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Research Article

Morphofunctional changes in the mitochondrial subpopulations of conceptus tissues during the placentation process

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Abstract. To establish the role of mitochondrial subpopulations in the mitochondrial maturation process, we studied morphological and functional changes in the mitochondria of different mammalian conceptus tissues during the organogenic and the placentation processes. Mitochondrial subpopulations of three different conceptus tissues, embryo and visceral yolk sac placenta on gestational days 11, 12 and 13 and placenta on days 12 and 13, were examined morphologically by transmission electron microscopy. Cytochrome oxidase activity and protein levels were also measured in each mitochondrial subpopulation. The results indicate two different mitochondrial subpopulation profiles: a homogeneous one, which corresponds to immature mitochondria, and a heterogeneous one, which represents the mature mitochondria. The three tissues studied show different morphologic and metabolic patterns of mitochondrial maturation during the placentation process, rendering them suitable as experimental models to establish the possible relationship between mitochondrial maturation and the mitochondrial subpopulations.

Key words. Embryo; visceral yolk sac; placenta; mitochondrial subpopulation; mitochondrial morphology; cytochrome c oxidase.

The mitochondrial pool in a specific tissue is not homogeneous. Previous studies have shown that mitochondrial populations from rat liver, skeletal muscle and both brown and white adipose tissues can be fractionated into different subpopulations, which vary in biochemical and morphological features [1–9]. These mitochondrial fractions show differential behaviour in several physiological and pathological conditions such as overfeeding, obesity, cold exposure/acclimation and fasting [3–5, 7].

There seems to be a connection between the process of mitochondrial maturation and the mitochondrial fractions, which are probably non-mature forms acting as precursors of the mature mitochondria, so there is a mitochondrial growth-maturation cycle in the cell, which is more or less apparent, depending on the cell function [3, 5–9].

The mammalian organogenic embryo is a good model to study mitochondrial development, because during early organogenesis, rat embryo mitochondria are smaller and contain only a moderate number of short distended cristae, while the fetal tissues in the following stages of development contain larger mitochondria with numerous lamellar cristae [10, 11]. Furthermore, the oxidative electron transport system located in the mitochondrial cristae has been shown to be relatively inactive during the early stages of rat embryo development, gradually maturing af-

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ter establishment of vascular circulation through the placenta [12–14]. Finally, a shift from anaerobic to aerobic energy metabolism has been observed in rat embryos during organogenesis [12–16]. Thus, embryo development seems to be an adequate system to study the mitochondrial maturation process.

There are other tissues in the rat conceptus at this stage of development that would be appealing as models for the study of mitochondrial subpopulations: the visceral yolk sac membrane (VYS) and the placenta.

The mammalian VYS plays an important role in embryonic development. It is composed of several visceral and parietal layers, and is able to function as a placenta, connecting mother and embryo from implantation, before the development of the chorioallantoic placenta [17]. During this period, the yolk sac (especially the VYS layer) is the only placental exchange site, making it critically important for nourishing the organogenic embryo [18].

The nutritional role of the VYS is transferred to the chorioallantoic placenta when the latter becomes available on gestational day 12 [17]. The specialized cells of the chorioallantoic placenta attach the embryo to the uterus and form the vascular connections necessary for nutrient transport [17, 19]. One of the most important achievements of the developing placenta is the onset of embryonic mitochondrial oxidative metabolism, somehow activated by the establishment of the allantoic circulation, which increases the oxygen delivery to the quickly developing embryo [17, 20–22]. However, the VYS and placental mitochondria have not been extensively studied.

These three tissues of the conceptus could be representative of different mitochondrial maturation statuses, because both growth and differentiation processes are coincident but they follow different schedules at this point of development [23]. Thus, these tissues would be appropriate for research into the role of mitochondrial subpopulations in their maturation process.

To our knowledge, there are no studies on mitochondrial subpopulations during development, so the aim of this study was to establish the morphological and some of the functional changes in rat embryo mitochondrial subpopulations during the onset of oxidative metabolism and the associated mitochondrial maturation, using as a reference both VYS and placental mitochondria.

Materials and methods

Animals

Three-month-old female Wistar rats (CRIFFA, Barcelona, Spain) were used. They were housed in individual cages in a controlled environment (22 °C) with a 12 h light/12 h dark period (lights on at 08:00 a.m.). They had free access to tap water and pelleted standard diet (Panlab, Barcelona). Vaginal smears were examined for sperm the morning after overnight mating. When intravaginal sperm were found, day 0 of the pregnancy was defined as the preceding midnight.

Sacrifice and isolation of samples

Pregnant female rats were sacrificed on day 11, 12 and 13. Uteri were dissected and transferred to a sterile culture dish containing physiological saline solution. Embryos, VYS and placentas were dissected, pooled and weighed (placentas could not be obtained on day 11, because they are undistinguishable). Several pregnant rats were used (four to six on day 11, three to four on day 12 and two to three on day 13) to combine the embryos, VYS and placentas for sufficiently large samples in each of the six to eleven experiments. Tissue samples were homogenized in MOPS/sucrose buffer (300 mM sucrose, 5 mM MOPS, 1 mM EDTA-K_3, 5 mM KH_2PO_4, 0.01% bovine serum albumi, pH 7.4) with a Teflon/glass homogenizer at 1000–1500 r.p.m. The homogenates were used for mitochondria isolation.

