

Oxygen metabolism in human placenta mitochondria

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Abstract Due to the high metabolic demands of the placental tissue during gestation, we decided to analyze the mitochondrial bioenergetic functions in the human term placenta. Different mitochondrial morphological parameters, membrane potential and cardiolipin content were determined by flow cytometry. Oxygen uptake, hydrogen peroxide production and cytochrome P450 content, were also measured. Some apoptotic mitochondrial proteins were also analyzed by western blot. Two isolated mitochondrial fractions were observed: large/heavy and small/light with different functional characteristics. Oxygen uptake showed a respiratory control (RC) of 3.4 ± 0.3 for the heavy mitochondria, and 1.1 ± 0.4 for light mitochondria, indicating a respiratory dysfunction in the light fraction. Good levels of polarization were detected in the heavy fraction, meanwhile the light population showed a collapsed $\Delta\Psi_m$. Increased levels of cytochrome P450, higher levels of hydrogen peroxide, and low cardiolipin content were described for the light fraction. Three pro-apoptotic proteins p53, Bax, and cytochrome *c* were found increased in the heavy mitochondrial fraction; and deficient in the light fraction. The heavy mitochondrial fraction showed an improved respiratory function. This mitochondrial fraction, being probably from cytotrophoblast cells showed higher content of proteins able to induce apoptosis, indicating that these cells can effectively execute an apoptotic program in the presence of a death stimulus. Meanwhile the light and small organelles

probably from syncytiotrophoblast, with a low oxygen metabolism, low level of $\Delta\Psi_m$, and increased hydrogen peroxide production, may not actively perform an apoptotic process due to their deficient energetic level. This study contributes to the characterization of functional parameters of human placenta mitochondria in order to understand the oxygen metabolism during the physiological process of gestation.

Keywords Human placenta mitochondria · Free radicals · Hydrogen peroxide · Cardiolipin · Apoptosis

Abbreviations

DiOC6	3,3'-dihexiloxocarbocyanine iodide
FCCP	carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone
FL-1	green fluorescence
MSH	Manitol-Sucrose-Hepes
FSC	forward angle light scatter
SSC	side angle light scatter
P450 _{ssc}	cytochrome p450 cholesterol side-chain cleavage enzyme
NAO	nonyl acridine orange
MnSOD	manganese-superoxide dismutase
$\Delta\Psi_m$	trans-membrane potential
H ₂ O ₂	hydrogen peroxide

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Introduction

The placenta plays a unique role in supporting the fetal allograft throughout gestation, protecting against immune rejection whilst also serving to supply oxygen and nutrients, and to remove carbon dioxide and waste products from the fetus (Carlin and Alfirevic 2008; Crocker et al. 2001). The specialized cells, called trophoblast, possess several

important functions (Wooding and Flint 1994), such as placental growth and development through a differentiation process into syncytiotrophoblast (Wooding and Flint 1994; Colleoni et al. 2010).

The villous cytotrophoblast are the replicating components of the trophoblast lineage. This mononuclear cytotrophoblast cells, exit from the cell cycle, gaining fusion competence to form the syncytiotrophoblast. One of the main functions of syncytiotrophoblast is the production of large amounts of progesterone, together with essential hormones and compounds required for the maintenance of pregnancy, transport, and immune function (Wooding and Flint 1994; Colleoni et al. 2010).

Mitochondria play a central role in the placental development, growth and function. In addition to ATP production, their functions include the vital role of steroidogenesis (Meigs and Sheean 1977; Martínez et al. 1996; Meigs and Ryan 1968; Klimek 1990). Oxidative phosphorylation is a fundamental mechanism in aerobic metabolism, being central for fetal and postnatal development (Klimek et al. 1998). It is known that the efficiency of the mitochondrial oxidative phosphorylation closely depends on the dimeric phospholipid cardiolipin, located at the inner mitochondrial membrane.

The mitochondrial ability to do steroidogenesis has important consequences not only for placental mitochondria but also for the placental tissue. It has been observed that mitochondria from the syncytiotrophoblast display different morphology and function compared to those present in the villous cytotrophoblast, in fact, syncytiotrophoblast are able to convert cholesterol into pregnenolone through the enzyme P450_{scc} which is inserted at the inner mitochondrial membrane. (Gellerich et al. 1994; Martínez et al. 1993; Martínez et al. 1997). The mitochondrial ability to do steroidogenesis, which is determinant for pregnancy, has important and dramatic consequences for placental mitochondria. Important to note, is the fact, that simultaneously to the ability to synthesize progesterone, the mitochondrial P450 system, is also able to generate superoxide anion (Klimek 1988; Klimek et al. 1998). Due to the high reactivity of this free radical molecule, important changes are induced to the mitochondrial lipid-membrane bilayer with harmful effects to the electron transport system and other mitochondrial functions. This “physiological” NADPH-dependent lipid peroxidation, observed in these human placental mitochondria could be similar to the “physiological lipid peroxidation process” described by Crivello et al. (1983) in adrenal cortex tissue. They reported that the peroxidative process starting at the glomerulosa zone, and continuing to the fasciculata and reticularis zone is just a consequence of its ability to synthesize mineralo- and gluco-corticoids mediated by the cytochrome P450_{scc}, with the concomitant generation of superoxide anion.

