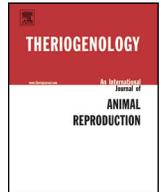




ELSEVIER

Contents lists available at SciVerse ScienceDirect

## Theriogenology

journal homepage: [www.theriojournal.com](http://www.theriojournal.com)

## Development and morphology of the inverted yolk sac in the guinea pig (*Cavia porcellus*)

Bruno Gomes Vasconcelos, Phelipe Oliveira Favaron, Maria Angelica Miglino, Andrea Maria Mess\*

Department of Surgery, School of Veterinary Medicine and Animal Science, University of Sao Paulo, Sao Paulo, Brazil

### ARTICLE INFO

#### Article history:

Received 15 February 2013

Received in revised form 5 June 2013

Accepted 6 June 2013

#### Keywords:

Yolk sac placentation

Caviomorphs

Hemotrophic nutrition

Histirotrophic nutrition

Reichert's membrane

### ABSTRACT

Although the guinea pig is an important animal model for human placentation, aspects of fetal nutrition are not fully understood, especially in regard to the yolk sac that is regarded to be essential for early development of the embryo. We investigated differentiation by means of histology, histochemistry, immunohistochemistry, and transmission electron microscopy. Data suggest that the guinea pig's yolk sac was not sufficiently developed to facilitate substantial fetal nutrition in early pregnancy. On Day 12, it was a flat, inverted, but avascular structure. This was followed by differentiation to form the typical, highly villous and vascularized condition of advanced gestation. Finally, the yolk sac degenerated toward term. We suggest that the guinea pig and other caviomorphs rely predominantly on hemotrophic nutrition via the placenta even in very early pregnancy. In contrast to the general pattern of mammals, histiotrophic nutrition via yolk sac routes seems to be most essential during mid-gestation.

© 2013 Elsevier Inc. All rights reserved.

### 1. Introduction

The guinea pig, *Cavia porcellus*, is an important animal model for human placentation, because placental structure and development are similar, especially in regard to trophoblast invasion and the hemomonochorial interface between the maternal and fetal blood systems [1–7]. However, there are other, important structures, such as the yolk sac, that differ. In general, the yolk sac is among the first extraembryonic membranes to form in mammals or vertebrates [8–10] and is regarded as essential for early embryonic survival and nutrition [11–14]. Later in gestation, it may or may not be involved in fetomaternal exchange as a choriovitelline or inverted yolk sac placenta [8,15]. In humans, the yolk sac is a temporary, small, and free-floating structure without placental function, but participates in early hematopoiesis. In contrast, a yolk sac is

present throughout pregnancy in the guinea pig, characterized by complete inversion of the germ layers with the endoderm directed toward the uterine tissues [15–19]. This fully functional yolk sac placenta may influence or support the functions of the chorioallantoic placenta [20–23]. However, there are significant gaps of knowledge, especially with regard to the early to mid-gestation differentiation [18,24,25]. The starting condition before inversion has been detected only recently [26]. The data show that the inverted yolk sac is derived from the visceral area only, whereas the outer, parietal layer along the yolk sac cavity is found in very early pregnancy but then disappears. Even the layer covering the placenta belongs to the former most visceral area and should better be called “placental yolk sac” rather than “parietal yolk sac” [26]. However, chronological interactions between yolk sac and placenta are not well resolved. Thus, further investigation will provide a more balanced understanding of the guinea pig's fetomaternal exchange processes, which may help us to understand the values and restrictions especially when it is used as an animal model for human placentation. We here

\* Corresponding author. Tel./fax: +55 11 3091 7690.

E-mail address: [drandrmss@aol.com](mailto:drandrmss@aol.com) (A.M. Mess).

describe the development and morphology of the yolk sac by conventional histology, histochemistry, immunohistochemistry, and transmission electron microscopy to clarify aspects of yolk sac transport functions during gestation. In addition to the study on very early gestation [26], we focus on the period after yolk sac inversion from Day 12 onward.