Chemicals

All enzymes, substrates and coenzymes were obtained from Sigma-Aldrich (Madrid, Spain). Routine chemicals used were supplied by Amersham Pharmacia Biotech (Barcelona, Spain), Panreac (Barcelona, Spain), Sigma-Aldrich and Cultek (Madrid, Spain).

Isolation of mitochondrial subpopulations

The mitochondrial fractionation process is based on a published method [1, 2], later modified in our laboratory [3] and further adapted to the conceptus tissue specifications as follows. Nuclei and cell debris were first removed by refrigerated centrifuge (Sigma-3K30) at 800 g for 10 min at 4 °C. The resulting supernatant was subjected to three 10-min sequential centrifugation steps, at 1000, 3000 and 8000 g, respectively, rendering the heavy (M1), medium (M3) and light (M8) mitochondrial fractions. The pellets were resuspended in the homogenizing buffer and kept on ice for further analysis.

Biochemical analysis

Cytochrome c oxidase (ferrocytochrome c:oxygen oxidoreductase, EC 1.9.3.1; COX) activity was determined in isolated mitochondrial fractions. A spectrophotometric method was used [24] to follow changes in optical density at 550 nm during oxidation of reduced ferrocytochrome. The activity was measured at 37 °C using a thermostatized spectrophotometer (Shimadzu UV-2100). Samples of mitochondrial fractions were taken for protein measurement [25].

Electron microscope analysis

Samples for transmission electron microscope (TEM) examination, (mitochondrial conceptus subpopulations)

were obtained under exactly the same conditions as indicated above and were carefully removed and placed in ice-cold fixative buffer (2.5% glutaraldehyde in 0.2 M trihydrated sodium cacodylate buffer, pH 7.2) for 2 h. The fixed pellets were then washed four times in 0.2 M trihydrated sodium cacodylate buffer, and postfixed (1% osmium tetroxide) for 2 h. They were then dehydrated in graded acetone steps, stained with 2% uranyl acetate overnight and embedded in Spurr's resin. Semithin sections for light microscopy, about 0.5 µm thick, were cut with glass knives, stained with methylene blue and examined under a light microscope (Olympus BX-60). Ultrathin sections for electron microscopy, about 50 nm thick, were stained with saturated lead citrate solution and examined by a Hitachi H-600 electron microscope, at 75 kV. TEM micrographs were obtained at magnifications of ×10,000.

TEM micrographs were transferred to a personal computer and were analysed by Scion Image software for morphometric studies, measuring mitochondrial area, perimeter and the minor and major axis.

Statistics

Protein and COX data are presented as group mean values ± SE. Differences between groups were assessed by one-way and two-way analysis of variance (ANOVA) and by LSD for post hoc comparisons ($p < 0.05$). For mitochondrial area data analysis, a normality distribution test (Kolmogorov-Smirnov) was applied. Since a normal distribution was not found, differences between groups were assessed by a non-parametric Kruskal-Wallis test ($p < 0.05$). All statistical analyses were performed with an SPSS 10.0 for Windows package.

Results

TEM analysis

Only the most relevant mitochondrial pictures are used in figures 1 and 2. Figure 1 shows M1, M3 and M8 mitochondrial subpopulations from embryo and VYS on gestational day 11, as an illustration of the differences between the subpopulation profiles in both tissues. The mitochondria were larger in the VYS than in the embryo, and showed many lamellated parallel cristae; on the other hand, embryo mitochondria showed a pattern with few, vesiculated cristae. Thus the VYS mitochondria seemed to be more mature than the embryo ones. In fact, the more lamellated the mitochondrial cristae are, the more mature the mitochondrion is [26, 27]. The characteristic form observed with embryo mitochondria has been documented previously in histological analysis of whole embryo tissue [10, 11, 14, 28].

Figure 2 shows the M1 fraction profile on gestational days 12 and 13 in the three tissues studied, as an

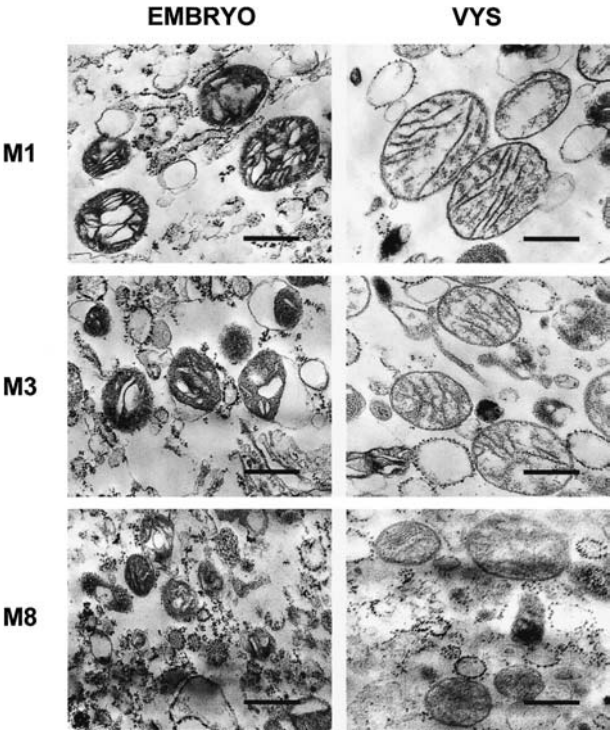

Figure 1. TEM micrographs of mitochondrial subpopulations in embryo and VYS on gestational day 11 (bar, 1 µm).