Apoptosis is an evolutionarily process that allows multicellular organisms to discard cells through a highly orchestrated cell demise program. It has been suggested that apoptosis and other death associated processes such as necrosis and autophagy could be determinant for normal placental development and their increased and early appearance may also be involved in the pathophysiology of pregnancy-related diseases. Mitochondria can undergo several typical and irreversible alterations during the apoptotic process. These alterations include early disruption of the electron transport chain, altering the ATP production, mitochondrial outer membrane permeabilization and the release of several proteins from the intermembrane space to the cytosol (Chipuk et al. 2006). Apoptosis can be controlled by different type of proteins that reside or translocate to the mitochondria where they can trigger the permeabilization of the outer and inner mitochondrial membranes releasing intermembrane space proteins. Among these proteins, the nuclear transcription factor p53, a cell death-inducing factor, can interact with multidomain proteins of the Bcl-2 family inducing mitochondrial outer membrane permeabilization. In addition, p53 can engage multiple pro-apoptotic pathways and “overkills” cells by transactivating a wide array of genes with apoptosis-inducing products (Vousden and Lane 2007). Bax, a pro-apoptotic member of the Bcl-2 family, due to its role in mitochondrial permeabilization processes, can create a special condition allowing the release of cytochrome *c* to the cytosol, causing the initiation of a caspase cascade leading to the ultimate cellular organized apoptotic degradation.

In this study we analyzed the oxygen metabolism and the ability to execute an apoptotic program of two isolated mitochondrial fractions, from human placental tissue. The two mitochondrial populations, isolated by differential centrifugation and characterized by flow-cytometry, were described as “heavy” mitochondrial fraction (H) formed mainly by particles with greater mass and size, and “light” mitochondrial fraction (L), formed mainly by particles with smaller mass and size.

Materials and methods

Samples

Central tissue portions from human term placenta were collected within 15 min of normal vaginal delivery from 5 pregnant healthy women, with a maternal age of 19.5 ± 3.4 years, 24.1 ± 4.5 of BMI, and a gestational age at birth of 39.8 ± 2.0 weeks. Fetal and placental weight was of 3013.2 ± 493.8 g, and of 439.4 ± 28.9 g, respectively. All participants entered the study at a gestational age ranging 16–20 weeks. They provided written informed consent and approval to take part in the study. The investigators were informed when any

study participant was admitted for delivery and were present to monitor labor and delivery and to collect data. Anthropometric measurement of newborns: birth weight and placental weight (SECA scale ± 10 g) were taken one hour after delivery by standard methods. Placental efficiency was defined as fetal weight/placental weight being 6.9 ± 0.7 . All the protocols were approved by the Ethics Committees of the Universidad del Valle, Cali, Colombia. The protocol was in accordance with the latest revision of the Declaration of Helsinki.

Mitochondrial procedures

Isolation of human placental mitochondria

Mitochondria were obtained from placental tissue with the modified protocol of differential centrifugation previously described by Martinez et al. (1993, 1996, 1997). Several central placental cotyledons were removed from the maternal side of the placenta, placed immediately into an ice-cold medium containing MSHE buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 5 mM Hepes, pH 7.4). All steps were carried out at 4 °C. Soft villous tissue (8–10 g) was free from connective tissue and minced into small pieces with scissors. The tissue was washed with the same solution and then filtered on a thin surgical gauze layer; this procedure was repeated three times. The tissue was resuspended in the same medium and homogenized with a Potter-Elvehjem homogenizer (7 up-and-down strokes). The homogenate was centrifuged at 1,500 g for 10 min in a refrigerated centrifuge. The pellet was resuspended in a minimal volume of MSHE buffer. The supernatant was recovered and centrifuged at 4,000 g for 15 min to obtain a first pellet described as “heavy” mitochondrial fraction. Then the supernatant was centrifuged again at 12,000 g for 15 min, the corresponding mitochondrial pellet was also resuspended in the previously named MSHE buffer and described as “light” mitochondrial fraction. The criterion used to classify both mitochondrial fractions, was based on the centrifugation conditions employed (Sorvall centrifuge, SS-32 rotor, and the specified sedimentation velocity), leading us to separate two mitochondrial fractions with different mass.

The level of the cytosolic contamination in our mitochondrial fractions was performed by the activity of the lactic dehydrogenase and the microsomal activity antimycin A-insensitive NADH-cytochrome c reductase, the results showed that the level of contamination was less than 1.8 and 2.4 % of the initial homogenate activity, respectively. Isolated mitochondrial fraction was frozen at -70 °C until the enzyme assays were performed. The isolated “heavy” and “light” mitochondrial fractions should belong mainly to the villous cytotrophoblast and syncytiotrophoblast respectively.

However, we cannot discard the possibility that minor contamination with endothelial and fibroblast mitochondria could exist. This method yields a total mitochondrial protein content estimated between 1 and 2 mg/g of wet placental tissue, which are free from contaminants from other parts of the cell. Determination of mitochondrial protein was performed by the Lowry assay (Lowry et al. 1951).

Flow cytometry characterization of human placental mitochondrial particles

Both “heavy” and “light” mitochondrial fractions, isolated from term placental tissue by differential centrifugation, were used for a fixed analysis of its mitochondrial morphology, by flow cytometry, using a three-color FAC-SCAN cytometer equipped with a 15-mW air-cooled $\lambda=488$ -nm argon laser (Becton Dickinson, USA) (Mattiasson et al. 2003). Mitochondrial size was determined by the light scattered at low angle using the FSC photodiode, and the response collected by a E-00 setting with logarithmic amplification gain of 5.39. The FSC intensity roughly equates to the particle’s size. The mitochondrial structure evaluated by the light scattered at the perpendicular direction detected by the SSC photomultiplier tube (PMT) using a voltage of 578 and a linear amplification gain was adjusted to 4.3. The side scatter channel (SSC) provides information about the granular content within a particle. Both FSC and SSC are unique for every cell or particle, and a combination of the two may be used to differentiate different cell types as well as particles in an heterogeneous sample. Both parameters are used routinely to characterize particle volume/size and granularity respectively of very small particles such as bacteria and mitochondria (Mattiasson 2004; Cossarizza et al. 1996). Differences in the observed FSC for the two mitochondrial samples were quantified in four independent experiments as the number of events which drop under the median value of the histogram distribution using a common marker (M1) (Bustamante and Lores-Arnaiz 2010; Karadayian et al. 2014). A higher value of FSC would reflect a larger size for the mitochondrial analyzed population. All the other mitochondrial parameters were performed in these two populations in order to investigate its metabolic function (Bustamante and Lores-Arnaiz 2010).

