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Sonographic evaluation of fetal cerebral structures correlated with histological aspects

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Abstract

Prenatal development of the human brain from undifferentiated neuroepithelium, crosses numerous steps towards primordial organization and subsequent cytoarchitectural layering, ascending and progressive from the lower cortical layers to the superior ones. Our study represents a systematic, comparative assessment of imaging studies and the histological evaluation of the prenatal development of the human brain. We evaluated 232 cases using 3D ultrasound. Histological study was performed on 17 cases aged between 8 and 32 weeks pregnancy and compared with imaging results. For the ultrasound study, we chose five anatomical landmarks: the choroid plexus, thalamus, cerebellum, hippocampus and island (Sylvian fissure). The histological study was performed on dissected brain specimens preserved in formaldehyde and was followed by immunohistochemical determination in order to complete the picture of the morphological evolution of the structures evaluated. We analyzed the accuracy of the description of marker elements (choroid plexus, thalamus, cerebellum, hippocampus and Sylvian fissure) in three-dimensional ultrasound evaluation. This showed a good correlation with the morphological evaluation as well as with the dimensional descriptions from the literature. Histological and immunohistochemical assessment helped complete the picture of the central nervous system development. Highlighting fetal cerebral structures by three-dimensional ultrasound, together with morphological examination helped us create a dynamic array of the central nervous system development.

Keywords: human brain, cerebral structures, sonographic evaluation, choroid plexus, immunohistochemistry.

Introduction

The development of the human brain represents a prolonged process that begins in the third week of pregnancy, with neural progenitor cell differentiation and extends at least until late adolescence [1]. Over the past decades, our understanding of the basic principles of human brain development has progressed, changing the fundamental models of brain development. Neuroimaging methods for investigating human brain can be used together with histology studies to answer specific scientific questions on human brain development and neurological disorders in pediatric pathology, caused by environmental or genetic factors [2]. The organogenesis of the central nervous system begins early in the intrauterine life, and stretches along until after birth, thus being subjected to a number of environmental factors [3]. Its origin lies in the neural tube and passes through a series of processes of differentiation. The data in the scientific literature on the complex process of brain development, both in terms of anatomical structures and cellular component is scarce [4]. The morphological changes of the brain structures are identified with high accuracy by using histological methods. Data obtained by this invasive method are effectively completed by the ones obtained by noninvasive methods. Ultrasonography represents a simple noninvasive method, which although not as exact as histological studies, is of great importance in routine practice (prenatal assessment) as well as starting point in neuroscientific studies. In our study, we examined the possibilities of assessment of fetal brain structures of different ages by three-dimensional ultrasound pregnancy and the anatomical and histological changes that occur at that age pregnancy, in miscarriage of independent causes (cervical incompetence, spontaneous rupture of membranes, etc.). The structures investigated in our study were chosen either because of their importance or because of their high degree of difficulty in neurosonographic examinations.

Materials and Methods

Sonographic techniques

We analyzed, using 3D ultrasound, 232 cases of fetuses with a gestational age between 8 and 32 weeks in patients that presented for sonographic screening or were admitted for various conditions (threatened abortion, bleeding, hypertension in pregnancy) in the Department of Obstetrics and Gynecology, Emergency County Hospital, Craiova, Romania.

The ultrasound examinations were conducted on Voluson 730 PRO, Voluson 730 Expert, Voluson E8 (GE Healthcare, Kretz Ultrasound, Zipf, Austria) for acquisition of volumes transabdominal (using RAB 4–8 probe), as well for acquisition of volumes transvaginal

(using a RIC 5–9 probe). Volumes were obtained in routine examinations that were part of a larger project.

For a good quality of the volumes, acquisitions were made in a state of fetal quiescence. The angle of acquisition was adapted to the gestational age and varied from 30^0 to 60^0, in order to include the whole head.

Volumes were analyzed "on the spot" (at the moment of the examination) and also later on the PC using the 4D view software (GE Healthcare, Kretz Ultrasound) for analyzing volumes – with the purpose of analyzing that the sections were right and reproducible.

We chose the following techniques and software to use in our volume manipulation:

▪ tomographic ultrasound imaging (TUI) – is a new method to display information from a 3D volume, allowing to display more 2D sections simultaneously, mimicking CT scans;

▪ volume contrast imaging (VCI) – this method allows obtaining a slice from a volume with a thickness defined by user;

▪ off-line analysis of the images (using 4D view software) allows the same manipulation on a PC as on the ultrasound machine used in the examination.

In obtaining all structures, we started from the axial transthalamic plane.

The choroid plexus were obtained on standard transventricular plane. We used the TUI software with sections on 1 mm or 2 mm distance depending on the gestational age to evaluate their integrity – visualization of iregularities in the contour could suggest intraventricular hemorrhage, modification of size reports could suggest hydrocephaly; also, choroid plexus can develop cysts or tumors – usually papillomas.