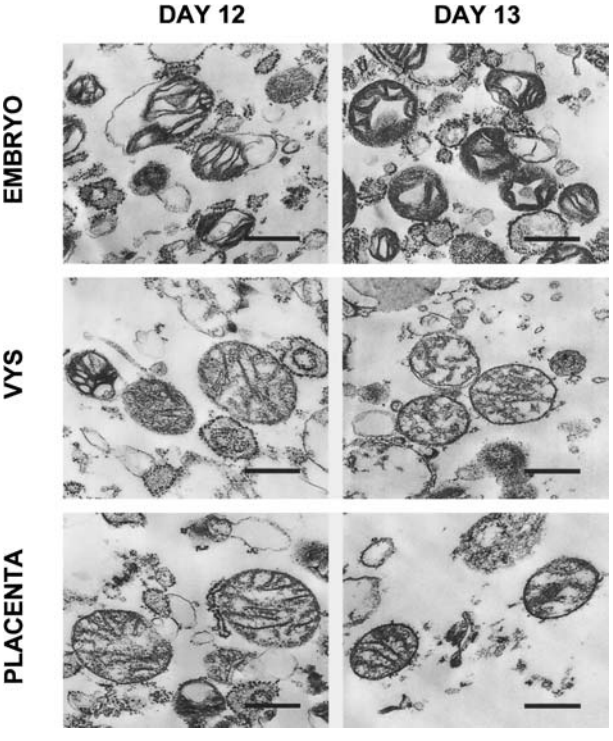

Figure 2. TEM micrographies of the M1 mitochondrial subpopulation in embryo, VYS and placenta on gestational days 12 and 13 (bar, 1 µm).

overview of the changes during the days studied (combined with M1 photos in fig. 1). In general, embryo M1 mitochondria looked different to placenta and VYS mitochondria, being, as indicated above, more vesiculated. There were very few differences in embryo mitochondria, on the days studied, which had an immature appearance. On the other hand, there were noteworthy differences in size and cristae profile between gestational days 12 and 13 in both placenta and VYS M1 subpopulations, although the mitochondria showed a quite mature appearance.

Morphometric analysis

We measured the area, perimeter and the minor and major axis of the mitochondrial subpopulations, but report only the area here. Mitochondrial areas of the M1, M3 and M8 subpopulations in the different tissues are shown in figure 3.

Despite the overall homogeneous profile of the embryo mitochondria, there were significant differences between the fractions, mainly associated with a trend towards a minor reduction in size over the days studied, in particular in the M3 population.

VYS mitochondria showed a different area pattern compared to the embryo, with M1 being the biggest and M8 the smallest. This profile seems to be representative of a developed tissue, as described in brown adipose tissue [7], while the embryo mitochondria profile would correspond to a tissue in the differentiation process. There was also a reduction in the area (about 30%) in all the fractions of VYS during the period of development studied, maintaining the same size pattern, probably related to the change in the nutritional functionality of this tissue.

Finally, the most striking feature of placental mitochondria was the change in the size profile between days 12 and 13, with a tendency to evenness in the mitochondrial size (40% reduction in M1), which may also be related to the increase in the capabilities of the placenta.

To further analyse the data of the range of areas in the different mitochondrial fractions, the distribution of frequencies for different area intervals is represented in figures 4–6. These distributions exhibited different profiles. Embryo mitochondria had a narrow range of size distribution with very few, large mitochondria during the period studied. However, VYS mitochondria showed a wider pattern of size distribution, with large as well as small mitochondria. This same profile was seen with placental mitochondria. Of interest is that all the embryo mitochondria subpopulations had about the same area as the M8 ones in the VYS (and with the same narrow size distribution), indicating again that all of the embryo mitochondria could be at an immature stage. In fact, the more mature VYS mitochondrial fractions M1 and M3 had a very heterogeneous size distribution profile.

