemia (mg/dl)	Triglyceridemia (mg/dl)	Total protein (mg/dl)	Insulinemia (µUI/ml)
94 ± 8	73 ± 10	65 ± 13	50 ± 24	51 ± 8	20,2 ± 9.3

The values are averages of glycemia, cholesterolemia, triglyceridemia, total protein and insulinemia affected the standard deviation of 8 *P. obesus* witnesses. The average body weight of the animals is 94 g. The glycemia, cholesterolemia, triglyceridemia, proteinemia and insulinemia are the parameters carried out on the animal in captivity after two weeks of adaptation. Under our experimental conditions, the animal is subjected to a hypocaloric diet rich in water and minerals and corresponds to the control group.

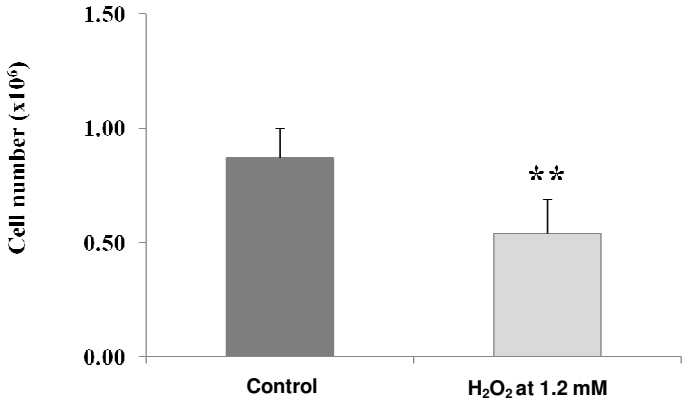

Figure 1. Rates of adventitious fibroblasts proliferation of *P. obesus* incubated in the absence or presence of hydrogen peroxide to 1.2 mM for 24 h. Values are mean of proliferation rate affected the standard deviation. **p ≤ 0.01. 6 wells of cells subjected to H_2O_2 *vs* 6 wells of control cells.

fluorescence increases in the presence of the oxidizing agent (H_2O_2). Moreover, compared to the control (Figures 5a and c), the fibroblasts exposed to hydrogen peroxide showed an intense fluorescence (Figures 5b and d) in the perinuclear area, marking a hypercondensation of chromatin and showing a budding of the plasmic membrane.

Proportioning of NO

The rate of total nitrites evaluated respectively in the extra and intracellular compartments of the aortic fibroblasts subjected to the H_2O_2 indicates an increase in 48% (P ≤ 0.05) and of 491% (P< 0,001) compared to the control (Figures 6 and 7).

Proportioning of the products of the lipidic peroxidation

Our results show a very significant growth of the concentration of MDA in the ECC of the cells subjected to the H_2O_2 compared to the control (p < 0,001) (Figure 8). The concentration of MDA in the ICC of the extracts

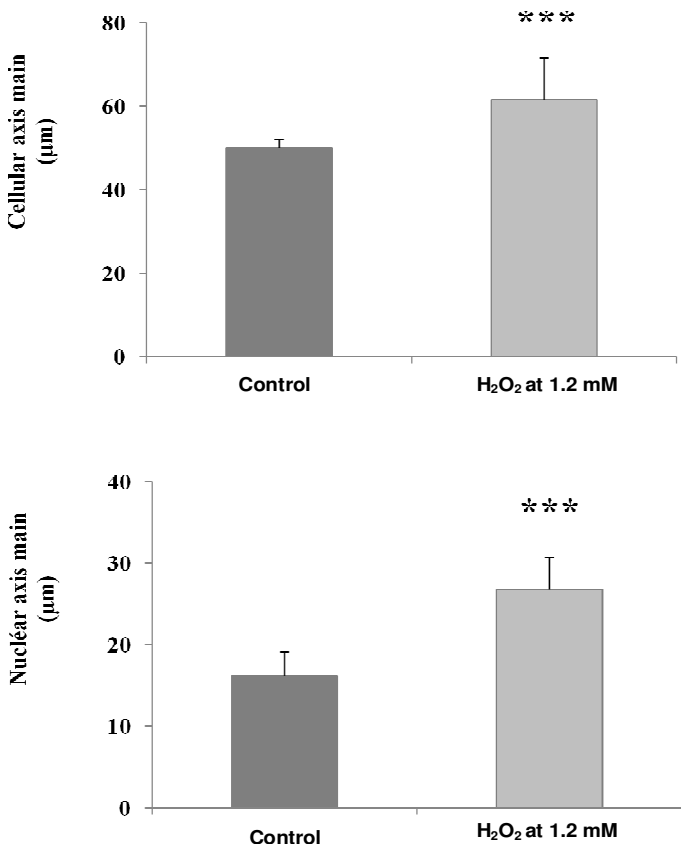

Figure 2. Morphometric analysis of cellular and nuclear main axis (µm) of adventitious fibroblast of *P. obesus* incubated in the absence or presence of hydrogen peroxide to 1.2 mM for 24 h. The values are averages of major cellular and nuclear main axis affected by the deviation measured on 100 cells in different fields and on several wells. ***p < 0.001. Cells subjected to H_2O_2 *vs* control cells.

subjected to H_2O_2 also increases; the statistical analysis is highly significant (p < 0.0001) (Figure 9).