The thalamus was shown using the TUI software that allows serial sections. To obtain a better contrast, from the surrounding areas of similar echogenicity, we used VCI.

Evaluation of the cerebellum was done on two planes – one axial, the transcerebellar plane for visualizing the cerebellar hemispheres and one median designed to show the integrity of the vermis. Starting from the transthalamic plane in plane A, we placed the marker at the level of the cavum septum pelucidum – with a slight rotation in the Y-axis we obtained the transcerebelar plane. In plane C, we obtained the median section of the vermis.

For the evaluation of the Sylvian fissure, we used two views: (a) the multiplanar mode – starting from plane A axial, transventricular, putting the marker at the level of the Sylvian fissure we will have in plane B a coronal section of the Sylvian fissure; VCI can be used to enhance the contrast; (b) TUI – allows serial sections of the Sylvian fissure.

For the evaluation of the hippocampusus, a rather difficult task, we chose two independent methods. The first described by Kier *et al.* [5] for examination during MRI (magnetic resonance imaging) scans that implies parallel coronal sections. The second, described by Gindes *et al.* [6], uses ultrasound 3D volumes and obtains the hippocampus together with the fornix.

Histopathological study

The histopathological study was conducted on a total of 17 brains obtained from aborted fetuses. The histological evaluation involved the following macroscopic and histological techniques: initially, a complete autopsy of the 17 fetuses was performed, along with examination of the placenta and umbilical cord. Brains were harvested and were initially completely sunk in 10% formalin until fixated and then parallel cross-sections were performed. For morphological analysis, the tissue fragments collected during autopsy were paraffin embedded, sectioned at 3–5-μm and were stained using standard stains – Hematoxylin–Eosin (HE), trichromic Goldner–Szekely (GS) –, which allowed the assessment of morphological features.

In addition to the standard staining, special stains have been used for some selected cases. We investigated the immunoexpression of CD34, GFAP, NeuN and Ki-67 in the study group of 17 subjects aged between 8 and 31 weeks of gestation. Immunohistochemical reactions were performed on 4-μm sections obtained from paraffin blocks. The detection and visualization system used was EnVision, Dako, Glostrup, Denmark, a two-staged method, based on conjugating a polymer with a secondary antibody.

The panel of antibodies used in our study is shown in Table 1.

Table 1 – ***Antibodies used in the immunohistochemical study***

Antibody	Clone	Dilution	Producer
Anti-CD34 (ab81289)	EP373Y	1:100	Abcam
Anti-GFAP (M0761)	6F2	1:2000	Dako
Anti-NeuN (ab128886)	polyclonal	1:1000	Abcam
Anti-Ki-67 (IgG1k)	MIB-1	1:100	Dako

The results were visualized by brown staining using the 3,3'-diaminobenzidine (DAB) chromogen. The reaction appeared positive in the membrane, the cytoplasm or nucleus, depending on the type of antibody.

Results

Sonographic evaluation

Standard exams were performed transabdominally. For a greater accuracy, transvaginal examinations were performed in cases were the fetus was in a vertex position and local conditions allowed it. All cases included in the study (with one exception) had a normal anatomy of the fetal nervous system demonstrated by a set of parameters accepted by current guides in the field (Table 2).

The gestational age of cases included in the study was between 8 and 32 weeks and was divided in four groups following the pattern designed in our larger project:

▪ first trimester (<13+6 weeks): 55 ultrasound evaluations – one case examined by pathology;

▪ early second trimester (15–19 weeks): 57 ultrasound evaluations – nine cases examined by pathology;

▪ second trimester (20–28 weeks): 83 ultrasound evaluations – six cases examined by pathology;

▪ third trimester (28–32 weeks): 37 ultrasound evaluations – one case examined by pathology; the case examined from the third trimester cohort was a case of pathology of the central nervous system, which was included in the study for its particular interest.

Table 2 – ***Standard elements/structures followed in routine anomaly scans of the central nervous system***

Gestational age	Standard elements/structures evaluated
<13+6 weeks	Symmetry of the cerebral structures Falx cerebri Choroid plexus
15–19 weeks	Symmetry of the cerebral structures Falx cerebri Thalamus Posterior horn of the lateral ventricles Cavum septum pelucidum Choroid plexus Cerebellar hemispheres Vermis Cisterna magna
20–32 weeks	Symmetry of the cerebral structures Falx cerebri Thalamus Posterior horn of the lateral ventricles Cavum septum pelucidum Cerebellar hemispheres Vermis Cisterna magna

The choroid plexus is an element accessible for examination at any gestational age, being easily obtained with 2D ultrasound as with 3D ultrasound (Figure 1). The only advantage noticed in our study in favor of 3D sonography appears in the possibility of a more precise localization of choroid plexus cysts with TUI and MPR (Figure 2).

The thalamus was investigated with 2D and 3D as well (Figure 3). We made evaluations with standard 3D techniques as well as with image improvement software – no significant differences were noted in the examination of normal structures.