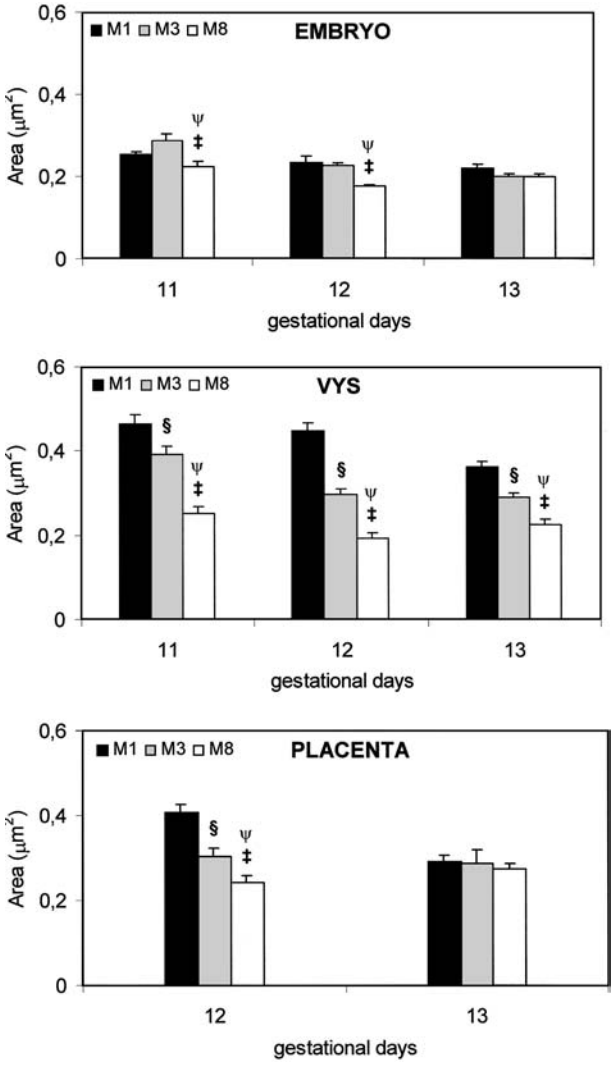

Figure 3. Area values of the mitochondrial subpopulations of embryo, VYS and placenta on gestational days 11, 12 and 13. Graphs represent the mean area ± SE and were analysed by Kruskal-Wallis test as described in Materials and methods. Significant differences ($p < 0.05$): § M3 vs M1; ψ M8 vs M1; ‡ M8 vs M3. The values for the three tissues are statistically different.

Mitochondrial protein content

Mitochondrial protein content expressed per total tissue in each of the fractions studied is shown in table 1. The scatter of the individual values is rather large, probably due to the high rate of conceptus growth at this particular stage of development, such that small differences in the developmental age of the conceptus (even in the same litter) would result in quite different protein values. Embryo mitochondria protein levels rose exponentially during the gestational period studied, especially in the M1 fraction. Placenta mitochondria showed the same profile, with the values in the three fractions studied increasing in the same way. The VYS mitochondrial protein content also exhibited a significant increase during development, but