Determination of mitochondrial cytochrome P450

Cytochrome P450 levels were determined in the two purified mitochondrial fractions described as heavy and light fractions, by the ability of this protein to bind in the reduced form to carbon monoxide resulting in a maximum absorbance at 450 nm ($\epsilon=91$ mM $^{-1}$ cm $^{-1}$). The determination was performed by differential spectrophotometry (Omura and Sato 1964).

Mitochondrial respiratory function

Oxygen consumption by “heavy” and “light” mitochondria was assayed by high-resolution respirometry (Oroboros Oxygraph, Paar KG, Graz, Austria). Mitochondrial protein (0.5–1 mg/ml) in a reaction medium consisting of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl, 1 mM EDTA, 5 mM malate plus glutamate, 5 mM $\text{PO}_4\text{H}_2\text{K}$, 4 mM MgCl_2 (pH 7.4), and 0.2 % bovine serum albumin, at 30 °C was used. State 3 was estimated by the addition of 1 mM ADP and the respiratory control ratio (RCR) was calculated from the ratio of the state 3/state 4 respiratory rates with and without ADP, respectively (Chance and Williams 1956; Estabrook 1967). The concentration of the different probes used in the cytometric assays, did not alter the respiratory mitochondrial function in these samples.

Mitochondrial transmembrane potential

Isolated mitochondria (25 $\mu\text{g}/\text{ml}$) were incubated at 37 °C for 20 min in MSHE buffer supplemented with 5 mM malate, 5 mM glutamate, 1 mM phosphate and 4 mM MgCl_2 in the presence of 30 nM of the potentiometric probe DIOC6, for direct measurement of the transmembrane potential ($\Delta\Psi_m$) (Bustamante et al. 2004). The fluorescence changes were determined by flow cytometry measurement. Fresh mitochondria were prepared for each experiment and samples were protected from light until acquired by the cytometer. Auto-fluorescence of the mitochondrial preparation was measured as a probe loading control, and 0.5 μM of the depolarizing agent FCCP as a positive control. A common marker, indicating the relative fluorescence intensity for the different mitochondrial populations was used to quantify the membrane potential values in each experiment.

Mitochondrial hydrogen peroxide production

Hydrogen peroxide (H_2O_2) generation was determined in intact isolated human placental mitochondria by the scopoletin-HRP method following the decrease in fluorescence intensity at 365–450 nm ($\lambda_{\text{exc}}-\lambda_{\text{em}}$) at 37 °C (Boveris et al. 2006). The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), 0.8 μM HRP, 1 μM scopoletin, 0.3 μM SOD (to ensure that all superoxide ($\text{O}_2^{\cdot-}$) was converted to H_2O_2) and 6 mM succinate plus glutamate were used as substrates. Calibration was made using H_2O_2 (0.05–0.35 μM) as standard to express the fluorescence changes as nmol $\text{H}_2\text{O}_2/\text{min}.\text{mg}$ protein. Hydrogen peroxide production was highly sensitive to catalase addition (3.500 U/ml).

Cardiolipin content

The mitochondrial staining probe NAO (nonyl acridine orange, λ_{em} 525 nm), specific for the phospholipid cardiolipin, located at the inner mitochondrial membrane, was routinely employed for the flow cytometry analysis of the two isolated mitochondrial fractions, in order to quantify in each mitochondrial sample their cardiolipin content (Bustamante and Lores-Arnaiz 2010).

Western blotting and chemiluminescence

P53 and Bax association to the mitochondrial fractions was determined in the H and L mitochondrial placental fractions (Bustamante et al. 2011): equal total protein amount of purified placental mitochondrial fraction (80 μg) were separated by SDS-PAGE (15 %), blotted into a nitrocellulose membrane (Bio-Rad, München, Germany) and probed primarily against goat polyclonal p53 (C-19) antibody and rabbit polyclonal Bax (N-20) antibody from Santa Cruz Biotechnology, in a dilution 1:500. The mitochondrial content of cytochrome c was also measured in both mitochondrial fractions and probed primarily against rabbit polyclonal cytochrome c (H-104) antibody (dilution 1:500) also from Santa Cruz Biotechnology. Then, the nitrocellulose membrane was incubated with a secondary anti-goat/rabbit antibodies conjugated with horseradish peroxidase (dilution 1:5,000), followed by development of chemiluminescence with the ECL reagent for 2–4 min. The evaluation of the Western blotting quantification of each pro-apoptotic protein was performed by its ratio to the mitochondrial content of Bcl-x_{S/L}, an anti-apoptotic protein present also in the mitochondrial membranes, using a rabbit polyclonal antibody (dilution 1:500) from Santa Cruz Biotechnology.

Statistical analysis

Values in Table 1 and figures are mean values \pm SEM, of 4–6 independent experiments. Results were analyzed using an unpaired Student *t*-test for group comparison.

Table 1 Oxygen uptake by human placental mitochondria

Mitochondrial sample	O ₂ consumption (nat g O/min mg protein)		
	State 4	State 3	RC
Heavy	3.33 \pm 0.2	11.5 \pm 0.2	3.4 \pm 0.3
Light	5.5 \pm 0.4*	6.1 \pm 0.5*	1.1 \pm 0.4*

Values are expressed as means \pm SEM. * $p < 0.05$, as compared with the H mitochondrial fraction