Proportioning of total proteins and pepsino-resistant proteins

Proportioning of total proteins: The evaluation of the quantity of total proteins contained in the two compartments

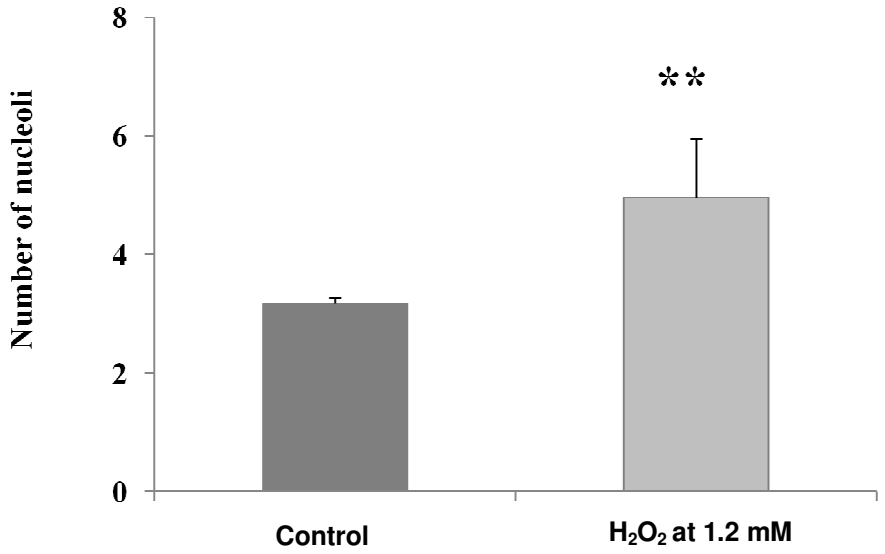

Figure 3. Number of adventitious fibroblasts nucleoli of *P. obesus* incubated in the absence or presence of 1.2 mM H_2O_2 for 24 h. The values are the averages of the number of nucleoli assigned and the standard deviation measured on 100 cells in different fields and on several wells. ** $p \leq 0.01$. Cells exposed to H_2O_2 *vs* control cells.

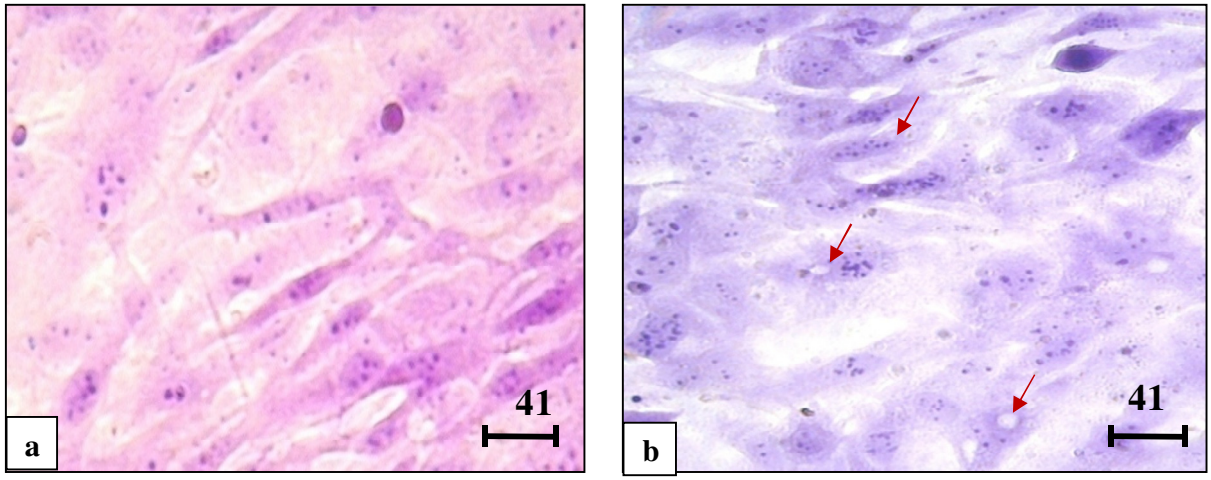

Figure 4. MGG staining of *P. obesus* aortic fibroblasts, incubated in the absence or presence of H_2O_2 for 24 h. (a) Aortic fibroblasts *P. obesus*, incubated in the absence of H_2O_2 for 24 h in secondary culture, fixed in aqueous Bouin and stained with May Grunwald Giemsa (MGG) (G × 246). (b) Aortic fibroblasts of *P. obesus*, incubated in the presence of 1.2 mM H_2O_2 for 24 h in secondary culture, fixed in aqueous Bouin and stained with May Grunwald Giemsa (MGG), showing cytoplasm vacuolization (red arrows) (G × 246).

(medium and cells) after 24 h of exposure to the H_2O_2 showed a very significant reduction compared to the control, this reduction is respectively of 70.6 and 69.9% ($p < 0,001$) (Figure 10 and 11).

Proportioning of total collagens: Our results relating to the quantification of the collagens contained in the ECC of the cells exposed to H_2O_2 have show a very significant reduction in 84.5% ($p < 0.001$) compared to the control (Figure 12). In the ICC, we also observed a 66.1% reduction of the rate of collagen, after the action of H_2O_2 ($P \leq 0.01$) (Figure 13).