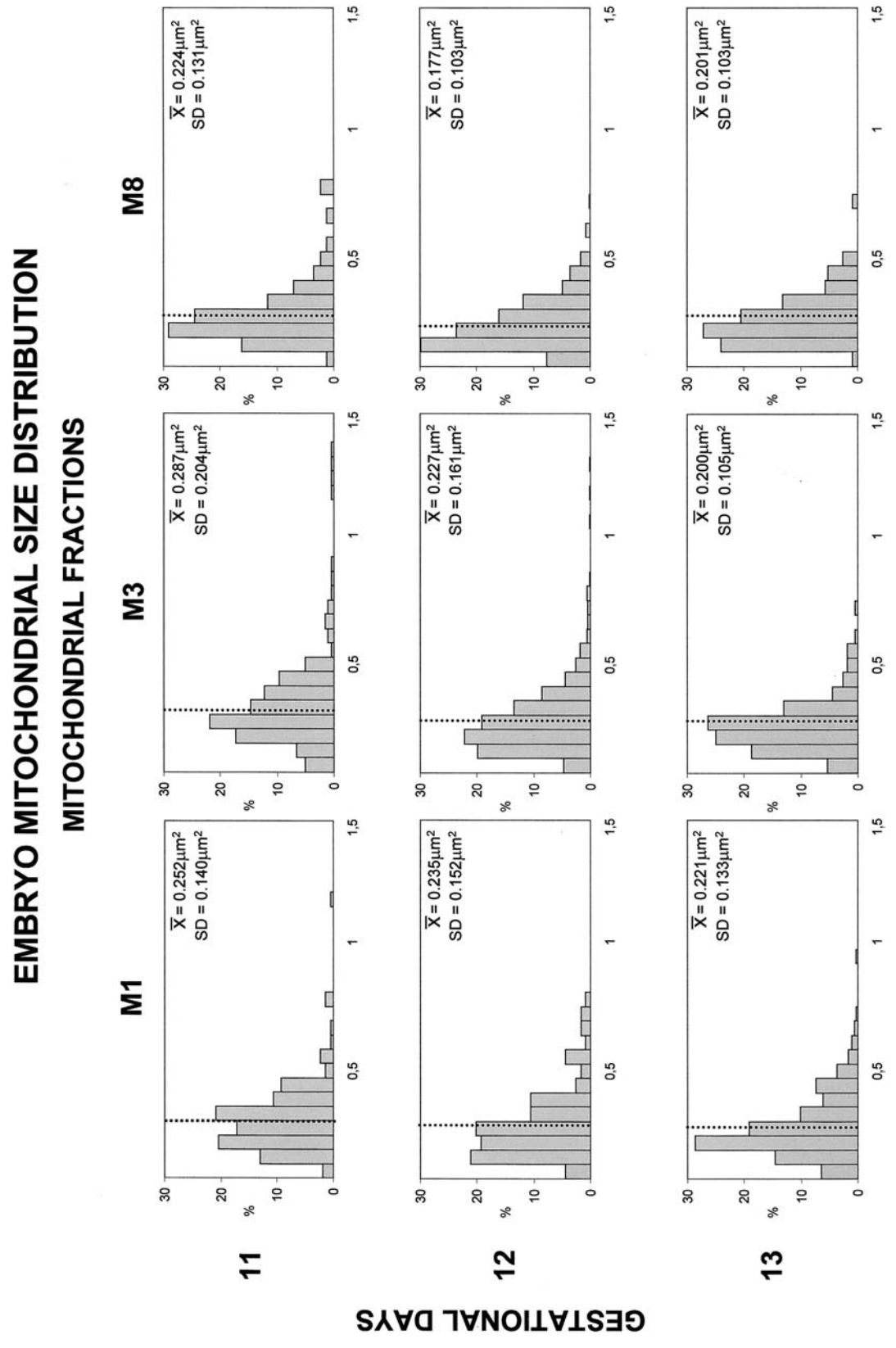

Figure 4. Area distribution of embryo mitochondrial subpopulations on gestational days 11, 12 and 13. Graphs show the population percent vs mitochondrial area (in μm^2 and categorized in 0.06-µm-increment columns). Data at the top of the graph show the mean value (also indicates as a vertical dashed line) and its standard deviation.