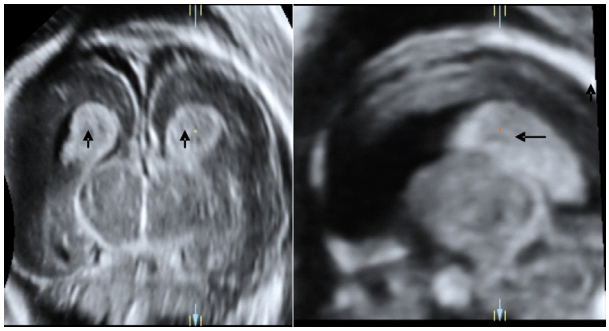

Figure 1 – ***Choroid plexus (arrow): normal appearance. 3D ultrasound – multiplanar imaging.***

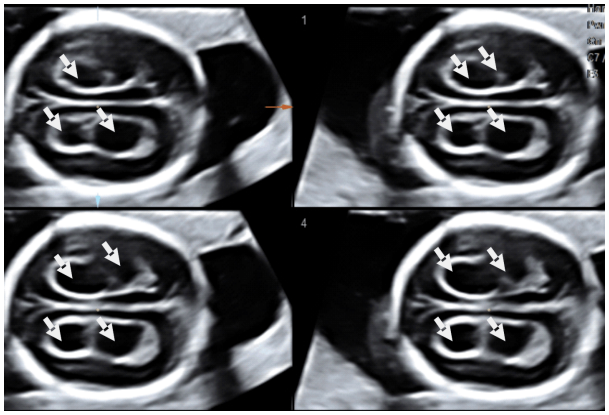

Figure 2 – ***Choroid plexus cysts (arrow). Tomographic Ultrasound Imaging (TUI).***

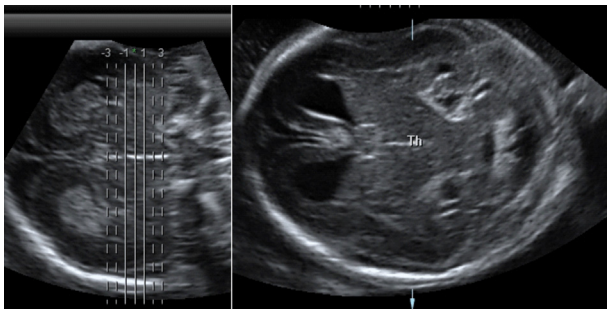

Figure 3 – ***Localizing the talamus (Th) on 3D ultrasound, TUI.***

The cerebellum can be seen, with difficulty, from 8 weeks – it was not visible in our eight weeks case. In cases of cohorts two and three, the cerebellum was accessible with visualization of the hemispheres and reconstruction of the vermis in multiplanar mode (Figure 4). We used in all cases above 19 weeks the VCI software for improving contrast. We recorded no difficulties in visualizing the primary fissure in any case above 19 weeks gestational age. We also noted changes in the echogenicity of the cerebellum. The echogenicity appears to change from hypoechoic to slightly echoic with marginal enhancement and to homogeneously hyperechoic near term. These changes could be mirroring the histological development of the fetal cerebellum during pregnancy.

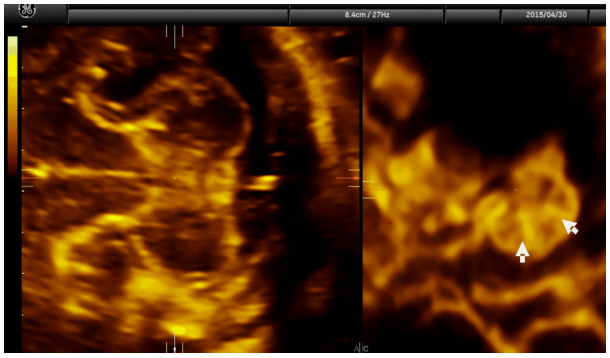

Figure 4 – ***3D evaluation of the cerebellar vermis, multi-planar mode: primary and secondary fissures can be noticed (arrow).***

The Sylvian fissure is the sonographic marker of the development of circumvolutions and neuronal migration. Its evaluation was possible in 176 cases (Figure 5). For a greater accuracy of the examination we measured in all the cases it was available the angle formed by the Sylvian fissure, this evolving from a simple indentation at 15 weeks, going through an obtuse angle stage, so that after 24 weeks it forms an acute angle.