## 2. Materials and methods

Yolk sac samples were obtained from 17 *Cavia porcellus* females at 12, 14, 16, 18, 22, 30, 40, and 55 days of gestation (term about 64 days). The study was approved by the Ethics Committee for the Use of Animals at the School of Veterinary Medicine and Animal Science at the University of São Paulo (USP, No. 2521/2012). Tissues for histology were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and in 4% methacarn (60% methanol, 30% chloroform, and 10% acetic acid), dehydrated and embedded in paraffine, and sectioned at 5 µm and stained with hematoxylin and eosin, toluidine blue, and the periodic acid-Schiff (PAS) reaction. Immunohistochemistry was performed for cytokeratin to identify the epithelial and trophoblastic cells (1:300 mouse anti-human monoclonal primary antibody; M0821, Dako, Carpinteria, CA, USA) and for vimentin to identify mesenchymal cells and endothelium (1:300 mouse anti-human monoclonal primary antibody; 0.N.602, sc-73259, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Proliferating cell nuclear antigen (PCNA) was used to stain proliferating cells (1:400 mouse monoclonal primary antibody; PC10, sc-56, Santa Cruz Biotechnology). We followed the protocols previously published by our group [27]. Slides were examined under an RX40 Olympus microscope (Zeiss KS400 3.4). Tissues for transmission electron microscopy were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, postfixed in 2% osmium tetroxide in phosphate buffer (pH 7.4) and embedded in Spurr resin. Ultrathin sections (Ultracut R, Leica Microsystems, Nussloch, Germany) were contrasted using 2% uranyl acetate and 0.5% lead citrate and examined under a transmission electron microscope (Morgagni 268D, FEI Company, the Netherlands; MegaView III camera; Soft Imaging System, Germany).

## 3. Results

### 3.1. Early gestation

Starting from the ectoplacental cone, where the invasive trophoblast established access to the maternal blood system, the disk-shaped, chorioallantoic placenta was covered by yolk sac tissue. On Days 12 and 14, this placental yolk sac had a pseudostratified, columnar structure including the nuclei with diffuse chromatin, distinguished from the placenta by a space filled with extracellular matrix in the area where later the Reichert's membrane (RM) developed (Fig. 1A). On Day 16, the yolk sac above the placenta was modified into branched and tuft like projections that were highly active in proliferation (Fig. 1B, C). An RM toward the placenta was present, characterized by an amorphous and acellular structure (Fig. 1C, D). Ultrastructurally, microvilli on the apical surface and large vacuoles with low density content characterized the endodermal cells

(Fig. 1D). Epithelial cells of the placental yolk sac reacted positively to cytokeratin. In addition, the endoderm of the remaining visceral yolk sac, facing the uterine cavity, was cytokeratin-positive (Fig. 1E), whereas the connective tissue and mesothelium, as well as the first developing fetal vessels, were positive for vimentin (Fig. 1F). Proliferation activity in the visceral yolk sac was low compared with the placental yolk sac (Fig. 1B), but was observed in all layers, especially in the connective tissue (Fig. 1G). On Days 12 to 16, the inverted yolk sac appeared to be flat all over (Fig. 1B, C, E, F), consisting mostly of simple columnar epithelium (Fig. 1E). Numerous vesicles occurred in the endoderm and the fetal vessels contained hemangioblasts on Day 16 (Fig. 1H). On Day 18, the initial formation of yolk sac villi occurred near the placenta.