Results

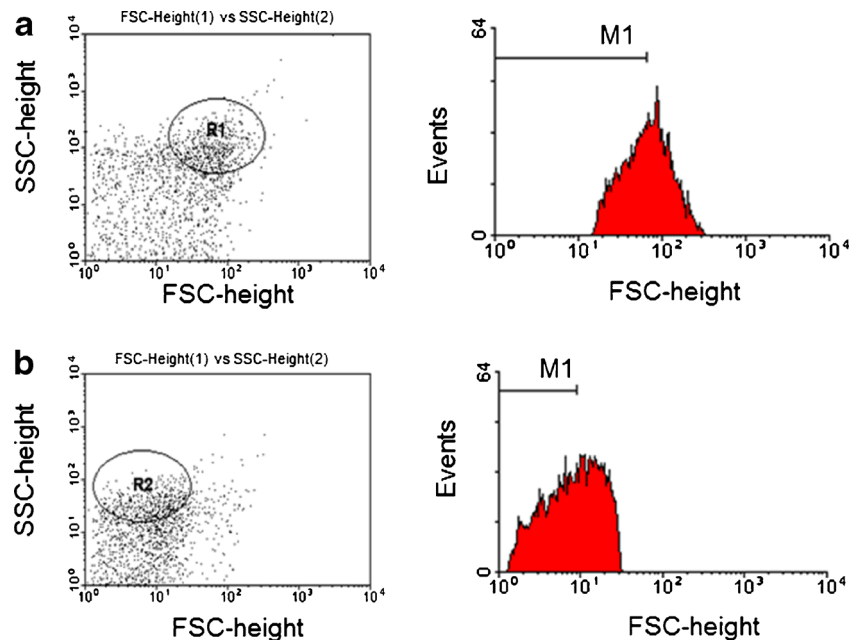
Flow cytometry characterization of human placenta mitochondrial morphology

The flow cytometry characterization of the mitochondrial morphology was performed analyzing the two isolated mitochondrial fractions by ultracentrifugation and described as “heavy” and “light”, depending on its FSC (size) and SSC (granulosity/complexity) (Fig. 1). The mitochondrial populations gated as R1 “heavy” (the analyzed mitochondrial particles) fraction clearly presented a FSC median of 67.2 ± 1.7 larger than the R2 “light” mitochondrial fraction, in which the FSC median was 11.9 ± 2.1 being clearly significantly lower ($t(\text{Bustamante et al. 2011})=20.55$; $p < 0.0005$). Both gated mitochondrial populations presented similar SSC (particles inside the ellipse) (Fig. 1a and b).

Content of mitochondrial P450

According to the classical methodology (Omura and Sato 1964) the levels of cytochrome P450 detected in the heavy and light isolated mitochondria from term human placenta, were 0.038 ± 0.009 nmol/mg protein and 0.15 ± 0.02 nmol/mg protein respectively, being significantly different ($p < 0.05$), and thus indicating that the levels of light mitochondria cytochrome P450 were 2.9 times higher than the cytochrome P450 content in the heavy fraction.

Fig. 1 Cytometric identification of mitochondrial populations. Two mitochondrial fractions from placental tissue were obtained by differential centrifugation, and described as “heavy” (a) and “light” (b). These mitochondrial samples were identified by flow cytometry as “large” R1 and “small” R2, corresponding to the studied mitochondrial subpopulations respectively (*left panel*). These subpopulations “heavy” and “light” were analyzed by their median FSC in the respective histograms, maintaining similar SSC as described (*right panel*)