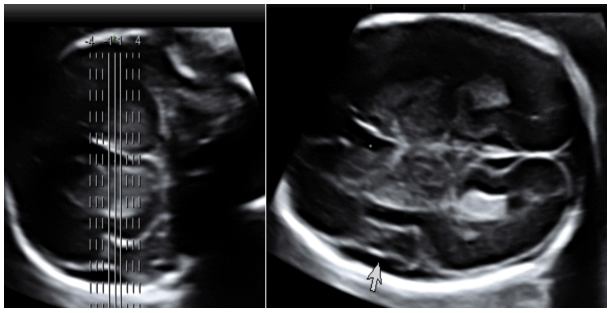

Figure 5 – ***The Sylvian fissure (arrow). 3D ultrasound – multiplanar mode.***

The evaluation of the hippocampus was the most demanding in terms of technical abilities. The fetal hippocampus begins to get an aspect similar to the adult one at around 19–20 weeks. The dentate girus and the horn of Ammon start to infold inside the temporal lobe. The hippocampus and the subiculum come closer being separated only by a narrow hippocampal sulcus. The visualization of the hippocampus after 19 weeks can be obtained by both techniques described – Kier & Gindes. The evaluation at 15 weeks was difficult to achieve, obtaining images with low quality due to the extremely delicate structures of the hippocampus at this age and the limitations of using post procession image improvements like thick slice. Images with good resolution were available from 21 to 28 weeks (Figure 6), in the third trimester the resolution decreasing for the transabdominal approach, the only good images were obtained with transvaginal probes, if the fetus had a vertex position.

A special case was included in cohort 4 – a fetus with acrania – anencephaly in which we showed the absence of the cranial vault, area cerebrovaculosa (the stroma that covers the cranial defect), protrusion of the eyes and excess amniotic liquid (Figure 7). The fetus initially presented probably only acrania, without total destruction of the supratentorial structures, hence the pathologist found structures of the choroid plexus with multiple cysts.

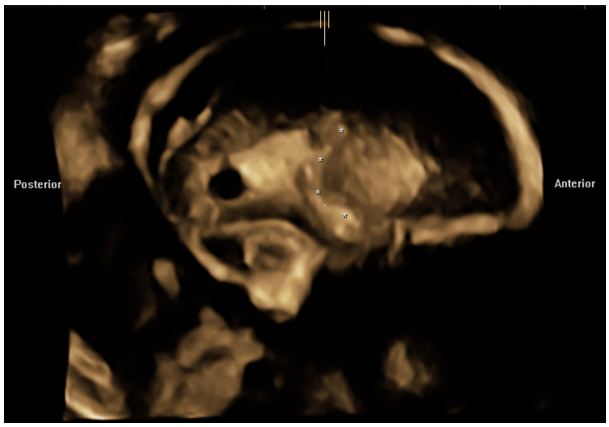

Figure 6 – ***3D evaluation of the hippocampus using the Gindes technique (hippocampus and fornix*).***

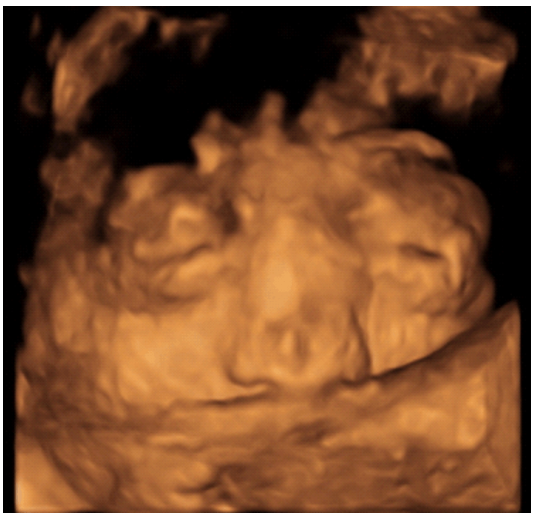

Figure 7 – ***Fetus with acrania. 3D rendering – surface mode.***

Histological aspects

The brain tissue showed different aspects depending on the pregnancy age. In the first trimester, after neural tube closure, neuroblasts begin to differentiate into neuroepithelial cells forming the periventricular germinal cell layer. Then, neurons migrate below piamater, where they will form the second layer of cells with the neurons located at this level, from which, starting with week 15, the grey matter will form. In our study, we had one case at the age of eight weeks, five cases of 15–17 weeks, four cases of 18–19 weeks, six cases between 20 and 24 weeks and one case of 31 weeks.

The histological evaluation of the 8 weeks embryo in our study helped to identify the onset of cyto-architectural development and organization of the brain. At this stage, we identified reduced morphological data, an immature brain with dilated lateral and 3rd ventricle. On serial histological sections, we have identified the hippocampus and dilated choroid plexus. All these changes had as macroscopic correspondents the presence of enlarged cephalic extremity, fact seen also on US examination.

The five fetuses aged 15–17 weeks showed large frontal and temporal poles separated by the wide central longitudinal fissure. On serial HE-stained sections, we identified the cells layers, with the presence of immature pyramidal cells, belonging to grey matter, which had a columnar pattern, alternating with acellular areas; no other cell type could be identified at this gestational age. By comparing sections of the distal areas to the proximal ones, we identified an increased number of pyramidal cells in the first one.