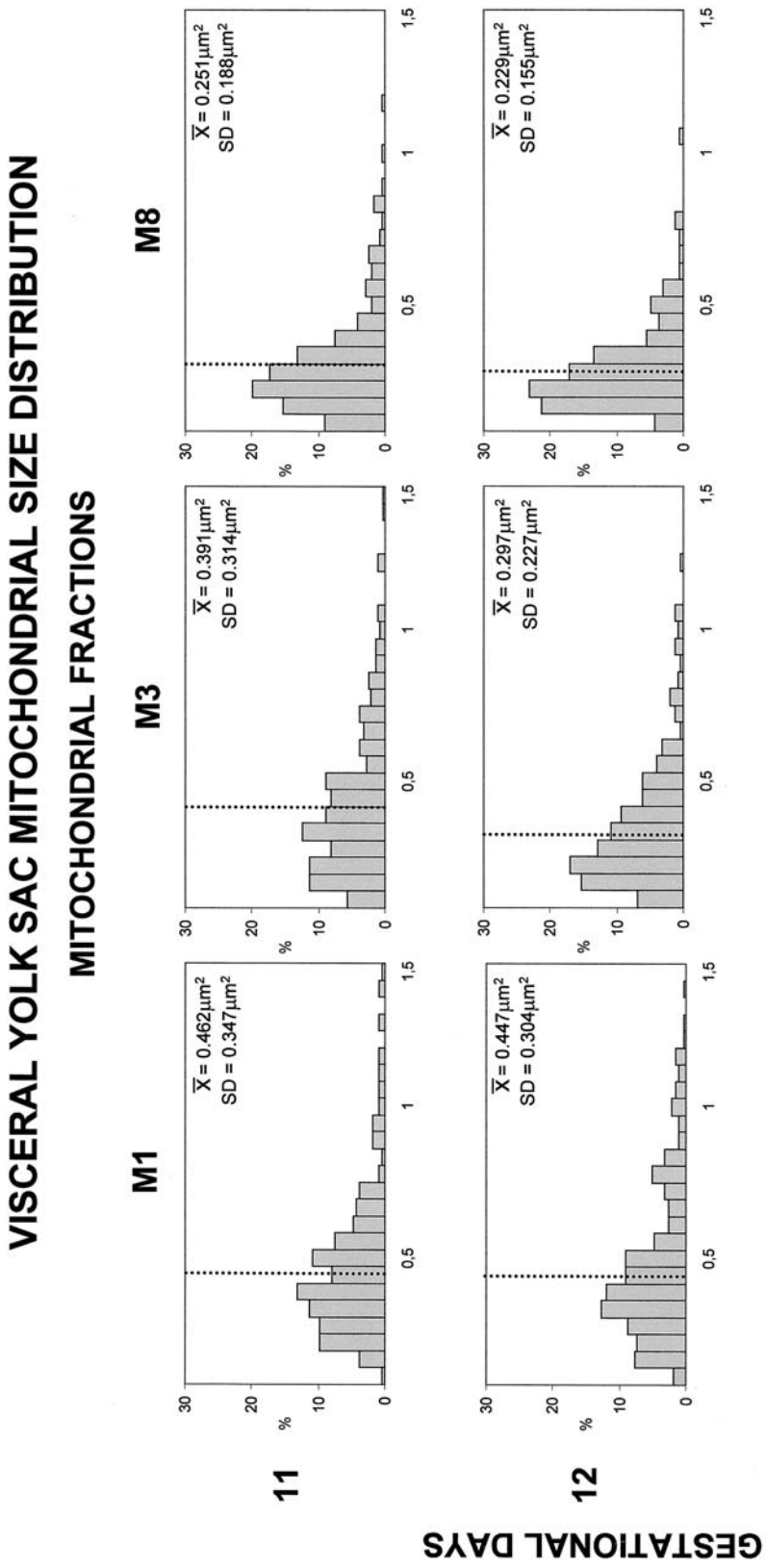

Figure 5. Area distribution of VYS mitochondrial subpopulations on gestational days 11, 12 and 13. Graphs show the population percent vs mitochondrial area (in μm^2 and categorized in 0.06-µm-increment columns). Data at the top of the graph show the mean value (also indicated as a vertical dashed line) and its standard deviation.

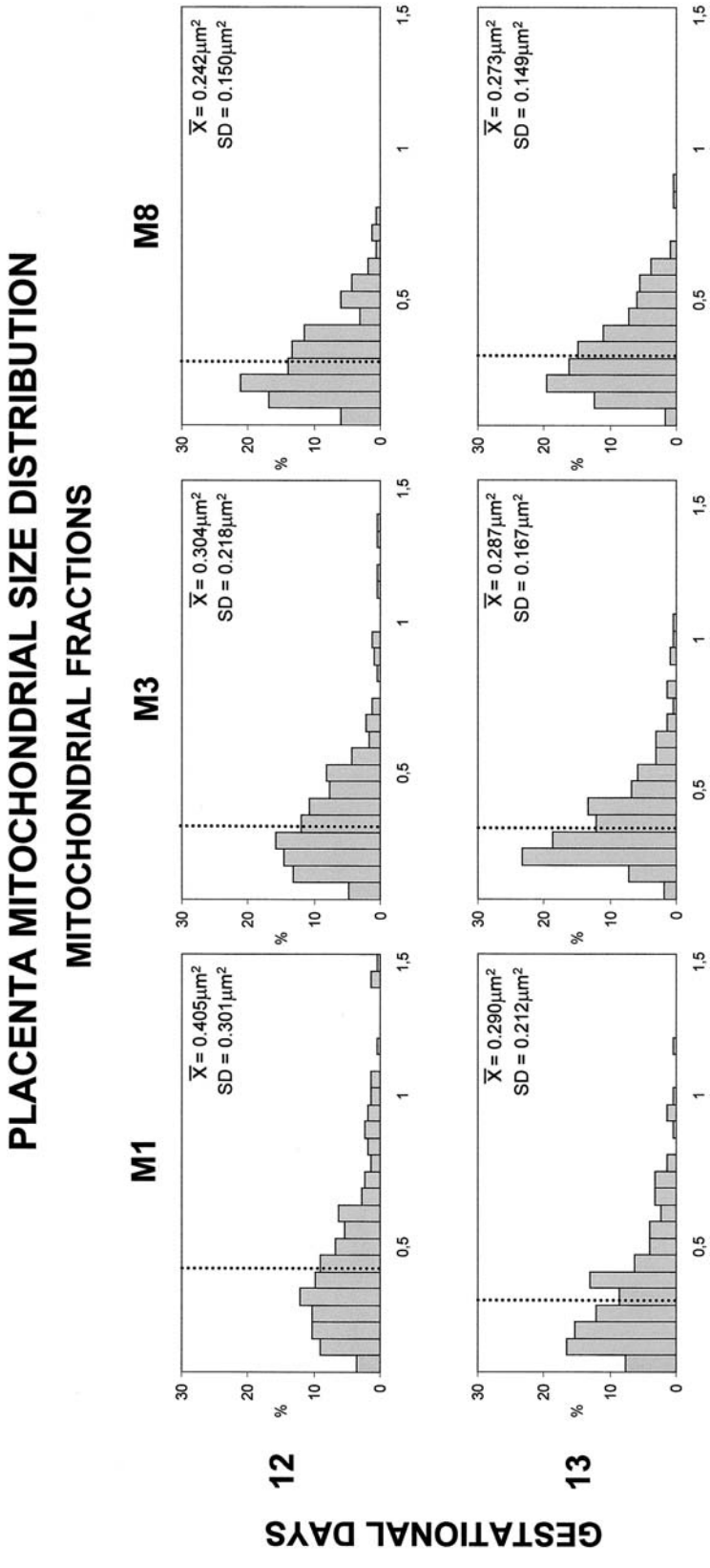

Figure 6. Area distribution of placenta mitochondrial subpopulations on gestational days 12 and 13. Graphs show the population percent vs mitochondrial area (in μm^2 and categorized in 0.06-µm-increment columns). Data at the top of the graph show the mean value (also indicated as a vertical dashed line) and its standard deviation.

it was linear, and VYS values were always lower than in the other tissues. This pattern could be related to the different functional changes taking place in these tissues at this point of development.