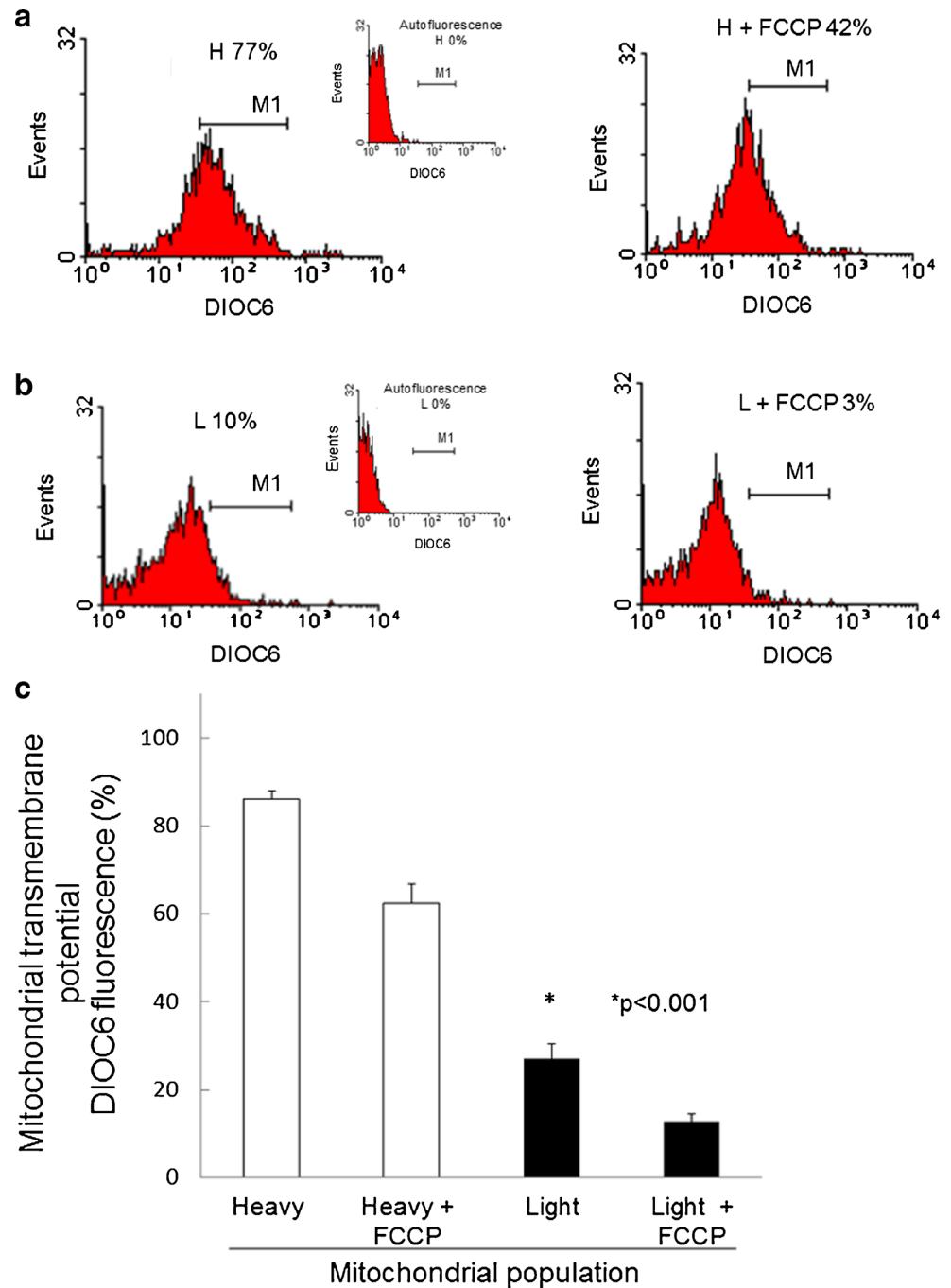
Mitochondrial respiratory function

The results of the oxygen uptake by the two mitochondrial populations described as heavy and light are presented in Table 1. In the light fraction, the resting state 4 was 65 % increased as compared with the heavy mitochondrial fraction. This same light fraction presented a 47 % decrease in state 3, ADP dependent respiration rates with a RCR of 1.1, characteristic of an impaired oxygen uptake and being 68 % lower as compared with the heavy mitochondrial population, showing a clear deficiency in the electron transport chain and as a consequence a low ability to generate energy necessary for executing a death program (Table 1). Meanwhile the heavy mitochondrial fraction showed a respiratory control rate (RCR) of 3.4 indicating that their respiratory function permits a proton motive force, able to generate enough energy for maintaining different cell functions, including its ability to execute a death program.

Mitochondrial transmembrane potential

Mitochondrial transmembrane potential studies from the heavy and light fractions were performed in the selected R1 and R2 gates, respectively, as described in Fig. 2a and b. The FL-1 (r. f. i, relative fluorescence intensity) histograms for each mitochondrial population were analyzed under M1 marker. Low levels of auto fluorescence from the unloaded (no probe) mitochondrial population are shown at the insets.

Fig. 2 Evaluation of $\Delta\Psi_m$. The mitochondrial membrane potential of the two human placental mitochondrial fractions, described as “heavy” (H) (a) and “light” (L) (b), were loaded with 30 nM DiOC6 for 20 min and immediately acquired by the cytometer. The right panel histograms showed the percentage in r.f.i. of the two analyzed populations, under the same marker (M1). Auto-fluorescence of the mitochondrial preparations is described in the inset. 0.5 μ M of the depolarizing agent FCCP was used as a positive control (right panel histograms). Quantification of the $\Delta\Psi_m$ is described by the bar graph in Fig. 2c. (* $p < 0.001$, as compared with H)



“Heavy” mitochondrial samples showed high DIOC6 relative fluorescence intensity, indicating a mitochondrial polarized fraction, as expected for coupled respiring mitochondria (Fig. 2a). The histograms of the “light” mitochondrial fraction showed an important decrease in DiOC6 fluorescence, as compared with the “heavy” fraction, indicating a clear dissipation of $\Delta\Psi_m$ (Fig. 2b), in agreement with the observed increase in state 4 respiratory rate in this mitochondrial population. Quantification of the transmembrane potential performed as the percentage values of the DIOC6 r. f. i. of four

different experiments is shown in Fig. 2c. A 70 % decrease in $\Delta\Psi_m$ was observed in the “light” mitochondrial sample, as compared with the heavy mitochondrial fraction. Depolarization after addition of the protonophore FCCP was registered for both populations. It is well known that the FCCP effect on mitochondrial depolarization depends on the dose employed. In this study 0.1 μ M FCCP was low enough to allow a minimal level of mitochondrial respiration; showing just a low level of polarization as compared with the FCCP untreated organelles.

Mitochondrial H₂O₂ production

Mitochondrial H₂O₂ production was determined in both isolated mitochondrial fractions as described in Fig. 3. “Light” mitochondrial samples showed higher levels of H₂O₂ production being 38 % more as compared with the “heavy” mitochondrial fraction.

Cardiolipin content

Both fractions were analyzed by the specific cardiolipin mitochondrial staining. The events within the gate R1 in the “heavy” fraction, and R2 in the “light” fraction, described by the dot blot graphs, were both positive for NAO, confirming that we were analyzing a mitochondrial sample, as presented in Fig. 4a and b. A negative unstained control is presented at the inset as autofluorescence. The mitochondrial events in R1 and R2 gates were analyzed for both heavy and light mitochondrial populations, respectively, for its intact cardiolipin content, and quantified in three different experiments. “Light” mitochondrial fraction showed 15 % lower cardiolipin content as compared with the “heavy” fraction (Fig. 4c). It has been stated the mitochondrial distribution of NAO, due to its positive charge, could be mediated by the mitochondrial polarization level (Mattiasson et al. 2003), thus, it is possible that the level of cardiolipin concentration observed in the “light” population could be influenced by the low mitochondrial membrane potential observed in this mitochondrial fraction.

p53, bax and cytochrome c content

The presence of the p53 transcription factor and the apoptotic member Bax, in the mitochondrial membranes was clearly observed in the heavy mitochondrial population. The Western blot assays showed a 53 kDa and 23 kDa proteins reacting