The fetuses with gestational age between 20 and 24 weeks, had an increased number of pyramidal cells, exhibiting longer dendrites, thus, by comparing with the sections from embryos of smaller gestational ages, we identified an expansion of grey matter represented by thickening of the cell layer.

In evaluating the histological features of the choroid plexuses we used the Netsky–Shuangshoti classification based on the aspect of the epithelium and stroma: stage I occurs between 7–9 weeks of gestation, stage II between 9–16 weeks, stage III between 17–29 weeks, and stage IV at more than 29 weeks. Most cases were included in stage II and III, and only one case belongs to stage IV, also being the only case with pathological changes (presence of choroid plexus cysts). One case showed tall pseudostratified neuroepithelium, with centrally, elongated nuclei, and loose stroma and belonged to stage I.

From the five cases, belonging to stage II (Figures 8 and 9), we identified the presence of choroidal epithelium, predominantly cylindrical, with basal nuclei and clear cytoplasm, due to the presence of glycogen, focally a pseudostratified appearance, especially in the interlobular clefts. Loose stroma showed a decreased number of connective fibers with a perivascular disposition, and congestive blood vessels.

From the 10 cases belonging to stage III, we identified the presence of a low glycogen content epithelium, with apical and focally central located nuclei, and epithelial cell clusters that have crossed the basal membrane. The

stroma had a greater amount of connective tissue and blood vessels (Figure 10).

One single case showed the presence of cystic structures lined by cubic epithelium, with focal cytoplasmic vacuoles, which supports the choroidal origin of the lesion and subepithelial collagen deposition (Figure 11). In this case, we identified the presence of an associated anatomical abnormality – anencephaly.

Identification of primary fissures was possible starting with the week 8 of gestation. Initially, we identified the primary longitudinal fissure, which separated the two cerebral hemispheres. Other primary fissures, such as the Sylvian or parieto-occipital fissure, were identified in three of the five fetuses of 15 weeks and in all the other fetuses with higher gestational ages.

To highlight glial cells in histological sections, we used GFAP, a cytoplasmic immunohistochemical marker for astrocytes, which showed positivity in 15 fetuses aged between 15 and 24 weeks (Figure 12). We have not identified GFAP reactivity at the level of the choroid plexus. Ki-67 is a nuclear marker, which indicates the cellular cycle and was identified in neuroepithelial cells associated with the phases G1, G2, G3 of the cell cycle. It was weakly positive in rare cells in the 8 weeks embryo (Figure 13), strongly positive in 15 cases aged 15–24 weeks and negative in one case.

The neuronal nuclear antigen (NeuN) is a specific protein for postmitotic neurons. In our study, we identified focal cytoplasmic and nuclear positivity in mature neurons (Figure 14).

The pial anastomotic capillary system showed nucleated red blood cells and was covering the entire cerebral cortex, which was avascular in early development stages. These changes have been visible since the 8th week of gestation and were highlighted using CD34. Starting with the histological sections of the 15 weeks embryos, we have identified the presence of anastomotic capillary plexus of the cerebral cortex consisting of perforating vessels that were crossing the grey matter to unite in the primordial pyramidal cell layer. Vascular marker CD34 was strongly positive in the vessels of the choroid plexus especially in stages II and IV, within piamater and focally positive in brain tissue, with vascular density variations (vessels number/mm^2), depending on the gestational age – with increasing gestational age, the number of blood vessels identified also increased (Figure 15).

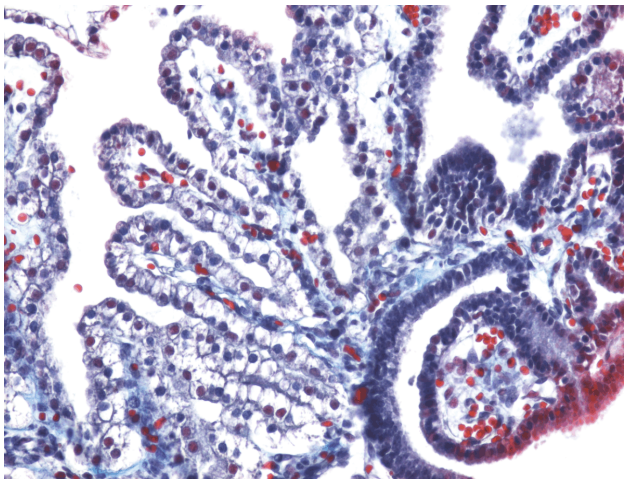

Figure 8 – ***Choroid plexus: cylindrical epithelium with clear cytoplasm basal and apical nuclei (Trichromic GS staining, ×200).***

