



Vulnerability of fourth ventricle choroid plexus in sudden unexplained fetal and infant death syndromes related to smoking mothers

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ABSTRACT

The human choroid plexuses in the ventricular system represent the main source of cerebrospinal fluid secretion and constitute a major barrier interface that controls the brain's environment. The present study focused on the choroid plexus of the fourth ventricle, the main cavity of the brainstem containing important nuclei and/or structures mediating autonomic vital functions.

In serial sections of 84 brainstems of subjects aged from 17 gestational weeks to 8 postnatal months of life, the deaths due to both known and unknown causes, we examined the cytoarchitecture and the developmental steps of the fourth ventricle choroid plexus to determine whether this structure shows morphological and/or functional alterations in unexplained perinatal deaths (Sudden Infant Death Syndrome and Sudden Intrauterine Unexplained Death Syndrome).

High incidence of histological and immunohistochemical alterations (prevalence of epithelial dark cells, the presence of cystic cells in the stroma, decreased number of blood capillaries, hyperexpression of Substance P and apoptosis) were prevalently observed in unexplained death victims ($p < 0.05$ vs. controls). A significant correlation was found between maternal smoking in pregnancy and choroidal neuropathological parameters ($p < 0.01$).

This work underscores the negative effects of prenatal exposure to nicotine on the development of the autonomic nervous system, and in particular of the fourth ventricle choroid plexus that is a very vulnerable structure in the developing CSF–brain system.

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1. Introduction

Properly-developing choroid plexus (CP), ventricular cerebrospinal fluid (CSF) and ependyma are essential to fetal brain formation (Redzic et al., 2005). Neurodevelopmental diseases occur when CP secretions and CSF dynamics do not adequately support neural network growth (Johanson et al., 2008; Miyan et al., 2003). A viable CP in the lateral, 3rd and 4th ventricles thus pivots brain development by providing trophic molecules for neurogenesis (Spector and Johanson, 1989), adjusting biogenic amine levels in CSF–brain (Dahlin et al., 2007), and generating intracranial pressure to sculpt central nervous system (CNS) architecture.

The CP of the fourth ventricle (4VCP) was in particular the focus of this study, due to considerations that neurotrophic secretions by 4VCP into CSF normally promote the development of pons, medulla and cerebellum (Yamamoto et al., 1996; Strazielle and Ghersi-Egea, 2000) and that specific nervous centers in these regions mediate autonomic regulation of ventilation and, more in general, of all the vital functions. In particular our purpose was to analyze how a deficient CP–CSF homeostatic system in the developing hindbrain likely could contribute to sudden intrauterine unexplained death and sudden infant death syndromes (SIUDS and SIDS, respectively).

In both SIUDS and SIDS, we have previously highlighted congenital abnormalities of the brain vital centers (Lavezzi and Matturri, 2008a,b; Lavezzi et al., 2004, 2006; Matturri and Lavezzi, 2007; Matturri et al., 2002) and specific developmental defects of the ependyma (Lavezzi et al., 2010) and area postrema (Lavezzi et al., 2012). This study is the first to focus on the development of the fourth ventricle in these pathologies, as there are no reports in the literature on this topic.

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Because the immature blood-CSF barrier (BCSFB) is susceptible to injuries (Rothstein and Levison, 2002), we also investigated a possible link between ontogenetic choroidal alterations and smoking in pregnancy. This anticipated linkage was prompted by defective maturation of the autonomic nervous system observed in sudden-death victims with smoker mothers (Lavezzi et al., 2005, 2007, 2010, 2012).

For various ages pre- and post-birth, we investigated the development of 4VCP and the related damages induced by perinatal smoke exposure. The study protocol included in all cases the examination, in serial brainstem histological sections, of the CP structure and the immunohistochemical detection of Substance P and apoptosis, often indicated as markers of defective permeability of brain barriers following exposure to injuries (Johanson et al., 2011a,b; Lavezzi et al., 2010). In particular Substance P, a neuropeptide readily released from perivascular axons following insults to central nervous system, compromises the capacity of the vasculature to regulate blood flow, thus evidently contributing to the increase of blood brain barrier (BBB) permeability (Donkin et al., 2007; Freed et al., 2002) and malfunction.

2. Methods

2.1. Study subjects

Clinical cases were organized into four groups (I–IV): I: fetal control for SIUDS; II: SIUDS; III: infant control for SIDS; and IV: SIDS. A total of 84 subjects, from 17 gestational weeks (gw) to 73 post-conceptual weeks (pcw) (8 postnatal months), were in depth analyzed for cytoarchitectural and functional alterations of the 4VCP. Premature sudden deaths were due to known and unknown causes. Table 1 breaks down the study groups according to gender, age and *n* values. For each subject of the controls (Groups I and III), a complete autopsy documented an anatomically-related cause of death. Specific diagnoses among the fetal control deaths were severe chorioamnionitis (*n* = 8) and congenital heart disease (*n* = 6). Infant control deaths were caused by congenital heart disease (*n* = 7), severe bronchopneumonia (*n* = 3), myocarditis (*n* = 1), pulmonary dysplasia (*n* = 2), pneumonia with acute respiratory distress (*n* = 1), and mucopolysaccharidosis type I (*n* = 1).

All these cases were sent to our Research Center and diagnosed according to the application of the guidelines stipulated by Italian Law n. 31/2006 “Regulations for Diagnostic Post Mortem Investigation in Victims of SIDS and Unexpected Fetal Death”. This law decrees that all infants suspected of SIDS who died suddenly in Italian regions within the first year of age, as well as all fetuses who died without any apparent cause (SIUDS), must undergo an in-depth anatomo-pathological examination, particularly of the autonomic nervous system. In about 30% of suddenly dead victims, a possible cause of death was recognized. This group includes the cases here used as controls, age-matched with the SIUDS/SIDS groups.

A recent study on ependyma (*n* = 24 SIUDS, 30 SIDS and 24 controls) (Lavezzi et al., 2010) used the same subjects as in the series of victims for this report. Here, these cases provide data for the first time on the role of CP in both SIUDS and SIDS, previously not contemplated. New cases include 2 fetal controls, 1 SIUDS, and 3 Infant controls.