DISCUSSION

The *in vivo* results confirm the result obtained by Marquié et al. (1991) and Aouichat et al. (2001) which showed that *P. obesus* is an experimental model of diabetes and atherosclerosis. Our *in vitro* study enabled us to analyze

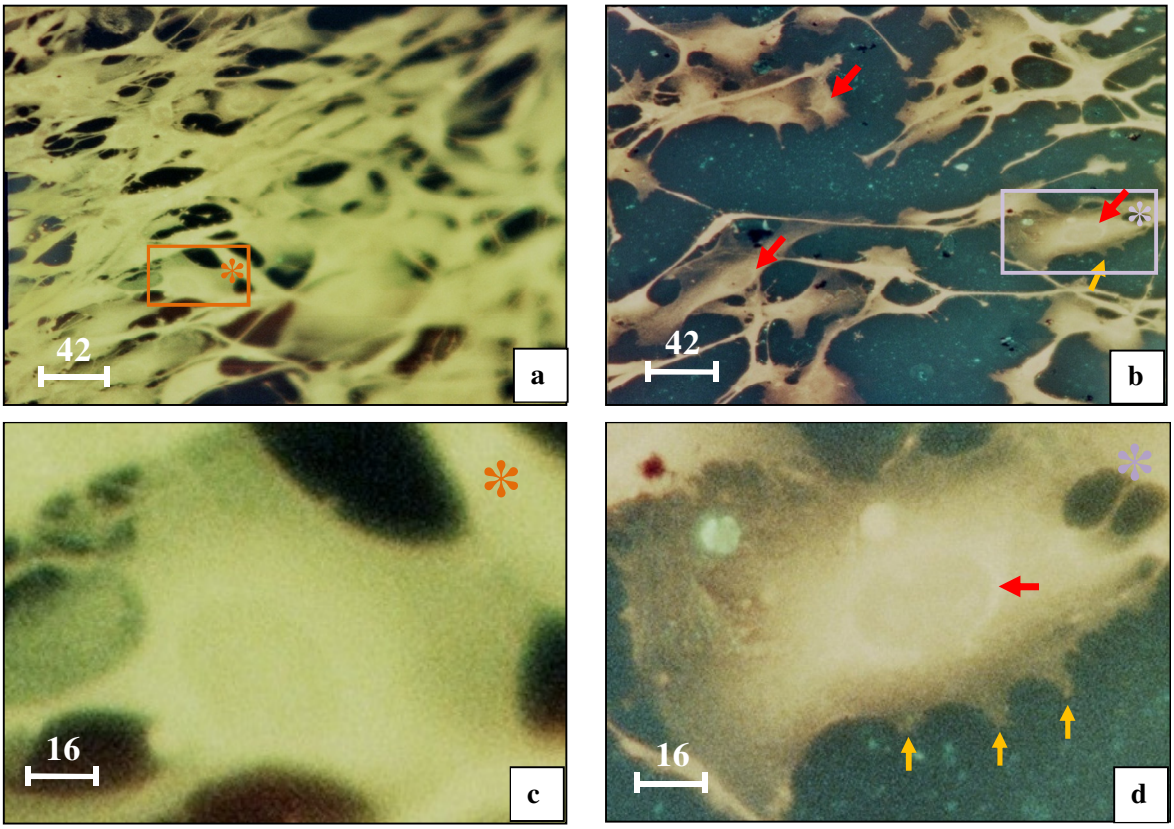

Figure 5. Acridine orange staining of aortic fibroblasts of *P. obesus* incubated in the absence or presence of H_2O_2 for 24 h. (a) Aortic fibroblasts of *P. obesus*, incubated in the absence of hydrogen peroxide for 24 h in secondary culture, fixed in aqueous Bouin and stained with acridine orange, low magnification (G × 237). The cell expansion is shown in (c). (b). Aortic fibroblasts of *P. obesus*, incubated in the presence of 1.2 mM H_2O_2, for 24 h in secondary culture, fixed in aqueous Bouin and stained with acridine orange, showing chromatin hypercondensation around the nuclear envelope, appears in bright color (red arrows) and plasma membrane budding (orange arrows), low magnification (G × 237). The cell expansion is shown in (d).

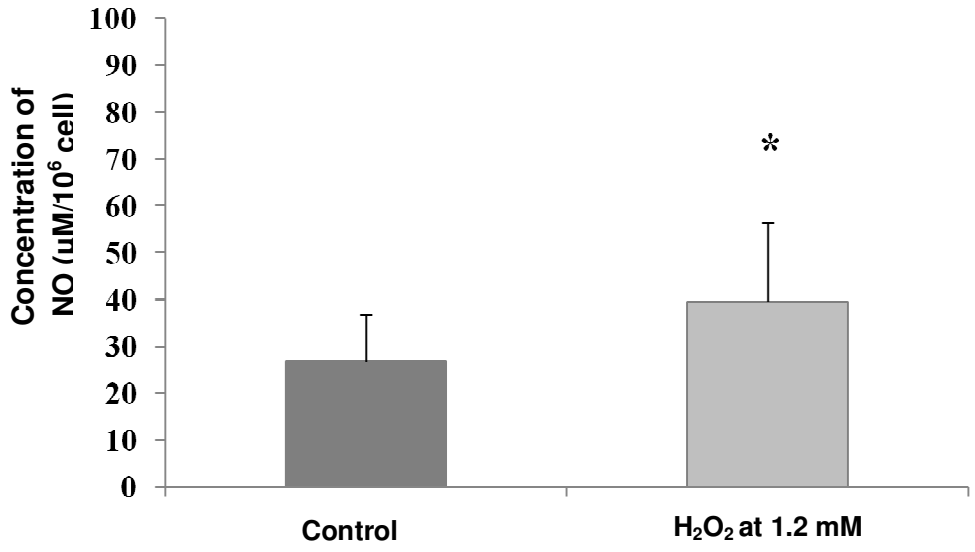

Figure 6. Evaluation of the concentration of total nitrite in the extracellular compartment of aortic fibroblast of *P. obesus* incubated in the absence or presence of 1.2 mM H_2O_2 for 24 h (µM / 10^6 cells). Values are averages of NO affected the standard deviation. *p ≤ 0.05. 5 extracts exposed to H_2O_2 *vs* 5 extracts witnesses.