### 3.2. Mid-gestation

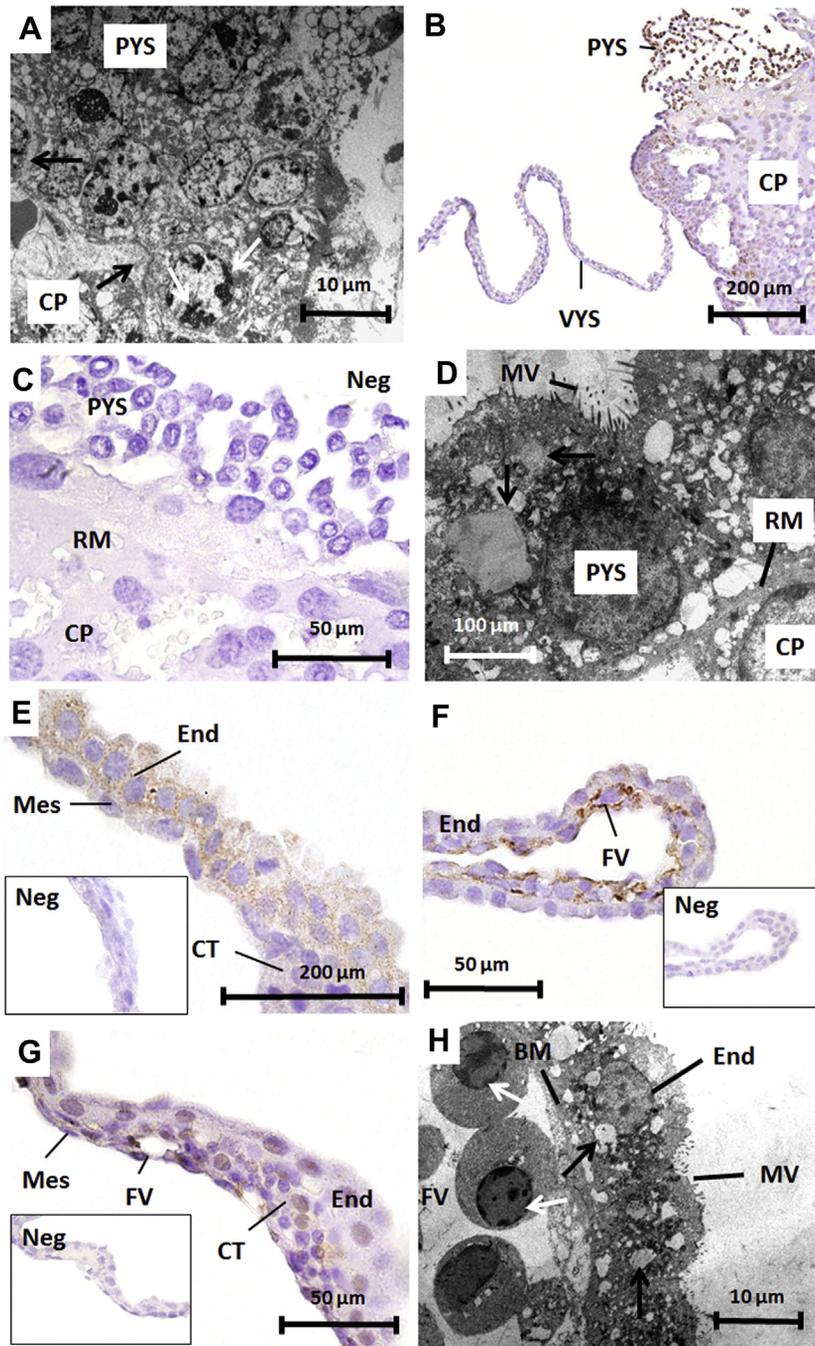
On Days 22 to 40, the first villi of the placental yolk sac were well developed. The RM toward the placenta had thickened (Fig. 2A, B) and was positive to cytokeratin. Local differences were evident with larger projections on the fetal side of the placenta and smaller ones on the maternal surface (Fig. 2A). Immunohistochemistry indicated strong proliferation activity of the endoderm, especially in cells at the apices of the projections (Fig. 2A). The placental yolk sac was fragmented or tufted and characterized by polyhedral cells with cytoplasmic projections, supported by some connective tissue (Fig. 2B). Apical microvilli and junctional complexes were frequent (Fig. 2B, C). Positive PAS staining suggested the presence of glycoproteins in the RM and at the apical surface of the projections (Fig. 2D). Apposed to the placenta, the visceral yolk sac contained lots of villi, characterized by connective tissue and fetal vessels lined by simple columnar epithelium (Fig. 2E). Microvilli occurred on the apical surfaces and vesicles inside the endoderm cells and the nuclei largely filled their cytoplasm. Fetal vessels were intimately associated with the basal membranes of the endoderm (Fig. 2F). On Day 22, all layers, especially the villous tips, showed positive response to PCNA, indicating proliferation (Fig. 2G), whereas on Days 30 and 40, predominately the endodermal cells were stained (Fig. 2H). In addition, the structures of the villous, visceral yolk sac reacted positively to PAS (Fig. 2E). Distal to the placenta, the visceral yolk sac was flat with mostly simple columnar epithelium (Fig. 2I).

### 3.3. Close to term gestation

On Day 55, the placental yolk sac had lost its treelike appearance and was drastically reduced to a flat, simple or pseudostratified, columnar layer. However, it still showed some proliferation activity. The visceral yolk sac was villous near the placenta and flat distally, consisting of simple cuboid epithelium, connective tissue, and fetal vessels. The visceral yolk sac was not proliferative.