2.2. Epidemiological information collection

Complete clinical histories were collected. Additionally, mothers completed a questionnaire on their smoking habit, detailing the number of cigarettes smoked before, during and after pregnancy. We defined a “smoking mother” when she smoked >1 cigarette/day. Twenty-two of 55 SIUDS/SIDS mothers (40%) were active smokers before and during the pregnancy, smoking >3 cigarettes/day. The remaining 33 mothers (60%) reported no history of cigarette smoking. Seven of the 29 mothers of controls (24%) reported a smoking habit. The remaining 17 control mothers (71%) were non-smokers.

2.3. Ethics considerations and informed consent

Ethical approval was granted by the Italian Health Ministry in accordance with Italian Law n. 31/2006. Parents of all subjects (controls, SIUDS and SIDS) provided informed consent for both autopsy and anatomo-pathologic analyses, under protocols approved by the L. Rossi Research Center Institutional Review Board of Milan University.

2.4. Tissue preparation for microscopy, histochemical staining and immunoprobing

Brain specimens were fixed in 10% phosphate-buffered formalin. After a standard period of fixation (with a mean value for all the four study groups of 2

weeks), brainstem and cerebellar specimens were then processed and embedded in paraffin, as previously reported (Matturri et al., 2005, 2008). Briefly, transverse serial sections were cut at 30-μm intervals. At each level, a dozen 5-μm sections were obtained. For light microscopy, two of these 12 sections were stained with alternately-applied hematoxylin–eosin and Klüver–Barrera. Selected sections were treated by Nissl (cresyl violet) stain to differentiate in particular CP light vs. dark epithelial cells. Additional sections at each level were used for immunohistochemistry.

2.5. Immunohistochemical techniques

2.5.1. Substance P

A specific primary antibody directed against Substance P was used with the avidin–biotin–peroxidase technique. Sections were deparaffinized and rehydrated in phosphate-buffered saline (PBS). After blocking endogenous peroxidase activity with 3% H₂O₂, slides in citrate solution (pH = 6) were heated in a microwave oven for antigen retrieval/epitope exposure. After further PBS washing, sections were incubated overnight with a rabbit polyclonal anti-Substance P primary antibody (C191, DBA, Segrate, MI, Italy) diluted 1:80. Immunohistochemical staining was done by the peroxidase-antiperoxidase method (ABC-Peroxidase kit, Vectastain, Vector Laboratories Inc., Burlingame, CA, USA) for 30 min at 20 °C. 3,3-Diaminobenzidine (DAB, Vector Laboratories Inc.) was used as chromogen. Sections were counterstained with light hematoxylin. Negative-staining controls for Substance P and other immunoprobes were verified by substituting PBS for the primary antibody.

2.5.2. TUNEL (DNA Nick End Labeling)

To detect cell death, immunohistochemical visualization of choroid epithelial cells was done by TUNEL (TdT-mediated dUTP-biotin nick end labeling). This identifies early nuclear DNA fragmentation by specific binding of terminal deoxynucleotidyl transferase (TdT) to 3'-OH ends of DNA. Deparaffinized sections were incubated with 20 μg/ml proteinase K (Sigma, St Louis, MO, USA). After blocking endogenous peroxidase activity with 3% H₂O₂, the enzyme TdT (0.3 U/ml) incorporated digoxygenin-conjugated deoxyuridine (dUTP 0.01 mM/ml) into DNA fragment ends. TUNEL signal was detected by an anti-digoxygenin antibody conjugated with peroxidase (Apoptosis Detection Kit, Oncor, Gaithersburg, MD, USA). Methyl green solution (10 min) was the counterstain. TUNEL positive cells were subdivided into Type 1 (TUNEL-positive necrotic) and Type 2 (TUNEL-positive apoptotic) cells, according to Rink et al. (1995). Precisely, Type 1 cells appear to lack morphological features characteristic of apoptosis (i.e., cell shrinkage, nuclear condensation and nuclear fragmentation) and displayed strong, uneven TUNEL staining predominantly in cytoplasm. Therefore, the morphological features of Type 1 cells are consistent with necrosis. Type 2 cells are identified by intense nuclear labeling, frequently fragmented in TUNEL-positive nuclear fragments. Taken together, these features of Type 2 cells point to apoptotic cell death.

2.6. Quantification of Substance P and TUNEL immunohistochemistry

For every case, only CP epithelial cells of Type 2 displaying clear apoptotic characteristics as nuclear condensation, cytoplasmic shrinkage and strong nuclear TUNEL immunolabeling, and CP epithelial cells with intense cytoplasmatic immunostaining for Substance P were considered.

Apoptotic index (AI) and Substance P index (SPI), defined as the number of cells with strong unequivocal immunostaining, divided by the total number of consecutive CP epithelial cells counted, expressed as percentage, were evaluated for every case.

Both AI and SPI were classified as: “Class 0” for no staining; “Class 1” when the index was <30%; “Class 2” with a percentage of immunopositive cells between 30 and 50%; “Class 3” with AI and/or SPI > 50% of the counted epithelial cells.

2.7. Markers for leukocytes, macrophages and fibroblasts in choroid interstitium

To identify cellular elements in the matrix, we probed for extracellular leukocytes and macrophages. Sections were selected for applying the CD18 antibody (rabbit monoclonal; Novocastra Laboratories Ltd Newcastle, UK) to react with leukocytes; and CD68 to bind macrophages (rabbit monoclonal; Santa Cruz Biotechnology, Santa Cruz, CA). Tissue fixation, blocking, sectioning and rehydrations were done as for Substance P detection. Sections were incubated overnight with the primary antibodies (1:50, in bovine serum albumin) at 25 °C. The ABC-Peroxidase Kit (Vector Laboratories) generated the immunoreaction product.