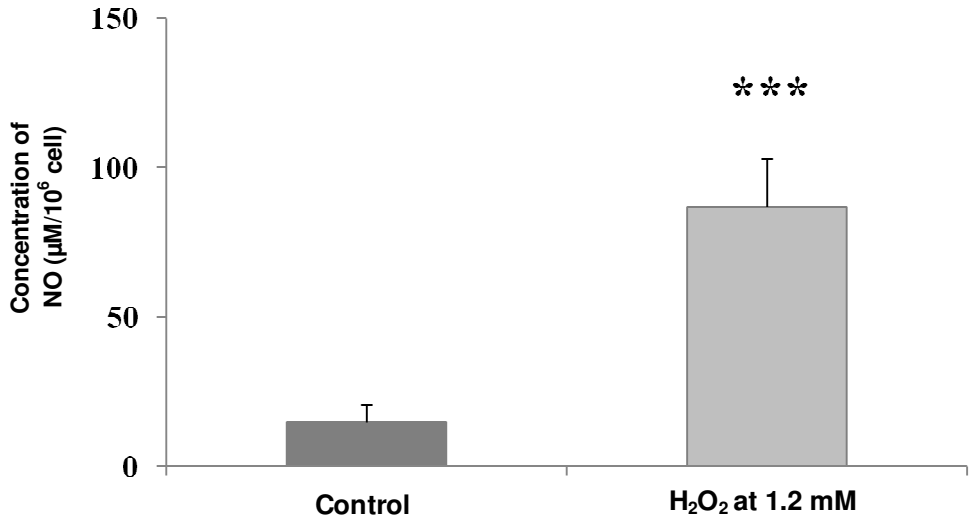

Figure 7. Evaluation of the concentration of total nitrite in the intracellular compartment of fibroblasts aortic *P. obesus* incubated in the absence or presence of 1.2 mM H_2O_2 for 24 h (µM/ 10^6 cells). Values are averages of NO affected the standard deviation. ***p < 0,001. 5 extracts exposed to H_2O_2 *vs* 5 extracts witnesses.

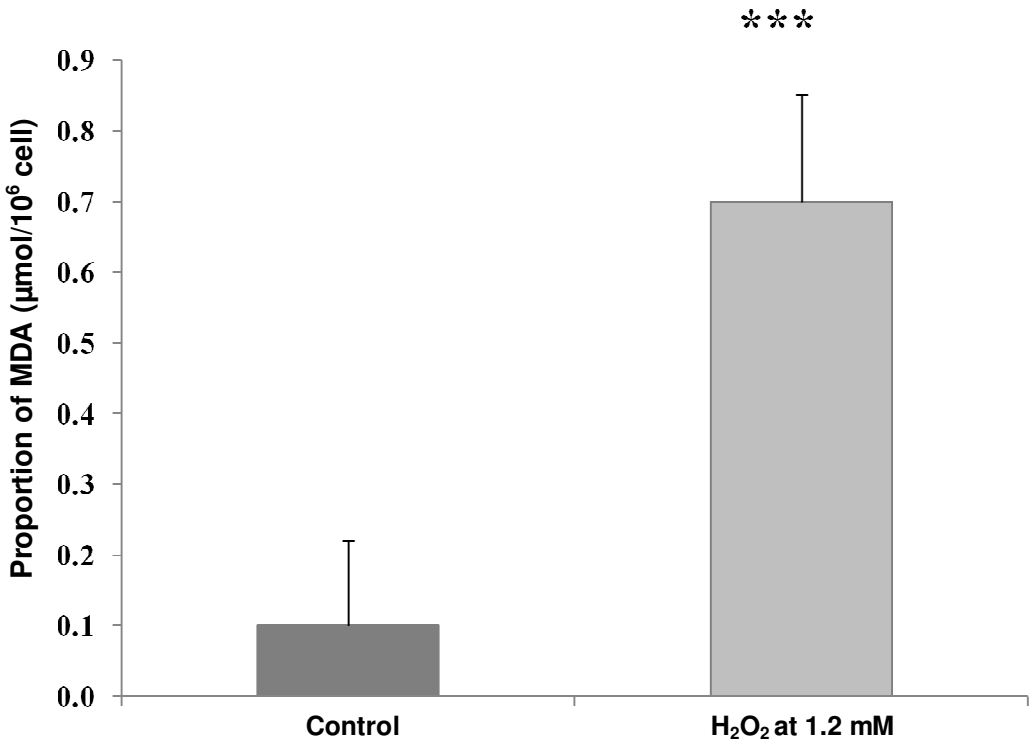

Figure 8. MDA levels evaluation (µM/ 10^6 cells) contained in the extracellular compartment of cultured aortic fibroblasts of *P. obesus* in the presence of 1.2 mM H_2O_2 for 24 h. Values are averages of MDA assigned standard deviation. ***p < 0,001. 5 extracts incubated in H_2O_2 *vs* 5 extracts witnesses.

some physiopathological aspects of the adventitial fibroblasts of *P. obesus* in culture under the influence of hydrogen peroxide. The fibroblasts exposed to H_2O_2 showed a decrease in the rate of proliferation compared to the control. Our results are in agreement with that of De Haan et al. (2004) who observed a decreasing of the proliferation rate in mice embryonic fibroblasts incubated in a concentration of 30 µM of H_2O_2. Moreover, Kang et al. (2008), studying hamster's fibroblasts incubated in a concentration of 1 mM of H_2O_2, also observed a decreasing