## 4. Discussion

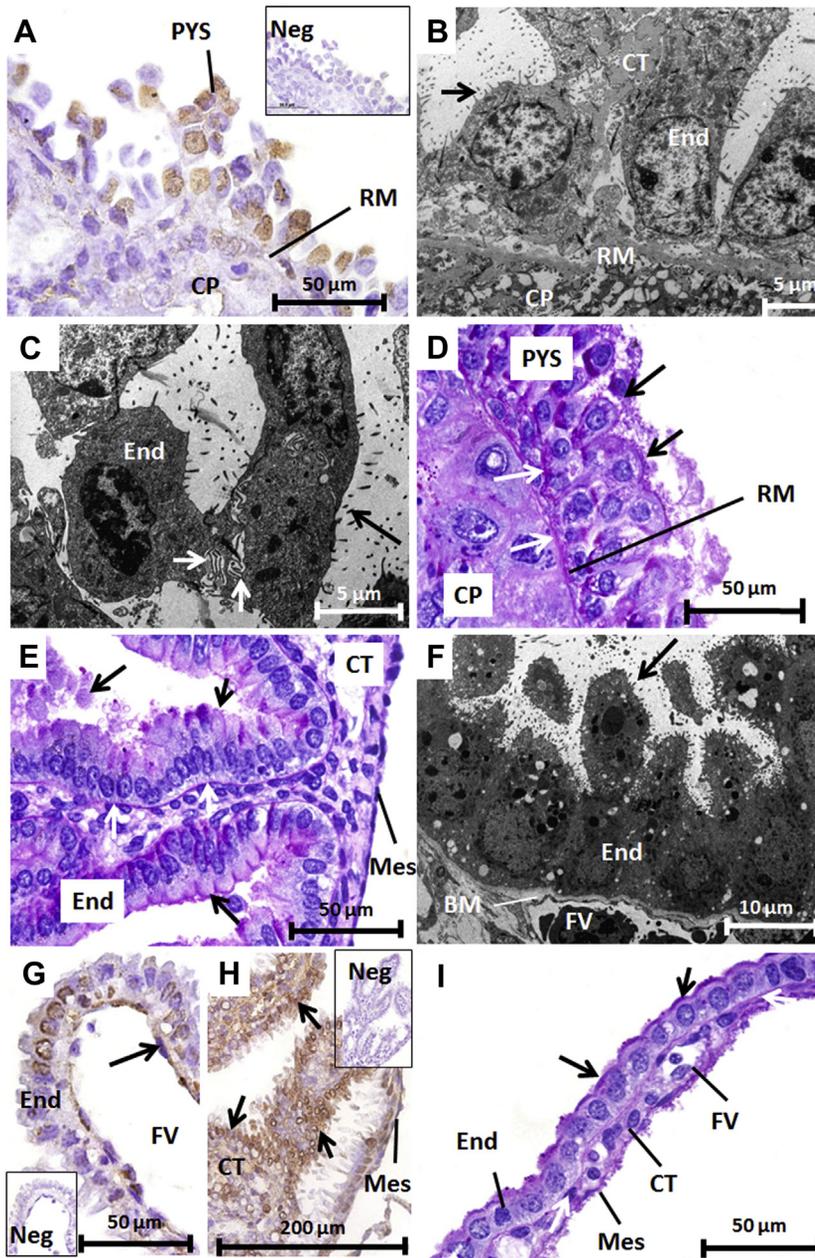
Eutherian mammals have a chorioallantoic placenta as the main region of fetomaternal exchange [8,10,15,28],



**Fig. 1.** Early gestation. (A–D) Placental yolk sac (PYS). (E–H) Visceral yolk sac (VYS). (A) TEM, Day 12. Pseudostratified epithelium, nuclei with diffuse chromatin (white arrows) and a space filled with extracellular matrix (black arrows) toward the chorioallantoic placenta (CP). (B) PCNA, Day 16. (C) Negative control of PCNA, Day 16. (D) TEM, Day 16. RM amorphous and acellular. Low-refringence vesicles (arrows) and microvilli (MV) were present. (E) Cytokeratin, Day 14; endoderm (End) was positive; connective tissue (CT) and mesothelium (Mes) were negative. (F) Vimentin, Day 16; fetal vessel (FV) endothelium was positive. (G) PCNA, Day 16. (H) TEM, Day 16, vesicles were present (black arrows); fetal vessels near the basal membrane (BM) of the endoderm, filled with hemangioblasts (white arrows).