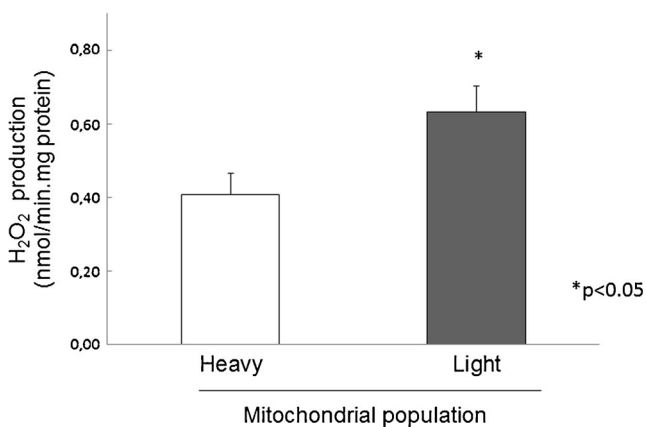


Fig. 3 Mitochondrial production of hydrogen peroxide. H₂O₂ generation was determined in the two isolated human placental mitochondrial fractions described as heavy and light. Bar graph describe the H₂O₂ generation in the heavy (H) and light (L) mitochondrial populations. (* $p < 0.05$, as compared with H fraction)

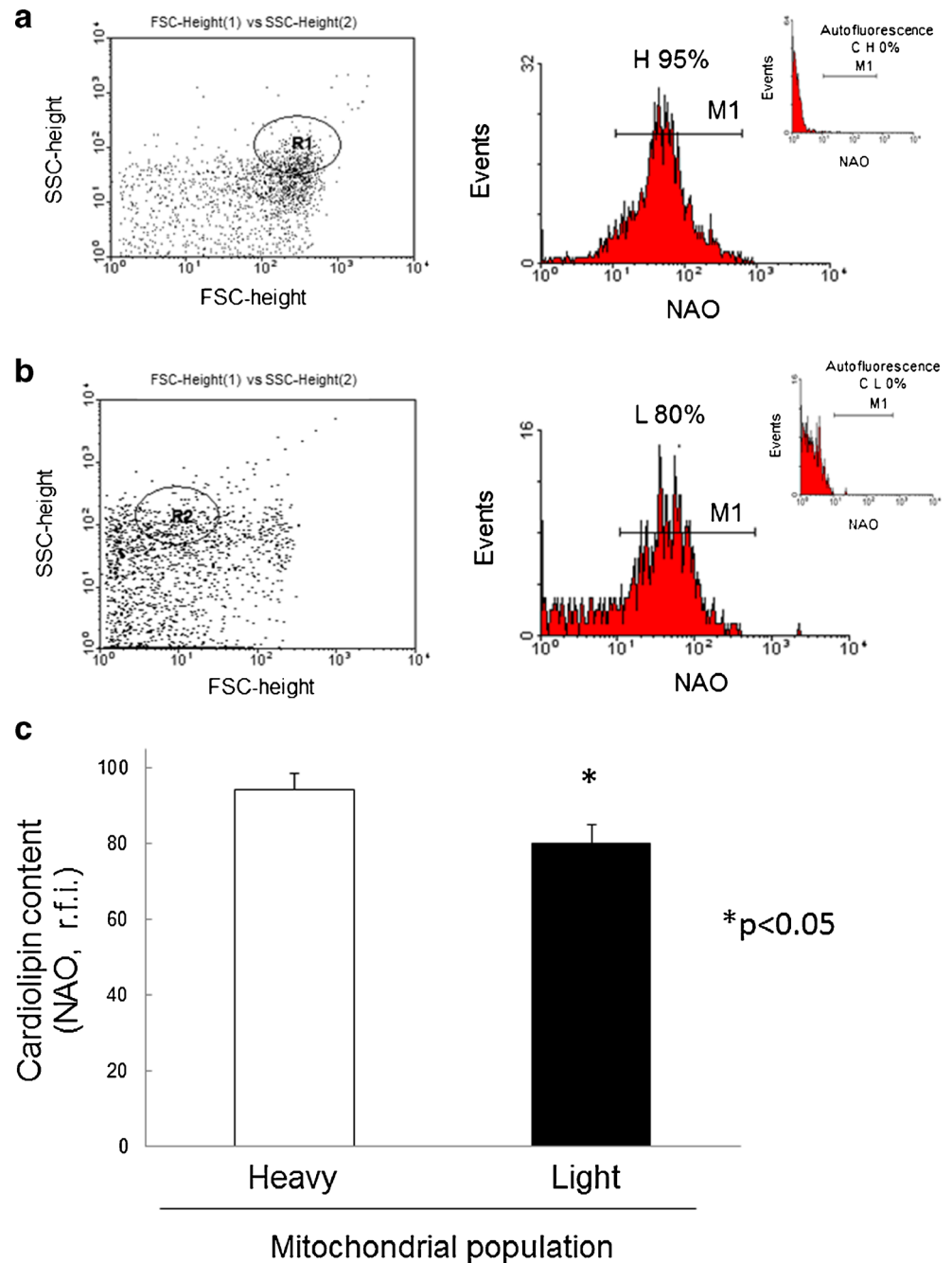
with antibodies directed against p53 and Bax respectively (Fig. 5a). The expression of these proteins was higher in heavy fraction as compared with the light mitochondria isolated from term placental tissue. In addition, a higher protein expression of cytochrome *c* was also observed in the heavy fraction as compared with that observed in the light mitochondrial fraction. The quantification was performed by densitometric analysis as the ratio of the transcription factor p53 and the pro-apoptotic proteins, Bax and cytochrome *c*) to the level of the mitochondrial porine, VDAC, present in the mitochondrial membranes. The results showed that p53 and Bax protein levels associated with mitochondria were 60 and 49 % respectively higher in the heavy fractions, as compared with the light mitochondria (Fig. 5b). The results of cytochrome *c* analysis showed also 53 % higher content in the “heavy” fraction, as compared with the “light” mitochondrial sample (Fig. 5b). No difference was observed in the content of the anti-apoptotic protein BCL-X_L in both mitochondrial fractions.