COX activity

COX activity indicates the respiratory chain capacity, i.e. mitochondrial oxidative metabolism functionality [29, 30]. Therefore, to establish changes in the oxidative capabilities of the mitochondrial subpopulations, COX activities were measured. These activities in the several mitochondrial subpopulations studied are expressed as specific activity (in table 2) and activity per total tissue (in table 3).

Embryo mitochondria COX specific activities showed similar values, but there was a significant differential behaviour in each of the different fractions during the days studied (especially in M1 and M8). COX values in the placenta were about the same, with lower levels in M8. In VYS, the activities in M1 were higher than in M3, which in turn were higher than in M8; this is the same profile seen for the mitochondrial area (fig. 3), and would also be related to the mitochondrial maturation status. There was also an interesting, significant pattern, of COX activities

Table 1. Protein levels in the mitochondrial subpopulations of embryo, VYS and placenta on gestational days 11, 12 and 13.

		Embryo	VYS	Placenta
Gestational day 11				
	M1	4.64 ± 0.45	2.29 ± 0.63	ND
	M3	5.70 ± 0.43	2.56 ± 0.18	ND
	M8	4.09 ± 0.46	2.51 ± 0.26	ND
Gestational day 12				
	M1	12.03 ± 1.55	6.94 ± 0.72	15.72 ± 2.36
	M3	11.16 ± 1.18	6.99 ± 0.74	15.16 ± 1.01
	M8	8.86 ± 1.14	7.37 ± 0.84	12.56 ± 0.66
Gestational day 13				
	M1	55.06 ± 14.88	13.07 ± 5.25	49.97 ± 6.53
	M3	46.15 ± 12.28	11.17 ± 1.89	58.48 ± 6.97
	M8	38.71 ± 6.73	9.69 ± 1.31	46.63 ± 6.11
ANOVA		gestation: $p < 0.05$ fraction: NS interaction: NS	gestation: $p < 0.05$ fraction: NS interaction: NS	gestation: $p < 0.05$ fraction: NS interaction: NS

Data are expressed as micrograms of protein per total tissue (mean ± SE) and analysed by two- and one-way ANOVA as described in Materials and methods. Values in the three tissues differ statistically. ND, not determined; NS, not significant.

Table 2. COX specific activity in the mitochondrial subpopulations of embryo, VYS and placenta on gestational days 11, 12 and 13.

		Embryo	VYS	Placenta
Gestational day 11				
	M1	5.89 ± 1.12	14.33 ± 2.99	ND
	M3	9.22 ± 1.21§	10.66 ± 1.48	ND
	M8	7.44 ± 0.96	2.51 ± 1.71ψ	ND
Gestational day 12				
	M1	10.55 ± 1.62	25.49 ± 8.59	13.23 ± 3.14
	M3	11.85 ± 1.17	18.05 ± 5.81	12.65 ± 1.55
	M8	10.27 ± 1.13	11.17 ± 2.74	7.79 ± 0.79
Gestational day 13				
	M1	9.33 ± 2.12	6.17 ± 1.72	12.50 ± 1.56
	M3	10.85 ± 1.34	6.37 ± 2.17	13.68 ± 1.69
	M8	6.44 ± 0.53‡	3.41 ± 0.83	9.34 ± 0.96‡
ANOVA		gestation: $p < 0.05$ fraction: $p < 0.05$ interaction: NS	gestation: $p < 0.05$ fraction: NS interaction: NS	gestation: NS fraction: NS interaction: NS

Data are expressed as µKat per milligram of mitochondrial protein content (mean ± SE) and analysed by two- and one-way ANOVA as described in Materials and methods. Post hoc significant differences ($p < 0.05$): § M3 vs M1; ψ M8 vs M1; ‡ M8 vs M3. NS, not significant; ND, not determined. Values in the three tissues differ statistically.

Table 3. COX activity per total tissue in the mitochondrial subpopulations of embryo, VYS and placenta on gestational days 11, 12 and 13.

		Embryo	VYS	Placenta
Gestational day 11				
	M1	22.36 ± 4.45	28.31 ± 10.59	ND
	M3	32.36 ± 12.15	27.71 ± 11.54	ND
	M8	24.86 ± 9.74	17.52 ± 6.37	ND
Gestational day 12				
	M1	71.33 ± 17.83	88.40 ± 28.47	213.86 ± 30.23
	M3	82.03 ± 13.51	50.23 ± 11.10	218.38 ± 25.40
	M8	56.58 ± 8.46	30.97 ± 7.60$^{\psi}$	104.69 ± 5.50$^{\ddagger,\psi}$
Gestational day 13				
	M1	430.62 ± 80.30	84.13 ± 27.39	620.68 ± 104.58
	M3	438.94 ± 70.74	65.88 ± 24.60	751.13 ± 93.96
	M8	238.47 ± 28.89$^{\ddagger,\psi}$	32.11 ± 11.49	407.23 ± 47.12‡
ANOVA		gestation: $p < 0.05$ fraction: $p < 0.05$ interaction: $p < 0.05$	gestation: NS fraction: NS interaction: NS	gestation: $p < 0.05$ fraction: NS interaction: NS

Data are expressed as nKat per total tissue (mean ± SE) and analysed by two- and one-way ANOVA as described in Materials and methods. Post hoc significant differences ($p < 0.05$): ψ M8 vs M1; ‡ M8 vs M3. NS, not significant. ND, not determined. Values in the three tissues differ statistically.

increasing from day 11 to 12, and then decreasing on day 13 to values lower than on day 11. This pattern could be related to the loss of VYS functionality, the VYS being replaced in its nutritional role by the chorioallantoic placenta [17].