whereas their yolk sac is regarded to be essential mainly for early pregnancy, that is, to provide embryonic nutrition before the placenta is fully developed [19,29]. However, analysis of yolk sac and placental differentiation in the

guinea pig suggests that it may be an exception to the rule. After implantation is achieved by Day 7, trophoblast of the ectoplacental cone [30] establishes access to the maternal blood system by Day 9 [7], followed by fusion with the



**Fig. 2.** Mid-gestation. (A–D) Placental yolk sac (PYS). (E–I) Visceral yolk sac (VYS). (A) PCNA, Day 22. Projections along placenta (CP), based on RM. (B,C) TEM, Day 22. Basal region of an endodermal (End) tree on acellular, amorphous RM with connective tissue (CT), extracellular matrix and secretion products, apical microvilli (black arrows), and junctional complexes (white arrows). (D) PAS, Day 40, suggesting glycoproteins in RM (white arrows) and at the apical surface of projections (black arrow). (E) PAS, Day 40. Positive response in endodermal cells (End, black arrows) and basal membrane (white arrows), not in mesothelium (Mes) and CT. (F) TEM, Day 22. Microvilli (arrow) and basal membrane (BM) of endoderm in contact to fetal vessels (FV). (G) PCNA, Day 22. Positive response, especially in cells of the endoderm and mesothelium (arrow). (H) PCNA, Day 40. Staining predominating in the endoderm (arrow). (I) PAS, Day 40. Distal to the placenta, the yolk sac was flat. Basal membrane (white arrows) and endoderm (black arrows) were PAS-positive.

allantois and the appearance of fetal vessels by Day 12 [18]. Starting from this early but functional condition, a highly lobulated labyrinthine placenta with countercurrent blood flow forms by extensive growing processes [5,18]. This placenta has one of the best fetal to placental weight ratios (placental efficiency) among mammals, resulting in excellent conditions for hemotrophic nutrition [31]. In contrast,

the yolk sac develops more slowly. On Day 12, it has a premature condition characterized as a flat, inverted, avascular structure. The typical villous structure started by Day 16 for the placental yolk sac (treelike projections) and Days 18 to 22 for the visceral yolk sac areas near the placenta, accompanied by intense cell proliferation. The first vessels in the visceral yolk sac are present on Days 15

to 16 [32]; however, areas distal to the placenta do not develop vascularization until the later phases of gestation. We suppose that in early pregnancy, the guinea pig's yolk sac is not adequately developed to provide substantial fetal nutrition, in contrast to the placenta. Among caviomorphs, early stages are known for another cavy, *Galea spixii* [26], and an echimyid, *Thrichomys laurentinus* [27], suggesting that a pattern of predominately hemotrophic nutrition in early pregnancy may be common for caviomorphs. The situation changes in mid-gestation. As shown here for Days 22 to 40, major differentiation takes place to transform both the placental and the visceral yolk sac near the placenta into villous structures with proliferative epithelia that participate in glycosylation processes. Such conditions, including the development of an absorptive system of the columnar endoderm of the villous part of the yolk sac near the placenta with membrane invaginations, endocytotic vesicles, large vacuoles, and yolk sac vessels are known for caviomorphs and are likely to facilitate substance transfer [17–19,24,25,27,33–39]. Indeed, it has been experimentally shown that the visceral yolk sac of the guinea pig is an important route for protein absorption and transport in advanced phases of gestation. Especially passive immunization of the fetus occurs across the visceral yolk sac and not via the placenta [20–22]. Consequently, in mid-gestation, histiotrophic nutrition via the placental and visceral yolk sac routes seems to be an important addition to hemotrophic nutrition by the placenta. This has to be borne in mind when choosing the guinea pig as a model species for human placentation, especially if exchange processes are in focus. Toward term, dramatic reductions takes place in the placental yolk sac that becomes very flat, and in the visceral yolk sac that changed into a mostly cuboid epithelium with reduced connective tissue. Data suggest that substance transport is significantly reduced then. In contrast, the chorioallantoic placenta reaches a maximum size to be fully functional; degeneration of tissues mainly affected areas responsible for invasion processes [5,6,18,24,25,32,40]. In conclusion, the data suggest that placentation in the guinea pig and other caviomorphs relies predominantly on hemotrophic nutrition via the placenta from very early pregnancy onward. In contrast to the general pattern of mammals, histiotrophic nutrition via the yolk sac routes seems to be not much involved for early embryonic development, but is a later feature essential during mid-gestation that is relevant for fetal purposes.