Immunostaining procedures for Fibroblast-specific protein-1 (FSP-1; 11 kDa) of the S100 superfamily were applied to identify in particular fibroblasts presenting as cystic-type cells with vacuoles. Skin samples were used as controls. After paraffine removal and rehydration, tissue sections were treated with 1:400 FSP-1 (Millipore #07-2274). Antigen in 0.01 M citrate buffer (pH 6.0) was retrieved by 98 °C-microwaving for 30 min. Sections were blocked with diluted serum for 1 h, then incubated overnight at 4 °C with primary antibody. Tissue peroxidase was blocked, then incubated with a biotinylated secondary antibody (1:200) for 30 min. Antigen–antibody complex was stained with DAB tetrahydrochloride. Counterstain was Mayer hematoxylin.

Table 1
Study subjects.

Group I fetal control				Group II SIUDS			
<i>F</i> =9	<i>N</i> =14	Age (gw):		<i>F</i> =15	<i>N</i> =25	Age (gw):	
<i>M</i> =5	17–41	Range – mean value ± SD	35 ± 1.2	<i>M</i> =10	17–41	Range – mean value ± SD	36 ± 2.0
Group III infant control				Group IV SIDS			
<i>F</i> =7	<i>N</i> =15	Age (pcw):		<i>F</i> =16	<i>N</i> =30	Age (pcw):	
<i>M</i> =8	34–71	Range – mean value ± SD	52 ± 2.4	<i>M</i> =14	34–73	Range – mean value ± SD	51 ± 2.0

gw: gestational week; pcw: postconceptional week; SD: standard deviation; SIUDS: sudden intrauterine unexplained death syndrome; SIDS: sudden infant death syndrome.

2.8. Statistical analyses

Histological and immunohistochemical analyses were done by two independent, blinded observers. Comparison among the results was performed employing Kappa statistics (Kappa Index-KI) to evaluate inter-observer reproducibility. Landis and Koch (1977) system of KI interpretation was used, where 0–0.2 is slight agreement, 0.21–0.40 indicates fair agreement, 0.41–0.60 is moderate agreement, 0.61–0.80 is strong or substantial agreement, and 0.81–1.00 indicates very strong or almost perfect agreement (a value of 1.0 being perfect agreement). This analysis revealed a very satisfactory value (KI=0.85).

Frequency distributions of CP impairments and their relation with maternal smoking were evaluated by Fisher's exact test. Rate difference (RD) and its 95% confidence interval (CI) of each CP alteration in SIDS and SIUDS vs. corresponding control was calculated.

Calculations were carried out with SPSS statistical software (version 11.0; SPSS Inc., Chicago, IL, USA). Significance was set at $p < 0.05$.

3. Results

3.1. Developmental stages of choroid plexus

We identified in control cases (groups I and III of Table 1) four stages of 4VCP development by histology criteria for the vascular, interstitial and epithelial compartments.

Stage I (about 17–18 gw) – The CP is well recognizable as symmetrical fringes that join at the medial line in the roof of the ventricle projecting into the ventricular lumen (Fig. 1). The CP epithelial layer, continuous with the ependymal cell layer that lines the ventricle, is at this stage prevalently stratified consisting of dark round cells with dense concentrated cytoplasm and central pyknotic nuclei. The epithelium interfaces with an inner interstitium on the basal side and contacts the CSF via a brush surface at the apical pole. A scanty amount of dense collagenized/myxoid

stroma, with numerous but not well-differentiated round cells and several capillaries with a very small luminal diameter can be observed.

Stage II (about 22–25 gw) – A large part of the epithelium changes to a columnar monolayer containing also some pale stained cells with a well evident basal oval nucleus. Stroma is wider than in Stage I and less compact. Compared to the first stage, the blood vessel number is strikingly increased in number and with enlargement of the vascular lumen (Fig. 2A).

Stage III (about 28–35 gw) – The CP becomes much more extensive and tortuously folded, showing a well demarcated inner vascular core. An increased number of pale (not dark) cells in CP epithelium is present. The intermediate zone between the vasculature and parenchyma now contains a loose connective tissue with several fibroblasts (Fig. 2B).

Stage IV (>35 gw) – The same proportion of the two types of CP epithelial cells, videlicet a light and a dark type, is reached in the last gws. This differentiation between light and intense stain of cells is well evident in sections treated with cresyl violet method (Fig. 3). A standard CP villus at this stage clearly shows in transverse section three compartments: epithelium, interstitium and blood capillary (Fig. 4). The vascularization of the CP appears to increase in the last gws, showing a very rich network of blood vessels.

The choroidal structure of Stage IV in humans undergoes minimal changes after birth. Therefore Stage IV in our study consists of the last gestational weeks through 73 post-conceptional weeks (8 postnatal months).

Immunohistochemistry revealed in control cases (groups I and III – Table 1) very few apoptotic cells (AI: Class 1) in CP epithelium

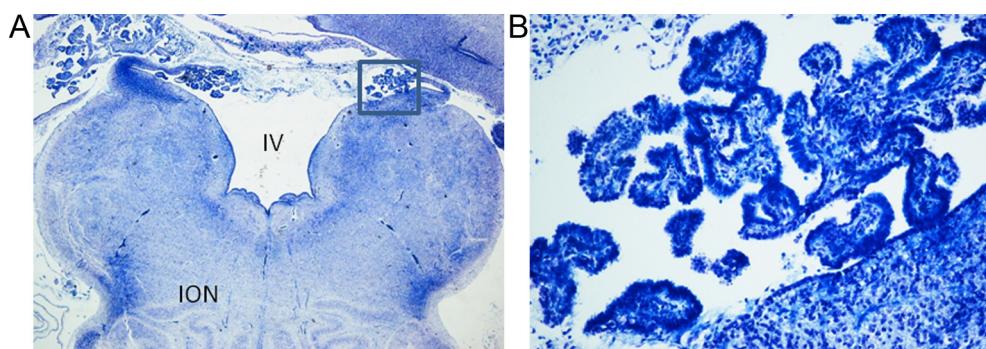


Fig. 1. Histological section of medulla oblongata in a control case at 17 gestational weeks, demonstrating early choroid plexus protrusion into 4th ventricle (Stage I of human CP development). In the framed area of A, showed at greater magnification in B, a dark stratified epithelium exists. Klüver–Barrera stain; magnification: (A) 2×; (B) 10×. IV: fourth ventricle; ION: inferior olivary nucleus.