Discussion

During labor, breathing is determinant, oxygen is going to the muscles allowing the body to work effectively with the contractions. Furthermore, it provides the baby with the oxygen he so vitally needs. Therefore, a proper breathing through all the labor stages results essential. In fact, a reduction in placental mitochondrial respiratory function during labor could compromise placental function and potentially fetal health. All the patients included in this study, maintained a deep abdominal breathing with relaxation during labor and no evident respiratory disturbances were observed.

In a previous study of human term placental mitochondria we focused our attention on the effect of exercise on the free radical generation by a heavy mitochondrial fraction (Ramírez-Vélez et al. 2013). In the present study, we compared the oxygen metabolism of the two mitochondrial fractions present in normal human term placenta and their content of pro-apoptotic proteins. The mitochondrial fractions were isolated by differential centrifugation as “heavy” and “light” fractions followed by a flow cytometry characterization as large mitochondrial particles with high FSC and small mitochondrial particles with low FSC respectively. It has been shown that during trophoblast differentiation to syncytiotrophoblast, important alterations in mitochondrial morphology are observed. Changes from large mitochondria in the cytotrophoblast into small structures in the syncytiotrophoblast, losing its lamellar cristae configuration for vesicular heterogeneous mitochondrial cristae, have been previously described by Martinez et al. (1993, 1996, 1997). Thus the two types of mitochondrial populations observed in this study, could correspond to the two main

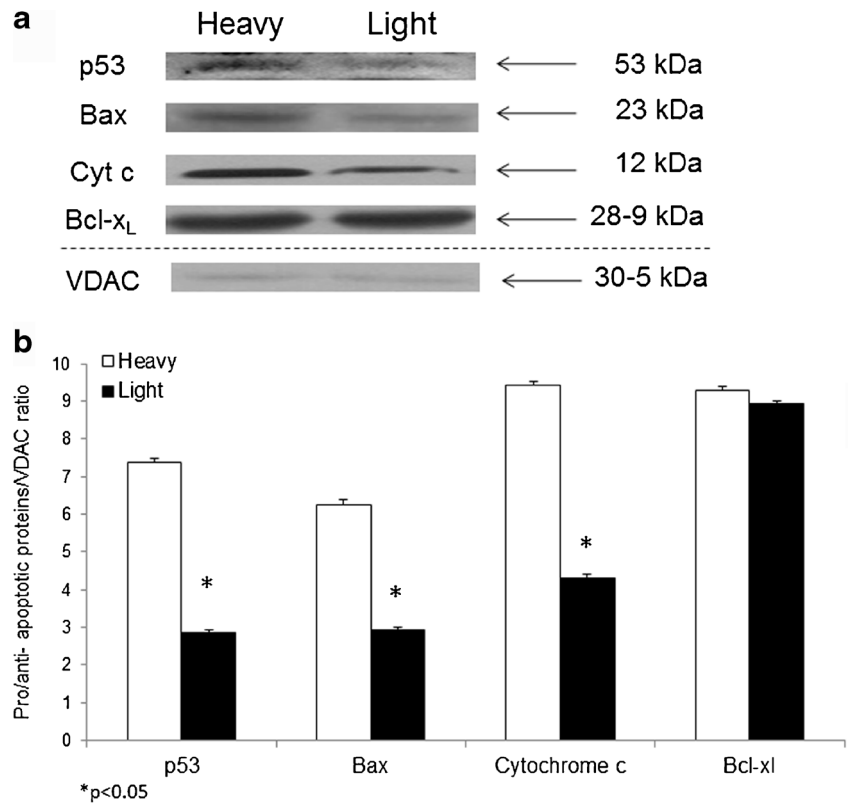
Fig. 4 Cardiolipin content in mitochondrial populations. The “heavy” (H) and “light” (L) mitochondrial populations, present in the R1 and R2 gates respectively (*left panel*), were stained with the specific cardiolipin sensor (NAO) (*right panel*). The levels of NAO r.f.i., are described in the right panel histograms of Fig. 4a and b. Insets describe the sample autofluorescence. Quantification of cardiolipin content was assessed by the difference in the median histogram, as showed in Fig. 4c. * $p < 0.02$, as compared with H fraction



throphoblast phenotypes present in normal term placenta: the syncytiotrophoblast as the outer epithelial layer with small mitochondria and the cytotrophoblast able to undergo cell division with large mitochondria as reported by electron microscopy studies (Martinez et al. 1993, 1996, 1997). In addition, the cytometric evaluation of NAO (nonyl acridine orange, λ_{em} 525 nm) staining reflects the purity of the used mitochondrial samples as well as its cardiolipin (Bustamante and Lores-Arnaiz 2010) content. The respiratory function studies evaluated as oxygen uptake, showed that oxygen uptake in the heavy/large population presented a RCR in agreement with coupled respiration, being higher

than the light/small mitochondrial population, which presented low RCR indicating an impaired respiratory function in this population. These results were in agreement with the lower $\Delta\psi_m$ observed in the light/small mitochondria as compared with the heavy mitochondrial sample. In addition, the characterization of the placental mitochondria showed that light/small mitochondrial fraction, presented higher content of cytochrome P450 than that observed in the heavy mitochondria, which was in agreement with Martinez et al. (1997), who described increased levels of P450_{scc} in the syncytiotrophoblast mitochondria by immunogold localization. This result strongly suggests that in fact, “light”

Fig. 5 Presence of proteins associated with the apoptotic process in the mitochondrial fractions. **a** Representative immunoblot analysis of the H and L mitochondrial fractions from placental samples, probed for the proapoptotic proteins p53, bax, cytochrome c. **b** Quantification of band intensity was performed by the ratio of the proapoptotic proteins, p53, bax, cytochrome c to the protein VDAC ($*p < 0.05$, as compared with H fraction)



mitochondria, with low FSC, and high cytochrome P450, showing an impaired electron transport chain, could belong to syncytiotrophoblast in term placental cells. It is well known, and interesting to note is that, in the syncytiotrophoblast cells during the process of steroidogenesis (Hanukoglu 2006), mediated by cytochrome P450, superoxide anion is being produced, inducing a potential deleterious effect on the inner mitochondrial membranes, and as a consequence an impairment in the electron transport system due to the decreased activity of its components (Meigs and Ryan 1968; Klimek 1990; Klimek et al. 1998; Hanukoglu 2006). In these conditions, the light/small mitochondrial population with high content of cytochrome P450 and increased superoxide production presents not only different morphology with vesicular cristae and swelling as described by Martínez et al. (1997) but a lowered proton motive force in their transmembrane space. In fact, it was observed in this study that the “light” mitochondrial populations clearly showed impairment in their respiratory function and decreased $\Delta\psi_m$; meanwhile the heavy mitochondrial fraction presented a good level of mitochondrial polarization.