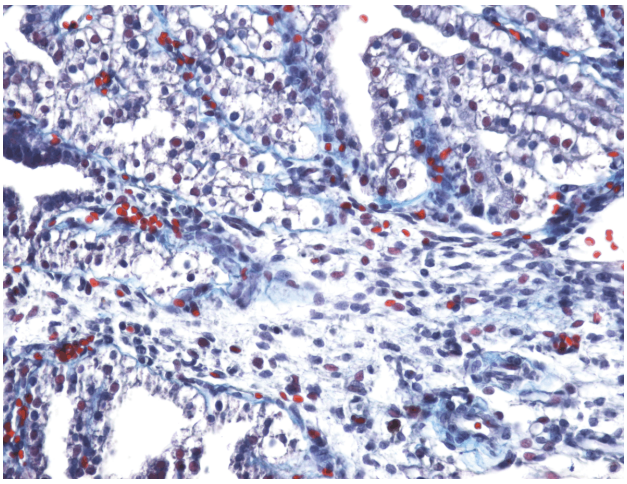

Figure 9 – ***Choroid plexus stage II with loose stroma (Trichromic GS staining, ×200).***

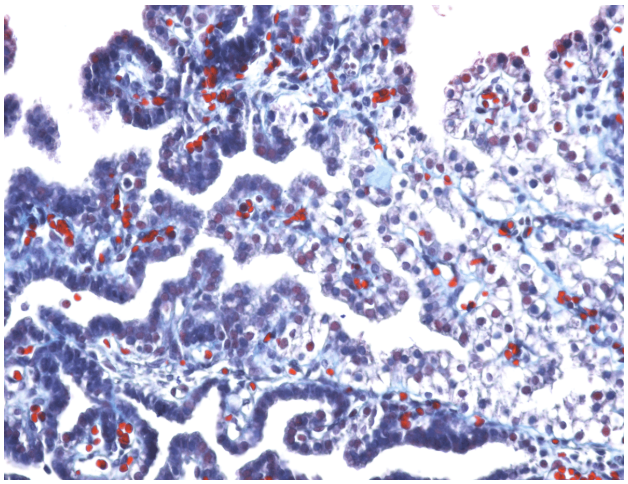

Figure 10 – ***Choroid plexus stage III with marked lobulation with the presence of primary villi (Trichromic GS staining, ×200).***

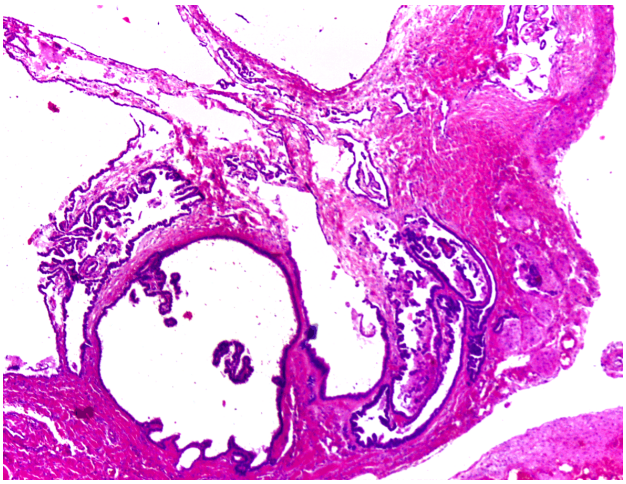

Figure 11 – ***Anencephaly: cystic structures lined by cubic epithelium (HE staining, ×40).***