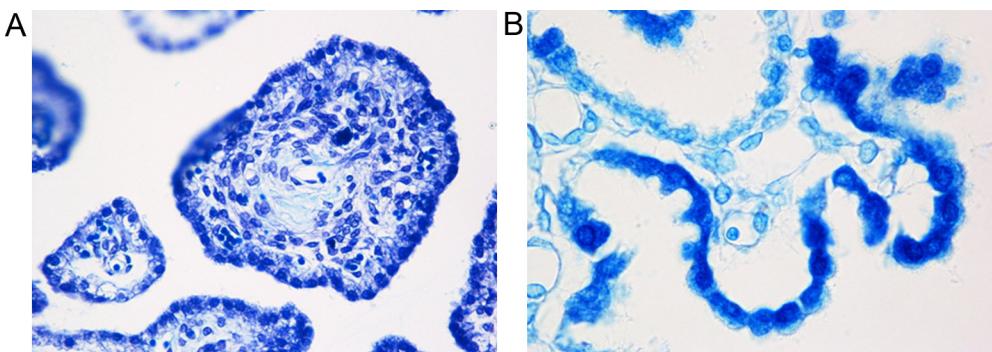


Fig. 2. Representative images of Stages II and III. In A (Stage II): CP with prevalently dark monolayered epithelium and wide compact stroma in a control fetus died at 25 gws; in B (Stage III): CP epithelium with increased number of pale cells and well demarcated inner vasculature (fetus of the control group aged 29 gws). Klüver–Barrera stain; magnification: (A) 20×; (B) 40×.

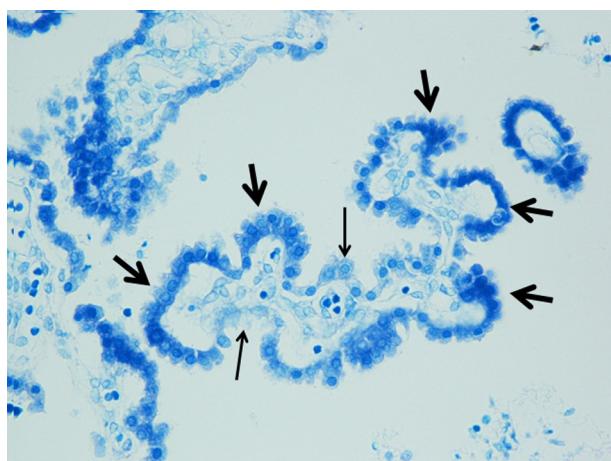


Fig. 3. Monostratified choroid plexus epithelium with both dark (thick arrows) and pale (thin arrows) cells in a dead fetus of the control group at 36 gestational weeks (Stage IV of human CP development). Cresyl violet stain; magnification: 20×.

from early fetus to 8 postnatal months. Minimal immunostaining also for Substance P occurred in controls. Only in 4 cases a SPI of Class 3 was observed. These results corroborate a general absence of BCSFB damage in control groups.

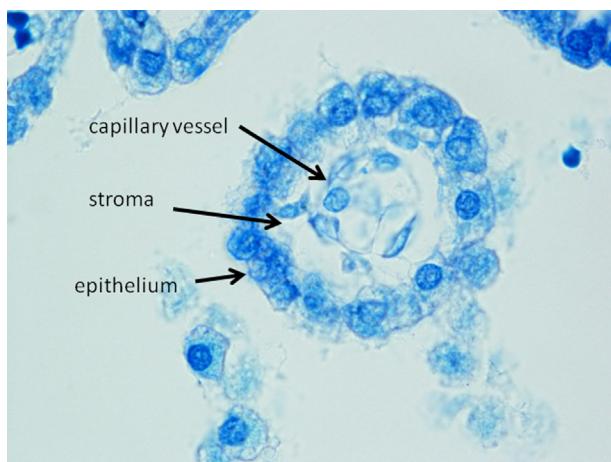


Fig. 4. Section of a choroid plexus villus in a late fetal death (38 gestational weeks) showing three major compartments: the inner vascular core, the intermediate loose stroma and the superficial ring of epithelial cells (Stage IV of human CP development). Klüver–Barrera stain; magnification: 60×.

3.2. Pathology of CP in fetal and infant death victims

On the whole SIUDS and SIDS cases displayed a significantly high incidence of histologic/immunohistochemical alterations of 4VCP, compared with age-matched controls. In fact 45 of 55 victims of sudden death (82%), but only 7 of 29 subjects in control groups (24%), showed CP modifications ($p < 0.05$).

There was marked pathology in 4VCP of nearly half of the SIDS victims. Comparatively, CP was less damaged in SIUDS victims than in SIDS subjects.

Structural and biochemical alterations, pointing to altered barrier, homeostatic and secretory functions at the BCSFB, are delineated below according to the three tissue compartments of 4VCP (choroidal vasculature, interstitium and epithelium) (Table 2).

3.2.1. Vasculature

In 29 control subjects (the entirety of Groups I and III), there was no evidence for maldeveloping capillaries in the plexus. Likewise, SIUDS victims did not display impaired microvessel development. One third of the 30 SIDS victims (Table 2), however, showed a diminished capillary number in comparison with the control group ($p < 0.01$, RD = 0.33; CI_{0.95} = 0.11–0.56). Along with attenuated vessel number, there was frequently desquamated epithelium in 4VCP.

3.2.2. Interstitium

Large cystic or stromal cells in extracellular space containing flattened peripheral nuclei in vacuolated cytoplasm, positive for FSP-1, were found in one fetal control (7% of subjects) and in just two infant controls (13% of subjects). These large fibroblast-like stromal cells abounded in 56% of SIUDS victims ($p < 0.01$, RD = 0.49; CI_{0.95} = 0.17–0.81) and a comparable 57% of SIDS subjects ($p < 0.01$, RD = 0.43; CI_{0.95} = 0.12–0.74), sometimes filling the whole interstitium (Fig. 5). The stroma in most of SIDS and late SIUDS cases, without a strong presence of cystic cells, is denser than that of the age-matched controls. Thus the collagenous interstitium in these SIUDS/SIDS cases resembles that of the early (Stage I) but not later controls. Moreover, the lack of choroidal tissue staining for CD18 and CD68, respectively, indicates that the interstitial cells were not leukocytes or macrophages.