It is possible that the stronger oxidative stress present in the light/small mitochondrial population is due to the level of the superoxide anion produced at the syncytiotrophoblast during the process of steroidogenesis. It is well known that the phospholipid cardiolipin is involved in the regulation of several bioenergetics processes optimizing the activity of key mitochondrial proteins associated to the electron transport chain

and the oxidative phosphorylation (Petrosillo et al. 2003). Because of its high content of unsaturated fatty acids and to its location near the site of free radical production, cardiolipin is particularly prone to peroxidative attack by different oxidant species, leading to inner membrane damage and contributing to the loss of the normal mitochondrial lamellar cristae structure (Petrosillo et al. 2003). All these facts are in agreement, with the low cardiolipin content observed in the light/small mitochondrial fraction, contributing to the mitochondrial respiratory dysfunction (Petrosillo et al. 2007).

It is well known that the oxidative stress can induce apoptosis which constitutes an important physiological process for the normal tissue development and homeostasis. Although in presence of a strong and persistent oxidative stress, is possible to induce a marked mitochondrial alterations blocking the energy supply, in this conditions the cell should be forced to find another death pathway different from apoptosis. Large variations in the level of placental apoptosis have been continuously reported, probably due in part to the difficulties to differentiate the type of trophoblast cell undergoing apoptosis (Straszewski-Chavez et al. 2005; Longtine et al. 2012a). This limitation can be explained by the fact that apoptotic cytotrophoblast could often be deeply interdigitated into the syncytiotrophoblast layer (Longtine et al. 2012a). Thus in the absence of a costaining for a membrane marker, an apoptotic cytotrophoblast could be misidentified as indicating a localized region of apoptosis in the syncytiotrophoblast (Longtine et al. 2012a). In addition it is well known that mitochondria play an important role in

the execution of the intrinsic apoptotic signaling cascade, in which the level of ATP production through the proton motive gradient, play an important regulatory role deciding how the cell should die. In fact, it is well known that low intracellular levels of energy determine the possibility to apply or not an apoptotic program. Deficiency of the intracellular level of energy, in presence of a death signal, induces other ways to die, different of apoptosis, such as necroptosis, necrosis or autophagy. The results described in this study, showed that key apoptotic markers such as p53, Bax and cytochrome *c* associated to intrinsic signaling pathways of apoptosis were mainly expressed in the heavy/large mitochondrial fraction. Meanwhile their content was almost undetected in the light and depolarized fraction. It should be clearly stated that a higher mitochondrial content of pro-apoptotic proteins is not enough as requirement to perform or execute apoptosis; many other conditions should be fulfilled by the cells in order to apply a specific death program.

In addition, in the light/small fraction, the higher peroxidative process observed could be the consequence of the increased steroidogenesis, inducing other types of cell death, different from apoptosis, which has been previously described for syncytiotrophoblast (Heazell and Crocker 2008). The observations in the present study are consistent with recent findings showing that apoptosis in the placental syncytiotrophoblast is strongly inhibited even under conditions of high cellular stress; meanwhile the cytotrophoblast can undergo apoptosis that easily propagates as a wave throughout syncytiotrophoblasts, (Longtine et al. 2012a, 2012b). Recently it has been observed that mitochondrial content and activity can be different depending on the placental cell lineage (Mandò et al. 2014). In addition caspase mediated apoptosis of trophoblasts in human term human placenta is restricted to villous cytotrophoblasts and absent from the multinucleated syncytiotrophoblast (Longtine et al. 2012c).

All these facts are in agreement with our results, showing that in the cytotrophoblasts cells, heavy/large mitochondria with a high performance of respiratory function, and enough energy, could easily regulate and perform an apoptotic program, in order to achieve normal physiological placental cell death. Oppositely in the syncytiotrophoblast, the presence of light/small mitochondria with impaired respiratory function, low cardiolipin content, and evidences of an increased oxidative stress, probably mediated by the mitochondrial P450 system, clearly induces an impaired energy supply. These observations together with the low level of apoptotic proteins founded in this “light” mitochondrial population, let us suggest, that these organelles should be involved with other signaling death pathways different from apoptosis, in order to execute a death program, such as necroptosis, necrosis or autophagy.

Conclusions

This study shows the morphological and functional characterization of two mitochondrial subpopulations present in the human term placental tissue, described as a heavy/large mitochondrial population with high FSC and a light/small mitochondrial population with low FSC. The “heavy” fraction showed an improved respiratory function, lower hydrogen peroxide production, low mitochondrial P450 and higher cardiolipin content. This fraction was characterized by the ability to concentrate higher levels of three proteins deeply associated with the apoptotic process being p53, Bax and cytochrome *c* content indicating its ability to execute the apoptotic program. Oppositely the light fraction was characterized by an impaired respiratory function, increased production of hydrogen peroxide, high P450 content and cardiolipin peroxidation, indicating a lower energetic capacity and as a consequence other cell death pathways such as necroptosis, necrosis or autophagy could be applied.

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Conflict of interest The authors declare that there are no conflicts of interest.

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