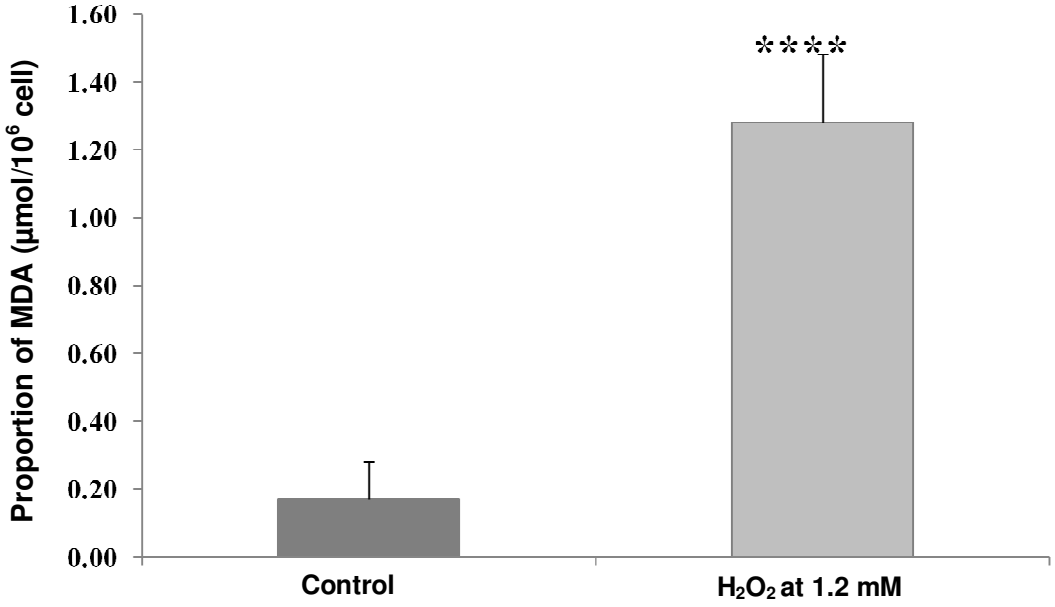

Figure 9. MDA levels evaluation (µM/ 10^6 cells) contained in the intracellular compartments of cultured aortic fibroblasts of *P. obesus* in the presence of 1.2 mM H_2O_2 for 24 h. Values are averages of MDA assigned standard deviation. ****p < 0,0001. 5 extracts incubated in H_2O_2 *vs* 5 extracts witnesses.

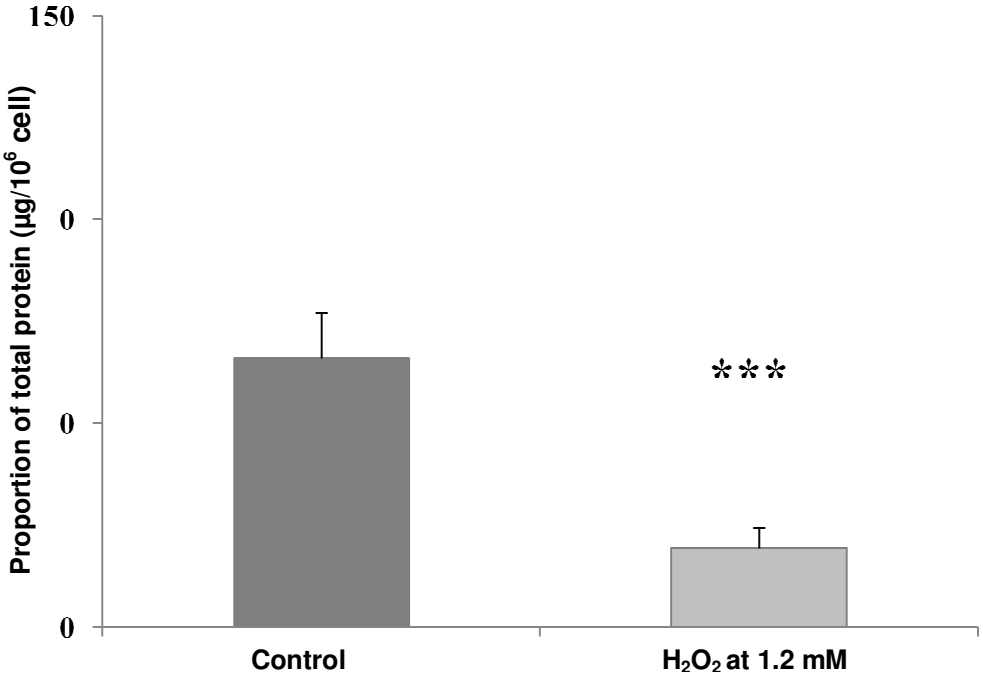

Figure 10. Total protein quantity evaluation (µg/ 10^6 cells) in the extracellular compartment of cultured aortic fibroblasts of *P. obesus* in the presence of 1.2 mM H_2O_2 for 24 h. Values are averages of proteins affected by the standard deviation. ***p < 0,001. 5 extracts exposed to H_2O_2 *vs* 5 extracts witnesses.

of the proliferation rate. According to Ohguro et al. (1999) and Colston et al. (2004), a high concentration of H_2O_2 (> 10 µM) is cytotoxic for the cells and leads to a fall of the cellular proliferation. This cytotoxicity is time dependant whereas a weak concentration (≤ 10 µM) stimulates proliferation and its effect is comparable to that of