3.2.3. Epithelium

In just 3/14 fetal controls and 1/15 infant controls, the epithelial lining was teased away from the choroidal matrix. However, in SIDS victims the frequency of epithelial stripping was substantially increased to 40% ($p < 0.05$, RD = 0.33; CI_{0.95} = 0.05–0.62) (Fig. 6). Stripped 4VCP epithelial cells were negligible in SIUDS (8%).

Table 2

Fourth ventricle choroid plexus (4VCP) alterations in SIUDS and SIDS victims compared to controls.

Choroid Plexus Alterations [§]	Group I fetal control	Group II SIUDS	Group III infant control	Group IV SIDS	K index	Interobserver agreement
<i>Vasculature</i>						
Capillary impoverishment ^a	0/14(0%) [0]	0/25(0%) [0]	0/15(0%) [0]	10/30(33%) ** [7]	0.89	97%
<i>Interstitialtum</i>						
Presence of cystic cells	1/14(7%) [1]	14/25(56%) ** [9]	2/15(13%) [2]	17/30(57%) ** [9]	0.90	95%
<i>Epithelium</i>						
Stripping from underlying matrix	3/14(21%) [2]	2/25(8%) [1]	1/15(6%) [1]	12/30(40%) * [8]	0.80	92%
Prevalence of dark cells	0/14(0%) [0]	0/25(0%) [0]	0/15(0%) [0]	20/30(66%) ** [10]	0.87	95%
Substance P immunopositivity (Class 3 of SPI) ^b	3/14(21%) [2]	9/25(36%) [8]	1/15(6%) [0]	12/30(40%) * [10]	0.74	89%
Apoptosis (TUNEL) immunopositivity (Class 3 of AI) ^c	0/14(0%) [0]	11/25(44%) ** [7]	0/15(0%) [0]	13/30(43%) ** [7]	0.91	96%

^a >30% of villi without an inner vessel.

^b Substance P index (SPI) >50%.

^c Apoptotic index (AI) >50%.

[§] Statistically significant differences, SIUDS or SIDS vs. corresponding control (Group II vs. I, or Group IV vs. III); *p<0.05 **p<0.01.

Individual victims may display any combination of the CP alterations. A total of 45 out of 55 SIUDS/SIDS cases had one or more developmental defects of the 4VCP. Nineteen of the 22 cases of SIUDS/SIDS with smoking mother belong to this group of 45 cases. In brackets are indicated, for every type of alteration the number of cases with smoking mother (always considering that individual victims may display any combination of the CP alterations).

Furthermore, only in SIDS victims we frequently observed the nearly exclusive presence of CP dark epithelial cells, rather than the same proportion of the two types of epithelial cells, videlicet a light and a dark type, observable in age-matched controls (Fig. 7).

Substance P immunostaining in 4VCP epithelium increased in both SIUDS and SIDS compared to controls (Fig. 8). In SIDS cases, 40% of the specimens displayed a SPI of Class 3 compared to just 6% of corresponding controls ($RD = 0.33$; $CI_{0.95} = 0.05–0.62$) ($p < 0.05$). In SIUDS, Class 3 SPI was substantial in 36% of the victims, while in fetal controls was 21%.

TUNEL staining showed a clear-cut difference between controls and sudden-death victims. Significant apoptosis, judged by TUNEL staining, was absent in the 29 controls. However, marked TUNEL staining in 4VCP (Class 3 of AI) was observed in 44%

($p < 0.01$, $RD = 0.44$; $CI_{0.95} = 0.17–0.71$) and 43% ($p < 0.01$, $RD = 0.43$; $CI_{0.95} = 0.18–0.69$) respectively, of SIUDS and SIDS victims (Fig. 9).

3.3. Correlation of findings with smoke exposure

On the whole the alterations of the CP were significantly related to maternal smoking ($p < 0.01$). In fact, in 19 out of 22 victims of sudden death with a smoker mother (86%) one or more developmental defects of the CP were present. To be precise, 4VCP pathology in SIDS exceeded SIUDS, likely due to longer cumulative effects of smoke exposure. Similarly, in the control group, 4 of the 5 (80%) cases with CP changes had a smoking mother, confirming the role of smoke absorption in the neuropathogenetic mechanism.

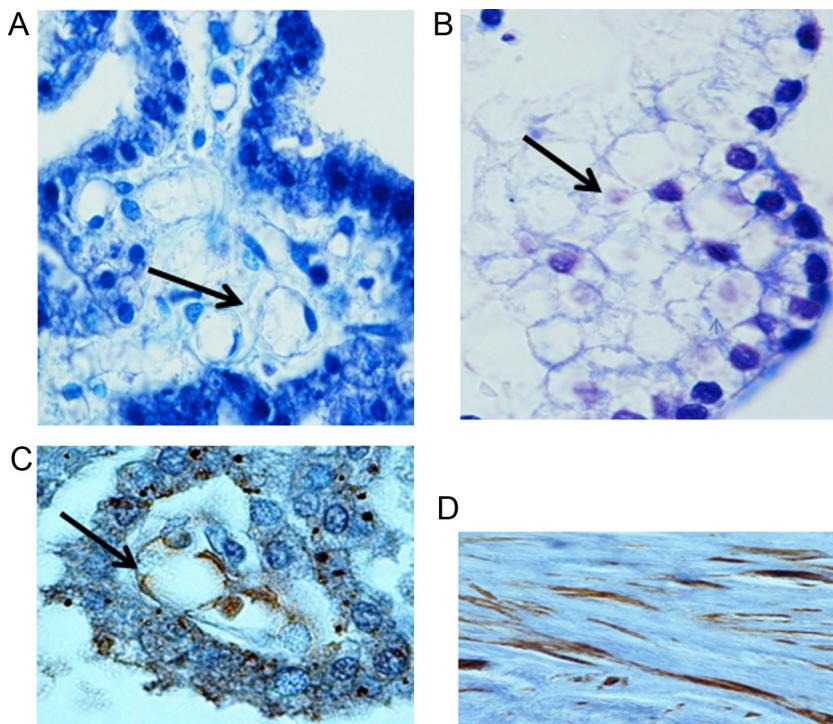


Fig. 5. Stromal cystic cells in 4VCP interstitium. In A: SIDS victim, died at 3 months (52 postconceptual weeks), revealing cystic cells in interstitial space. In B: SIUDS case (39 gestational weeks) showing numerous subepithelial vacuolated cells filling the stroma of the plexus. In C: identification of fibroblastic nature of cystic cells by FSP-1 immunohistochemistry (brown reaction product). In D: Skin control, positive for the presence of FSP-1-labeled fibroblasts in a 2-month old human infant. Arrows indicate vacuolated stromal cells. A and B: Klüver–Barrera stain; C and D: FSP-1 immunohistochemistry; magnification: 60× for A, B, C and D.