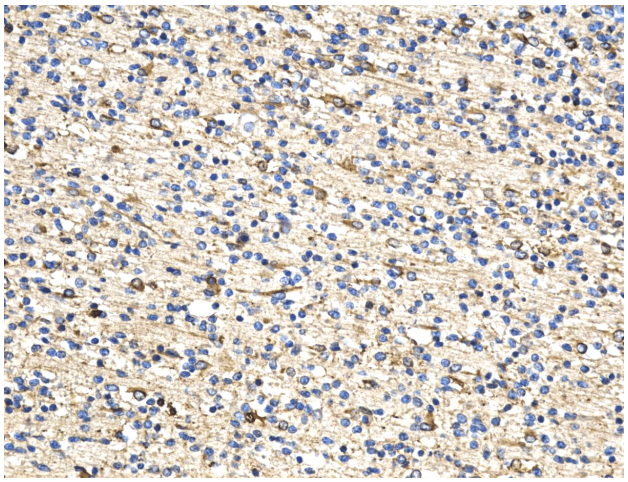

Figure 12 – ***Brain parenchyma: positivity for GFAP (IHC staining, ×100).***

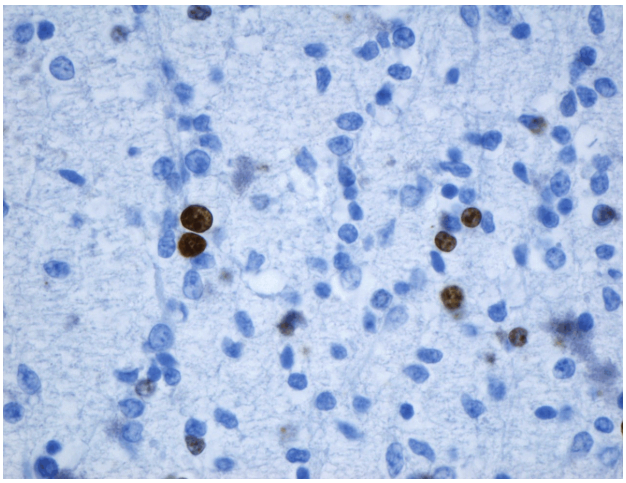

Figure 13 – ***Brain parenchyma: positivity for Ki-67 (IHC staining, ×400).***

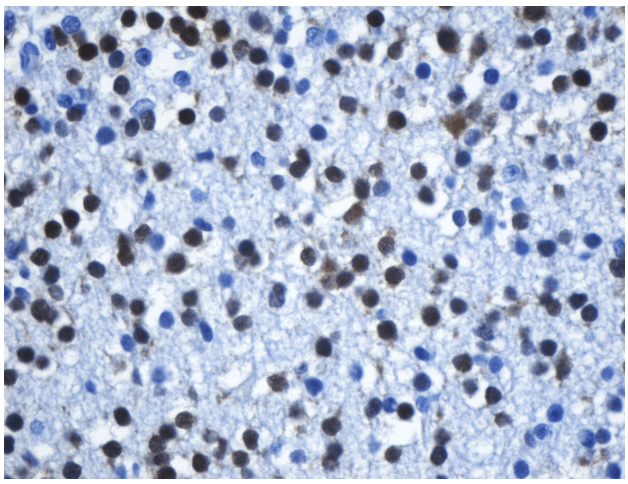

Figure 14 – ***Brain parenchyma: positivity for anti-NeuN (IHC staining, ×200).***

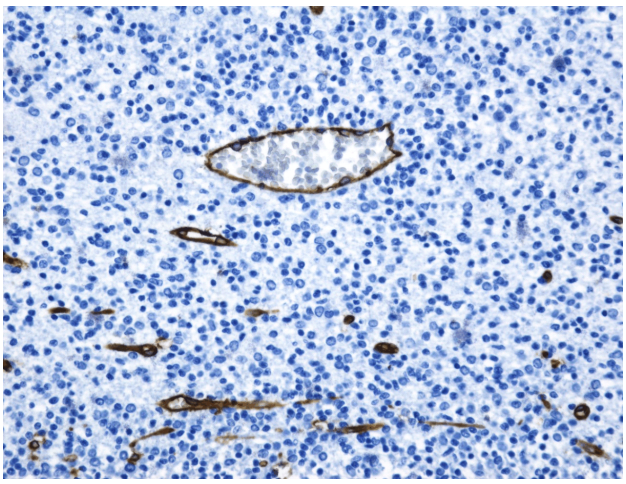

Figure 15 – ***Brain parenchyma: positivity for CD34 (IHC staining, ×100).***

Discussion

Brain development is a complex process influenced by intrinsic and extrinsic factors. Intrinsic factors are represented by the genetic code that shapes the development of neural circuits and extrinsic factors are represented by influences that start during intrauterine period and continue throughout life. The development begins with the formation of the neural tube followed by neuronal migration and differentiation process of synapse formation [8].

In the early stages of brain development, because of the action of a molecular signaling system in which a key element is the protein DPP/Bmp, the neuroectodermal tissue appears and will subsequently develops into the central nervous system (CNS) [9]. Ultrasonography is now a routine method in evaluating *in utero* fetal development, both due to increased sensitivity and to accessibility and low price, as compared with other imaging methods. In the case of the central nervous system, the evaluation in orthogonal planes with 3D sonography allows the analysis of anatomy by navigating in three planes (coronal, sagital and axial), which is impossible with 2D ultrasound [10, 11]. Many techniques of using 3D ultrasound in the investigation of the central nervous system have been described [12–14], but none were incorporated in routine examination.

The choroid plexus, the thalamus and the cerebellum represent standard elements in the second examination and can be easily viewed on 2D axial images – still a complete evaluation may need 3D ultrasound. The hippocampus and the Sylvian fissure are elements of advanced neurosonography, are not part of routine examination and may need 2D or 3D ultrasound and experience in examining the fetal brain. The central element is the ability to recognize even minor deviations from normal, because at the cerebral level can have a major impact on the fetus.

This imaging examination complemented by morphological study gives us important data about the temporal evolution of the brain tissue. However, histological study of the development of the human brain requires direct access to undamaged, postmortem harvested brain tissue and ethical considerations prevent experimental studies in this area, thus the data in the literature are more of descriptive than experimental nature. In our study, we used fragments of formalin-fixed brain tissue that give us information about the cytoarchitectural organization.

Cytoarchitectural organization of the human cerebral cortex begins in week 7 of development. The brain is quite immature, tertiary and lateral ventricles are enlarged, the cortical mantle is very thin and rudiments of the hippocampus and piriform lobe can be identified [15]. These changes were highlighted in our study in the eight

weeks embryo. Histological changes identified – expansion of the cerebral cortex and ventricle as well as of the hippocampus and choroid plexus – were translated into ultrasound appearance of enlarged head with frontal prominence.