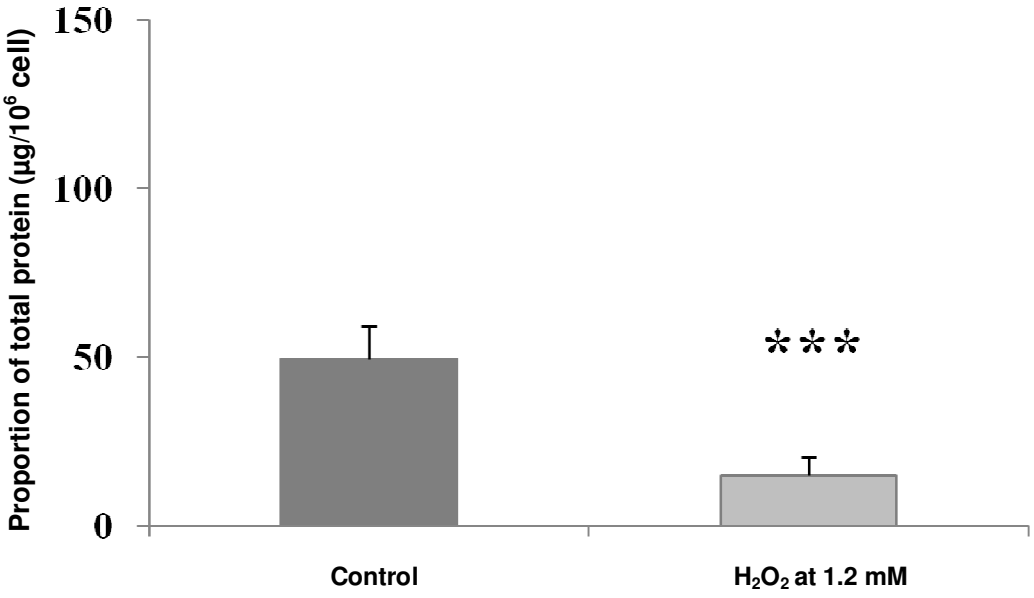

Figure 11. Total protein quantity evaluation (µg/ 10^6 cells) in the intracellular compartment of cultured aortic fibroblasts of *P. obesus* in the presence of 1.2 mM H_2O_2 for 24 h. Values are averages of proteins affected by the standard deviation. ***p < 0.001. 5 extracts exposed to H_2O_2 *vs* 5 extracts witnesses.

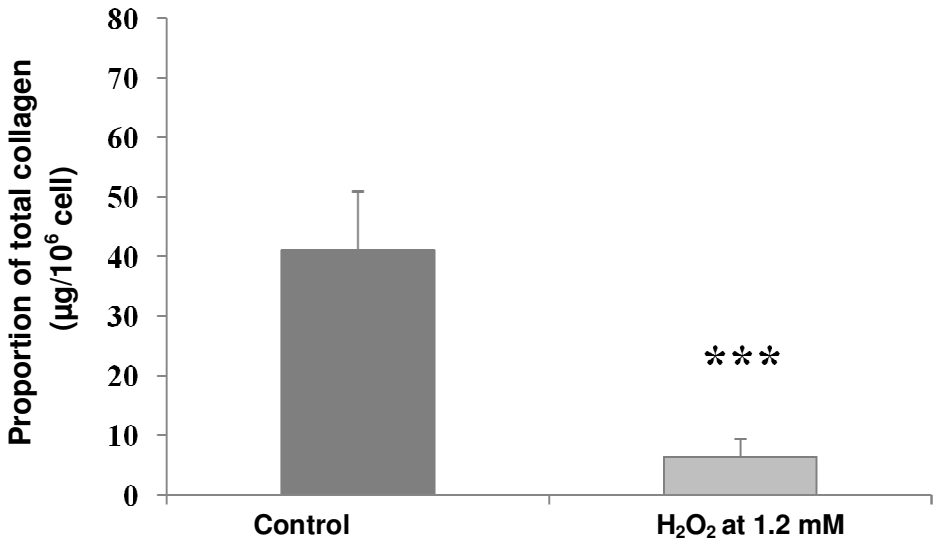

Figure 12. Total collagen quantity evaluation (µg/10^6 cells) in the extracellular compartment of cultured aortic fibroblasts of *P. obesus* in the presence of 1.2 mM H_2O_2 for 24 h. The values are the averages of the total collagen affected the standard deviation. ***p < 0.001. 5 extracts exposed to H_2O_2 *v*s 5 extracts witnesses.

growth factors, leading to cellular proliferation (Ohguro et al., 1999). At high concentrations (1 mM), the hydrogen peroxide induced damage to the cell membrane, one of the most important lesions to cells, responsible for the loss of cell viability (Kang et al., 2008). At low concentration, H_2O_2 can activate the factors of transcription (Esposito et al., 2004) controlling the proteins responsible of the stop of the cellular cycle (Vairo et al., 2000; Greider et al., 2002). According to Mocali et al. (1995), the exposure of the fibroblasts to the H_2O_2 involves a reduction in the activity of the cellular cycle by increasing the G0 phase and a loss of the replicative potential, another process which would explain the reduction in the proliferation of the adventitial fibroblasts