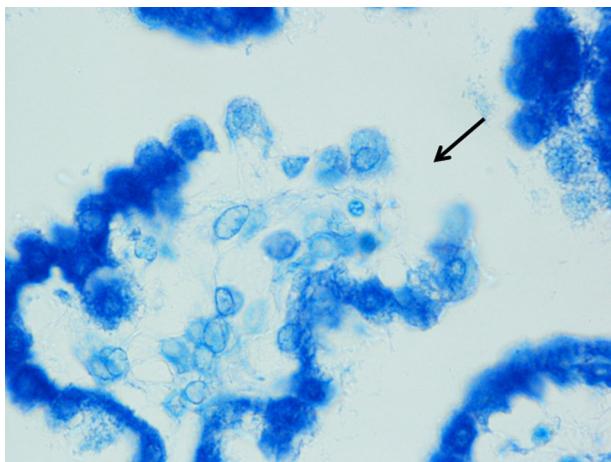


Fig. 6. Choroid plexus from a SIDS case (2 postnatal months; 45 postconceptual weeks) showing outer epithelial desquamation (see arrow). Klüver–Barrera stain; magnification: 100×.

3.4. Additional results on brainstem

The present choroidal analyses complement findings in our previous work delineating the ependymal alterations in this patient series (Lavezzi et al., 2010). These results include, in addition to a wide spectrum of pathological changes of the ependyma (such as desquamation, clusters of ependymal cells in subventricular zone, radial glial cells, unusual presence of neurons within and over the ependymal lining), developmental defects of different brainstem structures, namely hypoplasia/agenesis of the arcuate,

pre-Bötzinger, inferior olfactory, parafacial and serotonergic raphe nuclei.

A very high correspondence exists between the CP alterations reported here and those previously related to ependyma in SIUDS/SIDS. In fact, 27 of the 40 SIUDS/SIDS with CP alterations (65%) showed concurrent ependymal defects.

3.5. Examination of the CP of the lateral ventricles

The examination of the CP of both lateral ventricles (LVCP), performed to acquire a total view of the CP system, showed prevalently a normal structure in all the subjects of the study. Only a poor vascularization of the lateral plexuses was observed in 8 victims of sudden death, i.e., 2 SIUDS and 6 SIDS (15%), all with a smoking mother. Furthermore, in 4 of these SIDS victims there was intense nuclear TUNEL-immunopositivity detected in about 25% of LVCP epithelial cells (Class 1 of AI). Substance P immunostaining was negative in LVCP specimens. Overall, then, LVCP was spared the considerable damage observed in 4VCP of SIUDS and SIDS victims.

Alterations of the 4VCP were constantly present concurrently in the cases with the above reported LVCP defective development.

4. Discussion

Choroid plexuses, components of the brain ventricular system, besides representing the main source of CSF secretion (Oresković and Klarica, 2010; Jurado and Walker, 1990; Pollay, 2010), form a major interface between the blood and the CNS. The CNS is separated from the systemic circulation by the BBB, which is formed by endothelial cells of capillaries, and by the BCSFB, constituted by the epithelial cells of the CPs (Johanson et al., 2010,

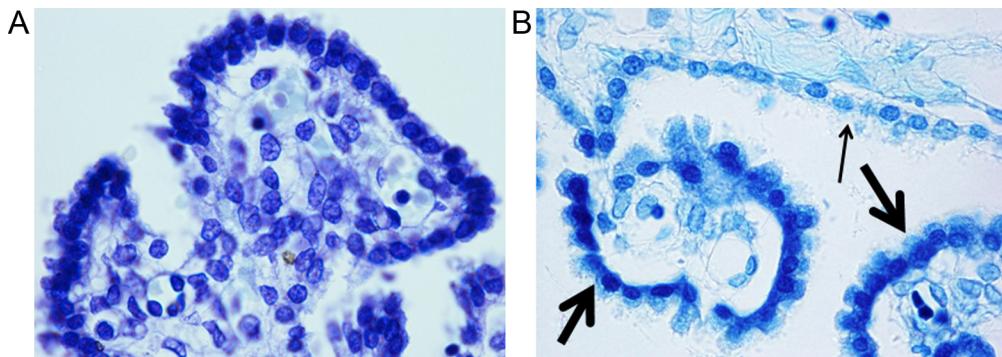


Fig. 7. In A: Prevalence of dark cuboidal epithelial cells with centrally-located nucleus in CP of a 3-month-old SIDS case (46 postconceptual weeks). In B: CP from a control case of similar age (3 months; 48 postconceptual weeks) showing the same proportion of the two types of CP epithelial cells (light-thin arrow/dark-thick arrows). Cresyl violet stain; magnification: 40×.