### Acknowledgments

We thank FAPESP (process number 2011/10714-5) and CAPES for funding as well as Anthony Carter for constructive comments on the article and help with the English. We want to address our gratitude for technical support to several members of the University of Sao Paulo.

### Competing interests

The authors declare that they have no competing interests.

Contributors: AMM and MAM devised the study and participated in its design. BGV analyzed the material and

wrote the article, supported by POF and AMM. All authors read and approved the final article.

### References

- [1] Verkesté CM, Slangen BF, Daemen M, Van Straaten H, Kohnen G, Kaufmann P, et al. The extent of trophoblast invasion in the preplacental vasculature of the guinea-pig. *Placenta* 1998;19:49–54.
- [2] Kaufmann P. Guinea pig *Cavia porcellus*. In: Benirschke K, editor. Comparative placentation. <http://placentation.ucsd.edu/guinea.htm>. 2004.
- [3] Carter AM, Enders AC, Jones CJ, Mess A, Pfarrer C, Pijnenborg R, et al. Comparative placentation and animal models: patterns of trophoblast invasion: a workshop report. *Placenta* 2006;27A:S30–3.
- [4] Carter AM. Animal models of human placentation. *Placenta* 2007;28A:S41–7.
- [5] Mess A. The subplacenta in *Octodon degus* and *Petromus typicus*: two hystricognath rodents without significant placental lobulation. *J Exp Zool B Mol Dev Evol* 2007a;308B:172–88.
- [6] Mess A, Zaki N, Kadyrov M, Korr H, Kaufmann P. Caviomorph placentation as a model for trophoblast invasion. *Placenta* 2007;28:1234–8.
- [7] Mess AM, Favaron PO, Vasconcelos BG, Oliveira MF, Miglino MA. Review: placentation in caviomorph rodents and their significance as animal models. In: Cabral RH, editor. XXII International Symposium on Morphological Sciences. Bologna: Medimond International Proceedings; 2012. p. 121–4.
- [8] Mossman HW. Vertebrate fetal membranes. New Brunswick: Rutgers University Press; 1987.
- [9] Steven DH. Comparative placentation. New York: Academic Press; 1975.
- [10] Ferner K, Mess A. Evolution and development of fetal membranes and placentation in amniote vertebrates. *Respir Physiol Neurobiol* 2011;178:39–50.
- [11] Fukuda T. Fetal hemopoiesis. I. Electron microscopic studies on human yolk sac hemopoiesis. *Virchows Archiv B Cell Pathology* 1973;14:197–213.
- [12] Nogales FF, Beltran E, Gonzalez F. Morphological changes of the secondary human yolk sac in early pregnancy wastage. In: Nogales FF, editor. The human yolk sac and yolk sac tumors. Heidelberg: Springer-Verlag Berlin; 1993. p. 174–94.
- [13] McGeedy TA, Quinn PJ, Fitzpatrick ES, Ryan MT. Veterinary embryology. London: Blackwell Publishing Ltd; 2006. p. 79–90.
- [14] Wooding P, Burton G. Comparative placentation: structures, functions and evolution. Heidelberg: Springer-Verlag Berlin; 2008. p. 1–13.
- [15] Mess A, Carter AM. Evolutionary transformations of fetal membrane characters in Eutheria with special reference to Afrotheria. *J Exp Zool B Mol Dev Evol* 2006;306B:140–63.
- [16] Dempsey EW. Electron microscopy of the visceral yolk-sac epithelium of the guinea pig. *Am J Anat* 1953;93:331–63.
- [17] King BF, Enders AC. The fine structure of the guinea pig visceral yolk sac placenta. *Am J Anat* 1970a;127:394–414.
- [18] Kaufmann P, Davidoff M. The guinea-pig placenta. *Adv Anat Embryol Cell Biol* 1977;53:5–91.
- [19] King BF. Comparative anatomy of the placental barrier. *Bibl Anat (Karger, Basel)* 1982;22:13–28.
- [20] Leissring JC, Anderson JW. The transfer of serum proteins from mother to young in the guinea pig. I. Prenatal rates and routes. *Am J Anat* 1961;109:149–55.
- [21] King BF, Enders AC. Protein absorption and transport by the guinea pig visceral yolk sac placenta. *Am J Anat* 1970b;129:261–88.
- [22] King BF. The permeability of the guinea pig parietal yolk sac placenta to peroxidase and ferritin. *Am J Anat* 1972;134:365–76.
- [23] Faber JJ, Thornburg KL. Fetal homeostasis in relation to placental water exchange. *Ann Rech Vét* 1977;8:353–61.
- [24] Davies J, Dempsey EW, Amoroso EC. The subplacenta of the guinea-pig: development, histology and histochemistry. *J Anat* 1961a;95:457–73.
- [25] Davies J, Dempsey EW, Amoroso EC. The subplacenta of the guinea-pig: an electron microscopy study. *J Anat* 1961b;95:311–24.
- [26] Oliveira MF, Favaron PO, Ambrósio CE, Miglino MA, Mess AM. Chorioallantoic and yolk sac placentation in *Thrichomys laurentinus* (Echimyidae) and the evolution of hystricognath rodents. *J Exp Zool B Mol Dev Evol* 2012a;318:13–25.
- [27] Oliveira MF, Vale AM, Favaron PO, Vasconcelos BG, Oliveira GB, Miglino MA, et al. Development of yolk sac inversion in *Galea spixii*