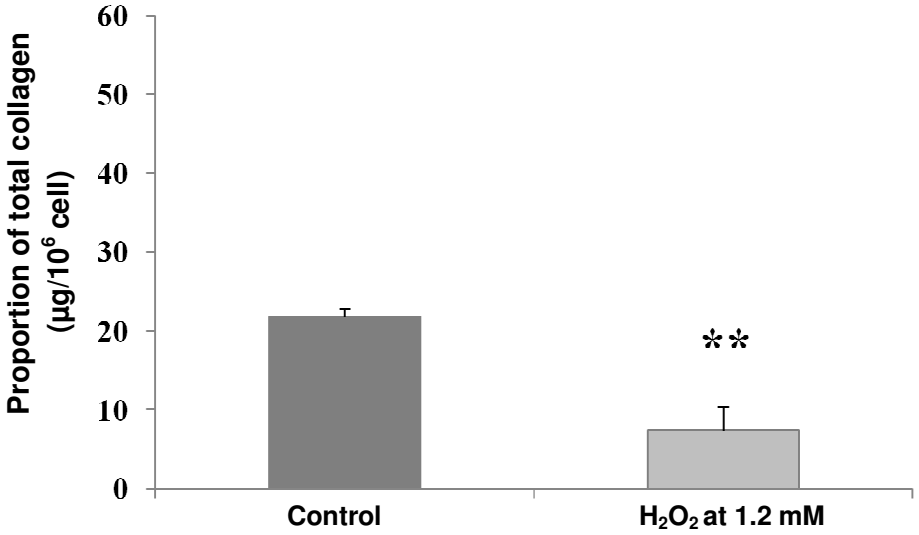

Figure 13. Total collagen quantity evaluation (µg/ 10^6 cells) in the intracellular compartment of cultured aortic fibroblasts of *P. obesus* in the presence of 1.2 mM H_2O_2 for 24 h. The values are the averages of the total collagen affected the standard deviation. **p ≤ 0.01. 5 extracts exposed to H_2O_2 *vs* 5 extracts witnesses.

in our study.

Subjected to a situation of oxidative stress by adding hydrogen, the adventitial fibroblasts of *P. obesus* exposed to H_2O_2 revealed an increase in cellular and nuclear main axis as well as in nucleoli quantity. Our results are in agreement with those found by De Haan et al. (2004) which showed an increase in the total cellular surface of mice fibroblasts incubated in the presence of 30 µM hydrogen peroxide for 6 h. According to the same authors, these morphological changes are similar to those of the senescent cells in culture. Landex et al. (2006) showed an increase in the general size of the thyroid epithelial cells incubated in the presence of H_2O_2 at 0.3 mM. According to Mocali et al. (1995), the increase in size of the fibroblasts exposed to H_2O_2 at 0.5 mM and their morphological deterioration would be due to a decrease of the proteins and DNA's rate of synthesis and would refer to compensatory cellular hypertrophy. Indeed De Haan et al. (2004) show that during the oxidizing stress induced by H_2O_2, the cellular proliferation decreases and the cells which remain alive tend to become hypertrophic and thus towards a phenomenon of compensation. Therefore, this way of resisting would mark their adaptation to the oxidative effect caused by hydrogen peroxide (Wiese et al., 1995). In addition to their implication in atherosclerosis, the ROS intervene in the development of cardiac hypertrophy, the apoptosis and the remodeling of the failing heart (Byrne et al., 2003). According to Chen et al. (2000), high concentrations of H_2O_2 induce the hypertrophy and the apoptosis of cardiomyocytes of adult rats. Indeed, the functional implication of ROS in cardiovascular cells shows that they are pro-hypertrophic (Wang et al., 2001); this phenomenon appears to be a process of adjustment (adaptation) to situations of oxidative stress marked by compensation (Chien, 1999). The ROS activate various kinase signal paths which are implied in the regulation of hypertrophy and/or of cellular apoptosis: ERK 1/2, JNK, p38 kinase and Akt. The signal path which would be activated depends on the concentration of H_2O_2. According to Kwon et al. (2003), a weak concentration of H_2O_2 (10 µM) leads to the hypertrophy of the cardiomyocytes of adult rats, stimulated following the activation of ERK 1/2 signal path. At high concentrations of H_2O_2 (100 µM to 1 mM), it is the JNK signal path which is activated, stimulating cellular apoptosis.

In our morphological analysis, we noticed deterioration in the cytoplasm vacuolization of fibroblasts subjected to hydrogen peroxide. Our results conform to those obtained by Weisburg et al. (2004) and Babich et al. (2006) in fibroblasts incubated in the presence of an oxidant which generates hydrogen peroxide. According to Weisburg et al. (2004) and Babich et al. (2006), hydrogen peroxide is responsible for this vacuolization which characterizes oncosis.

In our study, we observed signs of apoptosis in the fibroblasts exposed to hydrogen peroxide. Indeed, the cells subjected to the H_2O_2 and colored with orange acridine show an important perinuclear fluorescence marking the hypercondensation of chromatin as well as buddings of the plasmic membrane indicating an apoptotic state. These results are in agreement with that of Huot et al. (1998) in the endothelial cells exposed to H_2O_2. Moreover, Rabkin and Kong (2000) and Babich et al. (2007) studying respectively ; the cardiomyocytes and human's fibroblasts incubated in the presence of an agent

generating hydrogen peroxide, also observed an hyperfluorescence and a budding of the cells membranes. Orange acridine can penetrate in the cells without permeabilisation (Jajte et al., 2001) and preferably color the nuclear structures and lysosomal (Rabkin and Kong, 2000). According to Coleman et al. (2001), the budding of the plasmic membrane during the apoptosis is initiated following the activation of the caspases via the protein dependant Rho kinases (ROK-β). The induction of the apoptosis is associated both to a hypercondensation of chromatin (Weisburg et al., 2004) and to the expression and activation of specific protein that are responsible for the execution of the apoptotic program in the affected cells. H_2O_2 or the superoxyde radical or both cause the release of the apoptotic mitochondrial cytochrome C via the p53, Bad and Bax (Rüdiger von Harsdorf et al., 1999).