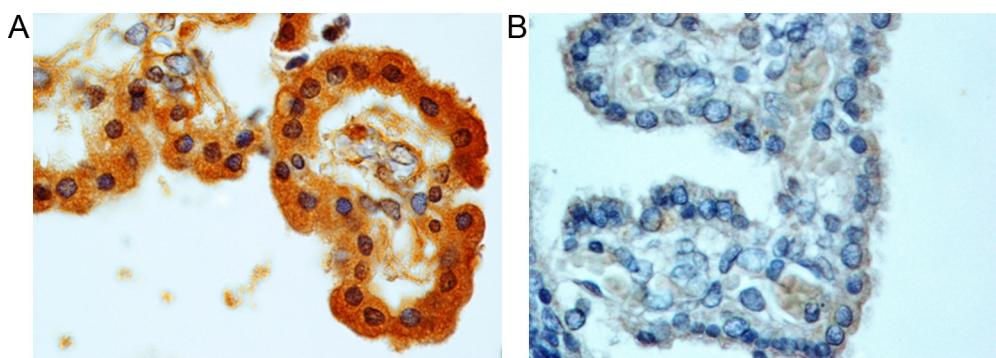


Fig. 8. Substance P immunostaining in 4VCP epithelium. In A: high immunopositivity (Class 3 of SPI) in a SIDS case (2-months; 40 postconceptual weeks). In B: negative Substance P immunostaining in CP of an age-matched control case (2 months; 39 postconceptual weeks). SP immunohistochemistry; magnification: 60×.

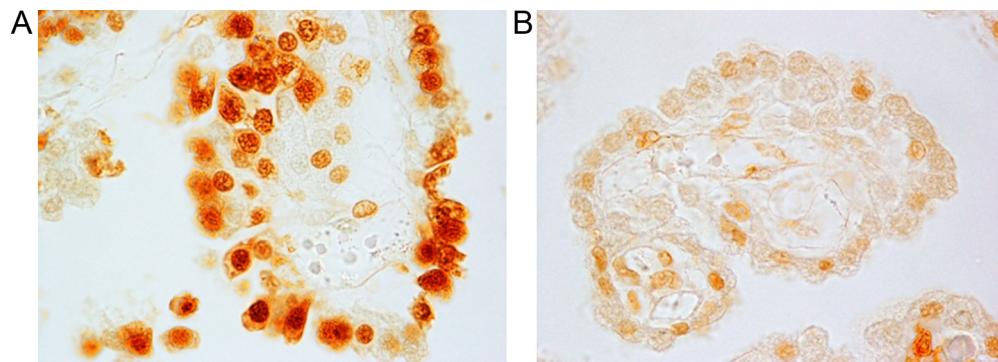


Fig. 9. TUNEL immunostaining in 4VCP epithelium. In A: high immunopositivity (Class 3 of AI), indicating DNA damage and cell death, in a SIDS case (3 months; 52 post-conceptional weeks). In B: absence of TUNEL-positive cells in CP of an age-matched control case (3 months; 44 postconceptional weeks). TUNEL (DNA Nick End Labeling) method; magnification: 60×.

2011a,b). Whereas the choroidal endothelial cells are fenestrated, the CP epithelial cells are intimately connected to each other by tight junctions. The barrier function of the tight junctions can undergo modulation to regulate the exchanges between CSF and CNS. Being located between two circulating fluids, the CP epithelium fulfills several functions such as protecting the brain against deleterious biochemical/xenobiotic-induced injuries (Ghersi-Egea and Strazielle, 2001). In addition, the CP epithelium acts as a filtration system removing metabolic waste, neurotransmitter excesses and foreign substances from the CSF-brain system to maintain the delicate extracellular environment required by the neurons to function optimally (Redzic et al., 2005; Johanson et al., 2011b; Spector and Johanson, 1989).

4.1. Development of the CPs in humans

Early in the 2nd trimester, the CPs appear at four sites of CSF formation. These include the cerebral ventricles in the roof of the neural tube, shortly after its closure, in the order of the fourth, lateral, and third ventricles (Dziegielewska et al., 2001). The CPs, given the sparse cerebral vascularization at this early fetal time, have a more important nutritional role in the immature brain than in the adult, in addition to contributing to the control of the internal milieu of the developing CNS.

In this study, focusing on 4VCP in victims of unexplained fetal and infant death vs. age-matched controls, we highlight that the CP increases in size and complexity rapidly through four developmental stages, according to the works of Dziegielewska et al. (2001) and Shuangshoti and Netsky (1966). From our earliest observations at 17–18 gestational weeks of a pair of bilateral ridges covered by a thick dark epithelium, the CP grows to form a large structure. The growth and differentiation of CP is subsequently accompanied by a change in the epithelium from stratified to simple columnar consisting of both light and dark cells, reaching approximately the same proportion by birth.

The presence of light and dark choroidal epithelial cells has already been reported by Wislocki and Ladman (1958) in a seminal electron microscopic study on CP. They suggested that the difference in cell density reflected different stages of the secretory cycle of the choroidal epithelium and that an increased number of dark epithelial cells in CP was associated with diminished production of CSF.

Interestingly, and perhaps significant for the onset of SIDS/SIUDS neuropathology, we identified a predominant or exclusive presence of dark cells in CP epithelium in a large proportion of SIDS victims. In age-matched controls, our study instead showed the same proportion of light and dark cells.

4.2. Physiology of the CSF

A firm fact is that, in physiological conditions, CSF secretion and absorption must be balanced to prevent alterations in intracranial pressure. This means that the decrease of CSF that is secreted inside the brain ventricles must be balanced by a decrease that is passively absorbed (Johanson, 2008; Johanson et al., 2008, 2010). Any other relationship will result in unbalanced CSF volume, and with time, an increase in pathological processes (Levine, 1987). In fact, a fluid turnover reduction necessarily affects the transport of many substances essential to maintain a stable brain interstitial fluid and to promote efficient transmission of impulses along axons and through synapses. Moreover, in case of reduced CSF formation, not only the delivery of trophic factors to neurons is altered, but also the CSF removal of brain catabolites is diminished, resulting in compromised homeostasis of the neuronal-interstitial compartments. Consequently, the imposing presence of choroidal dark epithelial cells observed in high percentage of SIDS victims of our study could be associated with CSF-brain dyshomeostasis, i.e., distortions of fluid volume, content and flow dynamics. Such a fluid imbalance likely affects regulatory mechanisms in the fourth ventricle-brainstem regions and may be part of the pathogenetic mechanism of sudden death.

4.3. CP pathology in SIUDS/SIDS

In addition to the prevalence of dark epithelial cells, we observed a wide spectrum of pathological changes of the CP in sudden fetal and infant deaths. These alterations consistently included: presence of cystic/vacuolated cells in the stroma, epithelial disruption, and a decreased number of inner capillaries.