The choroid plexus, part of the ventricular system, derive from the pia mater (leptomeninge), lay between two barrier systems (blood and cerebrospinal fluid) and have as a primary goal the production and regulation of the cerebrospinal fluid [16, 17]. From an embryological point of view, the choroid plexus develop in the medial and superior part of the initially anterior ventricles (future lateral ventricles). Though present from 6–7 weeks (menstrual age), they are not large and echogenic enough to be seen with ultrasound. In our study, they were identified in the 8 weeks embryo only by histological study. At 9 weeks, the plexus can be seen at the level of both lateral ventricles, with transvaginal ultrasound. Between 9 and 11 weeks, the choroid plexus cover most of the surface of the standard transventricular section. As the pregnancy progresses, the ratio between the choroid plexus and the ventricles decreases. As the ventricles develop, the choroid plexus occupy the atrium, hugging the thalamus [18]. A stable intraventricular environment is very important in maintaining normal neuronal function and play a significative role in the transport of electrolytes (Na^+, K^+, Cl^- and HCO_3^-) through the choroid plexus epithelium [19].

The immunohistochemical study of choroid plexus highlighted the presence of vascular structures by CD34 positivity and the absence of GFAP staining, according to the data in the literature [20]. GFAP is useful in the differential diagnosis between normal structures and choroid plexus tumors [21].

The thalamus is a cerebral structure, part of the diencephalon, with a multitude of motor, sensitive and neuropsychiatric functions. It can be seen by ultrasound starting at 10–11 weeks. In the second trimester, scan it is the landmark for the biometry of the fetal head. Anomalies of the thalamus detected in the first and second trimester is usually associated with severe cerebral anomaly. The thalamus was evaluated with 2D and 3D ultrasound – being an extremely accessible structure no improvement was noticed in the use of 3D. The evaluation of the cerebellum has the purpose of excluding pathology with unfavorable prognosis like Dandy–Walker syndrome, but also anomalies of vermis size (hypoplastic vermis) or rotation (Blake's pouch cyst). The elements that we followed in our study allowed the evaluation of both the cerebellar hemispheres and vermis with the purpose of performing as a screening tool for major anomalies of the posterior fosa. Also, noticed was the steady change in US appearance of the fetal cerebellum with advancing gestation, similar with the findings of Hashimoto *et al.* [22]. Evaluating the process of formation of the Sylvian fissure may help in appreciating the normal development of the cortex and lead to an earlier diagnosis of pathological alteration like lisencephaly. The visualization of the Sylvian fissure was described as early as 15–16 weeks pregnancy by high-resolution 2D ultrasound. It was also described at 15 weeks by nuclear magnetic resonance. Sylvian fissure changes shape with gestational age based on an ultrasound calendar: (1) slight indentation at 15–17 weeks; (2) at 17–20 weeks a sulcus fitting in an obtuse angle; (3) as the fissures deepens the angle changes from obtuse to acute at around 24 weeks. These elements were confirmed in our study – our angle measurements correlated with the observations made by two-dimensional ultrasound and MRI studies. Abnormalities in hippocampal formation has been described in a number of important pathological situations such as agenesis of corpus callosum, lissencephaly, holoprosencephaly, temporal lobe epilepsy, autism, Alzheimer's disease and generated an increasing interest increased its evaluation, beginning with its formation. One of the first studies regarding the fetal hippocampus, published by Kier *et al.* [5] in 1997, using MRI as a diagnostic tool, created a chronology of hippocampus development from 13 to 24 weeks. We performed the same task with ultrasound and achieved similar results – 3D ultrasound helped in obtaining an image of the hippocampus, difficult to assess with 2D ultrasound.

The absence of immunoreaction for NeuN in the cases studied translates not by loss of neurons but by their low viability, due to pathological conditions such as hypoxia or cerebral ischemia [23]. Evaluating vascular density using CD34 revealed the presence of a larger number of vessels in the cerebellum and thalamus, especially in sections from younger gestational ages, except for the choroid plexus where the number of vessels increased proportionally with advancing pregnancy age, until finally the gray mater becomes the most vascularized region of the human body [24]. The identification of capillary meningeal structures represents an important step in the assessment of the fetal brain due to their role in the development and structural organization of the cerebral cortex [25].

Conclusions

Prenatal brain development from the earliest phases is characterized by specific changes that may be identified with ultrasound but also by postmortem morphological techniques. Knowledge of the correlations between histological and ultrasound features is useful in appreciating the normal development and identifying abnormalities of brain structures. Our study on fetal brain development covered different levels of neural organization from the ultrasonographic appearance to the cellular one, highlighting a series of different, changing processes and thus providing a new platform for investigating the structures capable of influencing neural development.

Conflict of interests

The authors declare that they have no conflict of interests.

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