When COX mitochondrial activity is expressed per total tissue, the values in embryo and placenta showed the same significant increase described for the protein levels (table 1). COX activity in VYS had a very different profile, with a tendency to increase between days 11 and 12, and to maintain the same levels on day 13, in accordance with the already indicated loss of functionality of this tissue.

Discussion

The present results indicate large morphological and functional differences between the mitochondria of the tissues studied. Mitochondrial subpopulations show different profiles that are specific for embryo, VYS and placenta.

Previous studies on mitochondrial fractions in adult tissues such as like liver [1, 2, 6, 9], skeletal muscle [8], brown adipose tissue [7] and white adipose tissue [9] have demonstrated a specific size profile in these subpopulations: the heavy fraction is usually the largest, while the light fraction is the smallest, coincident with the possibility that the light mitochondria are the immature form in the growth cycle of mitochondria, while heavy fractions would be the mature forms. This profile seems to be the same in the developed tissues. In the present results, this pattern is discernible in mitochondria of the VYS, a quite well-developed tissue at this stage of development [18, 31, 32], while the embryo mitochondria have about the same area during the period studied, which is characterized by the onset of oxidative metabolism [12–14].

The individual areas of embryo mitochondrial subpopulations show a narrow distribution (as seen in fig. 4), as that found in the M8 fraction in the VYS (and with the same area distribution), while the VYS mitochondrial fractions M1 and M3 have a very heterogeneous size distribution profile. On the whole, there seem to be two major size distribution profiles: a wide one, which would be characteristic of mature mitochondria (in placenta and M1 and M3 of VYS), and a narrow one, which would be distinctive of the immature mitochondria (in the embryo and M8 of VYS).

This immaturity of the embryo mitochondrial subpopulations is also supported by their appearance, with few and vesiculated cristae (figs. 1, 2). In fact, previous ultrastructural studies of whole embryo mitochondria during early organogenesis have shown that there is an increase in cristae, which appear tubular or vesicular after the end of neurulation, but with the onset of vascular circulation through the placenta, the cristae gradually become lamellated, and have a more mature appearance [14, 28]. Our ultrastructural study would suggest that the mitochondria in the differentiating tissues are at an immature stage.

On the other hand, in the present results, there are no important differences between embryo mitochondrial COX specific activity on the 3 days studied, a profile coincident with the mitochondrial-area profile discussed above.

Consequently, COX activities also point to the same lack of maturation for these mitochondrial subpopulations.

The changes in VYS mitochondrial areas indicate a perceptible slowing-down of mitochondrial turnover, characterized by an overall size reduction in these organelles from day 11 to 13, which could be related to the loss of functionality of VYS, which is being replaced in its role by the chorioallantoic placenta [17]. Moreover, embryo mitochondrial protein levels show an exponential increase from day 11 to 13, as does placental mitochondrial protein. However, VYS protein does not show an exponential, but rather a linear increase, suggesting a different tissue development pattern, probably also related to the indicated role change for this tissue.

Placental mitochondrial fractions show a distinctive pattern. On day 12, there are size differences between the mitochondrial fractions, which show the same mature profile discussed above for developed tissues. However, on day 13, the mitochondria are all the same size. This can be related to the change in placental development which begins differentiation on day 12, when the distal portion of the allantoides reaches the ectoplacental cone to form with it the definitive chorioallantoic placenta [17].

Mitochondrial biogenesis is the combination of two different processes: proliferation and differentiation. Proliferation is an increase in the mitochondrial population, while differentiation can be defined as an augmentation in the functional capabilities of pre-existing mitochondria (with the incorporation of newly synthesized proteins and lipids) to acquire the ultrastructure and functional features of mature mitochondria [33–39]. These processes are especially well illustrated during postnatal development of the liver [34, 35], and also during the cold-induced recruitment of brown adipose tissue [3, 40].

Taking this background into account, our results indicate that the different embryo mitochondrial subpopulations seem to be in a proliferative stage, but are not yet differentiated; these mitochondria can incorporate more proteins, enough to increase their density (M1 being higher than M8), but not enough to grow in size and improve their functionality, so they are still at an immature stage. This would be the immature mitochondrial subpopulation profile in the developing tissues.

In conclusion, the three tissues studied (embryo, VYS and placenta) show different mitochondrial morphologic and metabolic patterns of maturation during the placentation period, making them suitable models to establish the possible relationship between mitochondrial maturation and the mitochondrial fractions.

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