Moreover, CP demonstrates in SIUDS/SIDS a characteristic immunocytochemical profile that differs from that of CP in controls. Intense Substance P immunopositivity (Class 3 of SPI) and strong hallmarks of apoptosis (DNA fragmentation, chromatin condensation, Class 3 of AI) were in fact present in CP epithelial cells of almost the half of the victims of sudden death and in about 14% of subjects who died of a known cause.

4.4. Correlation of the CP alterations with smoke absorption in pregnancy

These choroidal neuropathological parameters were significantly correlated to tobacco smoke exposure in utero. In fact, a very high percentage of SIUDS/SIDS victims with CP developmental alterations had a smoker mother.

Maternal smoking has been reported as a primary determinant of hypoxic/ischemic brain damage particularly in victims of

unexplained death (Lavezzi et al., 2005, 2007, 2010, 2012). Here is one possible explanation: the carbon monoxide, a gaseous combustion product of nicotine, easily crosses the placental barrier by passive diffusion, where it binds to hemoglobin. Consequently, carboxyhemoglobin, that is present in the fetal compartment at concentrations that are generally 15% higher than the maternal levels, inhibits the release of oxygen into fetal tissues, causing hypoxia with consequent delayed maturation of all the organs, especially those most susceptible to hypoxic damage, including the brain (Levin and Slotkin, 1998; Lichtensteiger et al., 1988). Besides, nicotine is one of the few lipid-soluble substances that can go beyond the blood-brain barrier and act directly on the expression of genes modulating the developing brain, i.e., by inducing specific molecular alterations in the DNA, RNA, and proteins of the nervous cells (Johns et al., 1982; Gressens et al., 2003).

Each of the developmental defects observed in this study in victims of sudden death can be interpreted as primary response to maternal smoking in pregnancy.

As regards the Substance P, a neuromodulator belonging to a group of neuropeptides that are widely distributed in the CNS (Datar et al., 2004; Mantyh, 2002), it has been reported its involvement in regulation and generation of many physiological and pathophysiological conditions (King and Barr, 2003; Higa et al., 2009; Jiménez-Corral et al., 2006; Mauborgne et al., 1983) including breathing acceleration in hypoxic events (Lagercrantz et al., 1991; Hedner et al., 1985; Lavezzi et al., 2011b). This function in particular is mediated by Substance P-binding to the NK1 receptor and subsequent transport across the BBB with its release in periphery (Chappa et al., 2006). Mapping of Substance P immunoreactivity in the human CNS has been reported as mainly localized in fiber-structures of the brainstem (Jordan et al., 1995; Ribeiro-da-Silva and Hökfelt, 2000; Lavezzi et al., 2011b). Noteworthy in the present study, a great degree of SPI was confined to almost all the epithelial cells of the CP in victims of unexplained death. Increased expression of Substance P has been demonstrated in experimental investigations in different epithelia (e.g., airway, intestinal, urothelial, epithelial cells) after exposure to toxic, infectious or hypoxic insults (Birder et al., 2010; Chu et al., 2000; Mezey, 1998). Likewise, we ascribe the high SPI observed in the CP epithelium to a primary response to maternal smoking in pregnancy.

The presence of high percentages of apoptotic epithelial cells in CP (Class 3 of AI) can be interpreted similarly. It has been demonstrated that CP epithelial cells in rodents die by apoptosis after several hours of cerebral ischemia (Gillardon et al., 1996).

Also the observation of vacuolated cells in CP interstitium in over half of the victims of sudden death could represent a direct toxic effect of the smoke absorption in the developing brain. Toxicology studies on CNS, indicating as an intense vacuole formation in CP usually reflects excessive accumulation of xenobiotic material (Roth and Krinke, 1994; Wells and Krinke, 2008), give credence to our supposition. In particular, Roth and Krinke (1994) demonstrated that, following intraperitoneal injection of toxicants (e.g., methylcellulose) in rats, numerous vacuolated cells appeared within the CP interstitium, thereby distorting CP structure and function. The vacuolation phenomena in CP observed here, suggests an attempt to absorb and break down the nicotine and carcinogenic catabolites, thus preventing their passage into brain.

We highlighted that regional differences occur in the CP response to smoking. In fact, there is greater involvement in developmental alterations of human CP of the fourth ventricle vs. lateral ventricle. In SIUDS/SIDS cases, 86% of victims demonstrated histological/immunohistochemical abnormalities of 4VCP, likely related to maternal smoke absorption, but only 15% presented slight defects of LVCP in telencephalic forebrain.

This differential response cannot depend merely on 4VCP formation before the lateral ventricles (Dziegielewska et al., 2001).

Our observations provide biological evidence of variable responses to prenatal smoke exposure in altering development of similar structures in different CNS areas. Therefore we can more generally postulate that the hindbrain is primarily more vulnerable to smoking in pregnancy than forebrain, at least in the protective choroido-meningeal and vascular interface membranes.

Our hypothesis is supported by the research of Gospe et al. (1996). In pregnant rats exposed to sidestream smoke they demonstrated decreased DNA concentration and increased protein/DNA ratio (indicative of cell number reduction and cell hypertrophy, respectively) only in the hindbrain, proving that environmental tobacco primarily affects the development of this brain region.

This differential embryonic susceptibility deserves further exploration and should open new research fields in neurotoxicology. A key question is whether the 4VCP breakdown in SIDS/SIUDS is a primary or secondary pathology. Would prevention of CP damage or breakdown in early development confer protection to the developing brainstem? Application of molecular techniques will expedite study of initial stages of CP differentiation to reveal genes involved in plexus morphogenesis and in epigenetic regulation of specific gene expression at the BCSFB.

5. Conclusions

Our research continues with the aim of confirming the involvement of the mother's smoke in causing serious neuronal and neuroepithelial damage in victims of SIUDS and SIDS. In view of strong evidence that maternal smoking is a main contributor to CNS dysgenesis, particularly in the exquisitely-sensitive CP, it is vitally important to warn women that smoking places their fetus at serious risk of CNS developmental abnormalities leading to sudden death of babies and infants.

Conflicts of interest

All authors declare that they have no conflicts of interest, financial or otherwise.

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