The oxidative stress we have induced by incubating aortic fibroblasts in the presence of hydrogen peroxide, led to an increasing of the production of nitric oxide in both the extra and intracellular compartments (ECC, ICC). Our results confirm those of Rabkin and Kong (2000) who observed an increase of NO in cardiomyocytes incubated in the presence of a H_2O_2 generating agent for 24 h. According to Colston et al. (2004), exposure of cardiac fibroblasts to hydrogen peroxide activates the endogenous production of the superoxide radical and the NO. A significant concentration of the superoxide radical produced by H_2O_2, in combination with nitric oxide leads to peroxynitrite ($ONOO^-$) which causes cell and tissue damages (Beckman and Koppenol, 1996; Endeman and Schiffrin, 2004). Furthermore it should be noted that the presence of NO in the ECC is probably due to the deterioration of cell membranes by the peroxidation of membrane lipids, leading to its release outside the intracellular compartment. NO is a key molecule in various pathophysiologies. In fact, the activity dysregulation of NO synthase (NOSs) and NO metabolism seem to be responsible for various heart diseases (Fogli et al., 2004). However, the direct and physiological effect of NOs is predominant at low rates (< 1 µM). They are synthesized once the constitutive isoforms are activated, the eNOS and the nNOS activating the cyclase guanylate that ensures the production of cGMP (Balligand and Cannon, 1997). When the iNOS were induced, large amounts of NO (> 1 µM) were synthesized. They interacted with both the molecular oxygen and the superoxide anion, forming RNS (reactive nitrogen species) such as peroxynitrite, thus causing the nitrosylation, the oxidation and the nitration of amine and thiol groupments from hydroxyaromatic groups and tyrosine residues (Davis et al., 2001). An important expression of iNOS is observed in human cardiac diseases (Lefer et al., 1991). Koglin et al. (1999) showed that NO can induce apoptosis and thus cardiac dysfunctioning via an over-activation of iNOS.

In our study, we have analyzed the effect hydrogen peroxide has on the production of MDA, a lipid peroxidetion marker, in both the extra and the intracellular compartments (ECC, ICC) of *P. obesus* cultured advent-tious fibroblasts. In the presence of a high concentration of H_2O_2, adventtious fibroblasts show a significant production of MDA compared to reference. Our results confirm those of Okuma and al. (1969) who observed an increase of MDA levels in rat blood platelets, those of Kang et al. (2008) on hamster fibroblasts incubated in the presence of H_2O_2 at 1 mM for 24 h. According to Nakagawa et al. (2002), H_2O_2 ensures the formation of free radicals such as the superoxide radical ($O_2^{\bullet -}$) and the hydroxyl radical ($OH^{\bullet}$) which have an important role in the peroxidation of lipids. According to Levy et al. (1992) and Meriga et al. (2003), lipid peroxidation would be the consequence of an oxidative deterioration of membrane polyunsaturated fatty acids by H_2O_2 generated free radicals. This would explain the results we have obtained, a decrease of MDA in the ICC of fibroblasts exposed to H_2O_2, following its release in the ECC, in which the rates of MDA increase. Lipids are extremely sensitive to oxidation mediated by ROS, particularly polyunsaturated fatty acids localized at the level of cell membranes.

The latter are more susceptible to attacks that generate toxic lipid peroxides and conjugated dienes (Halliwell and Gutteridge, 1998) which are responsible for the inactivation and the destruction of enzymes, for the disruption of metabolic pathways and for the inhibition of cell division (Heath and Packer, 1968), given the interaction of ROS with the various molecules (Favier, 2003).

In our study, we found a decrease of total protein and total collagen rate in the ECC and the ICC of fibroblasts incubated in hydrogen peroxide. Our results confirm those of Mocali et al. (1995) which showed a significant decrease of total protein rate in human fibroblasts incubated in 0.5 mM H_2O_2 for 24 h. Similarly, according to Kumar and Knowles (1993), the products from the peroxidation of lipids (hydroperoxides, MDA) reduce the synthesis of proteins and can induce an accumulation of amino acids. The works of Kwon et al. (2003) showed that exposure of adult rats' cardiomyocytes to low concentration of H_2O_2 (10 to 30 µM) for 48 h, increases the total amount of proteins and the rate of their synthesis when a high concentration (100 µM to 1 mM) leads to their decline following the reduction of cell proliferation. According to Mocali et al. (1995), hydrogen peroxide at 0.5 mM causes cell membrane damages, the soluble proteins can then diffuse freely and independently from their relative molecular mass.

Of all proteins contained in the extracellular matrix, collagens represent the largest proportion produced by adventitious fibroblasts. The results obtained for collagen were similar to the results obtained by Kato et al. (1992) who observed a collagen degradation after incubation with H_2O_2 at 5 mM for 4, 8 and 24 h and those of Siwik et al. (2001) who observed a decrease of production of total collagen in the cardiac fibroblasts of rats after incubation

with H_2O_2 for 24 h. The decrease of collagen synthesis caused by the ROS is due to the transcriptional decrease of mRNA and/or their stability (Siwik et al., 2001; Cox and al., 2002). According to Tyagi et al. (1996) and Siwik et al. (2001), an important oxidative stress leads to a direct activation of the proenzymes of the metalloproteinases (pMMP) in *in vitro* cardiac fibroblasts, activating the metalloproteinases (MMPs), especially MMP-13, MMP-2 and MMP-9, which induce the degradation of fibril collagens in the fibroblasts and which may play a role in the development of cardiovascular diseases by regulating the quantity and the quality of the extracellular matrix.

Conclusion

The oxidative stress induced by adding hydrogen peroxide to cultured adventitious fibroblasts induces a decrease in cell proliferation, an increase of the total surface of cells and nuclei indicating compensatory hypertrophy, a vacuolization of the cytoplasm, which is typical of oncosis, a chromatin hypercondensation and a budding of cell membranes, which is typical of apoptosis. Moreover, we have noted an increase of products causing lipid peroxidation, especially MDA, which is responsible for the loss of cell viability following the damages caused to membranes, an overproduction of ROS especially of nitric oxide (NO•). Finally, hydrogen peroxide leads to very significant metabolic disorders of the extracellular matrix of adventitious fibroblasts, particularly stressed by a decrease in total protein and collagen production.

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