- and *Cavia porcellus* (Rodentia, Caviidae). *Placenta* 2012b;33: 878–81.
- [28] Wolf E, Arnold GJ, Bauersachs S, Beier HM, Blum H, Einspanier R, et al. Embryo-maternal communication in bovine: strategies for deciphering a complex cross-talk. *Reprod Domest Anim* 2003;38: 276–89.
- [29] Enders AC. Reasons for diversity of placental structure. *Placenta* 2009;30A:S15–8.
- [30] Duval M. Le placenta des rongeurs. IV. Le placenta Du cochon d' Inde. *J Anat Physiol* 1892;28:58–408.
- [31] Leiser R, Kaufmann P. Placental structure: in a comparative aspect. *Exp Clin Endocrinol* 1994;102:122–34.
- [32] Carter AM, Tanswell B, Thompson K, Han VK. Immunohistochemical identification of epithelial and mesenchymal cell types in the chorioallantoic and yolk sac placentae of the guinea-pig. *Placenta* 1998;19:489–500.
- [33] Perrotta CA. Fetal membranes of the Canadian porcupine, *Erethizon dorsatum*. *Am J Anat* 1959;104:35–59.
- [34] Bonatelli M, Carter AM, Machado MR, Oliveira MF, Lima MC, Miglino MA. Placentation in the paca (*Agouti paca* L). *Reprod Biol Endocrinol* 2005;3:1–12.
- [35] Oliveira MF, Carter AM, Bonatelli M, Ambrosio CE, Miglino MA. Placentation in the Rock Cavy, *Kerodon rupestris* (Wied). *Placenta* 2006;27:87–97.
- [36] Mess A. Development of the chorioallantoic placenta in *Octodon degus*: a model for growth processes in caviomorph rodents? *J Exp Zool B Mol Dev Evol* 2007b;308B:371–83.
- [37] Miglino MA, Francioli AL, Oliveira MF, Ambrósio CE, Bonatelli M, Machado MR, et al. Development of the inverted visceral yolk sac in three species of caviids (Rodentia, Caviomorpha, Caviidae). *Placenta* 2008;29:748–52.
- [38] Kanashiro C, Santos TC, Miglino MA, Mess AM, Carter AM. Growth and development of the placenta in the capybara (*Hydrochaeris hydrochaeris*). *Reprod Biol Endocrinol* 2009;7: 1–13.
- [39] Flamini MA, Portiansky EL, Favaron PO, Martins DS, Ambrósio CE, Mess AM, et al. Chorioallantoic and yolk sac placentation in the plains Viscacha (*Lagostomus maximus*): a caviomorph rodent with natural polyovulation. *Placenta* 2011;32:963–8.
- [40] Bjellin L, Sjöquist PO, Carter AM. Uterine, maternal placental and ovarian blood flow throughout pregnancy in the guinea pig. *Z Geburtshilfe Perinatol* 1975;179:179–87.