Alterations of Biological Features of the Cerebellum in Sudden Perinatal and Infant Death

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Abstract: This article intends to show how the cerebellum, a structure ordinarily not considered in mediating breathing or cardiovascular control, may play a critical role in compensatory responses particularly to hypoxic insults occurring pre and/or postnatally and thus may be involved in the sudden unexplained perinatal and infant death. Besides the ontogenesis of the cerebellar cortex in man, we reported alterations of biopathological features (neuronal immaturity, altered apoptotic programs, negative expression of somatostatin and EN2 gene, intense c-fos expression positivity, astrogliosis) in the cortex and in the dentate nucleus of the 63% of sudden deaths, and only in 10% of the controls. The correlation of these results with the mother's smoking habit was highly significant. Therefore, we support the hypothesis, already expressed in previous studies on brainstem, of a close relation between maternal cigarette smoking and a wide range of morpho-physiological defects of the brain, leading to unexplained sudden death in stillbirths, newborns, and Sudden Infant Death Syndrome (SIDS) victims.

Keywords: Sudden unexplained perinatal death (SUPD), sudden infant death syndrome (SIDS), sudden intrauterine unexplained death (SIUD), sudden neonatal unexplained death (SNUD), cerebellum.

INTRODUCTION

Sudden infant death syndrome (SIDS) and unexpected perinatal death are fundamental social and health problems which have yet to be resolved.

Essentially, these types of death are characterized by the lack of any evident syndrome, and by their unexpected nature and rapidly fatal outcome.

The Sudden Infant Death Syndrome (SIDS) is defined as a sudden infant death, between 1 and 12 months, completely unexpected and unexplained after a thorough case investigation, including performance of a complete autopsy with in-depth histological examination of the cardiorespiratory innervation and specialized myocardium, investigation of the death scene, and a review of the clinical history [1,2]. SIDS affects one of 700-1,000 infants and is the foremost cause of death in the first year of age.

The sudden unexplained perinatal death (SUPD), which remains unexplained after a complete autopsy investigation, comprises **1)** the Sudden Intrauterine Unexplained Death (SIUD), when a fetus died suddenly after the 25th gestational week, before complete expulsion or retraction from the mother, and **2)** the Sudden Neonatal Unexplained Death (SNUD), when a newborn died suddenly before the end of the 1st postnatal week of life [3-5].

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Altogether, the frequency of the unexpected perinatal death, which has ranged from 5-12‰ in the last 20 years, has not declined despite modern advances in maternal-infant care [6].

Our researches performed up to now on sudden unexpected perinatal and infant death [7-18], according also to studies by other authors [19-22], have focused upon structures prevalently assigned to breathing as well as to cardiovascular, upper digestive and arousal control mechanisms that are located in the brainstem. Our histopathological and immunohistochemical examinations have revealed the presence in these locations of many neuromorphological and/or functional alterations, such as arcuate nucleus hypoplasia, often associated to reticular formation and pulmonary hypoplasia, as well as hypoplasia of the hypoglossus nucleus and parabrachial/Kölliker-Fuse complex, lack of tyrosine-hydroxylase in neurons of the locus coeruleus, an abnormal distribution of somatostatin in the hypoglossus nucleus.

These observations have led us to propose a unifiable theory, that is the SUPD should not be regarded separately from the SIDS, since the same congenital abnormalities of the autonomic nervous system are present in both.

Recently, we aimed to extend our research to structures ordinarily not considered in mediating breathing or cardiovascular control, precisely the cortex and the deep nuclei of the cerebellum.

This article intends to present our new results that show as also the cerebellum may play a critical role in compensatory responses particularly to hypoxic insults occurring pre and/or postnatally and thus may

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be involved in the sudden perinatal and infant death.

In experimental studies many authors have focused their attention on the cerebellum. Its simple structure and its gradual maturation from the early embryonic stage, lasting a long period of time after birth, make this structure a favorite field for research, particularly on the development and fiber connections of the central nervous system [23-28].

Ontogenesis of the Cerebellar Structures

The cerebellum arises bilaterally from the alar layers of the first rhombomere [29]. The formation of the cerebellar cortex is ascribed to two main cell populations (granule cells and Purkinje cells) that have different times of development and distinct patterns of migration and differentiation.

The Purkinje cells undergo terminal mitoses in the ventricular zone of the metencephalic alar plates [24,30] and only in the postmitotic phase they migrate from the germinal zone into the cerebellar wall, probably following radial glial guide [31], where they aggregate to form an immature layer 10-15 cells thick. Around birth the Purkinje cells form a row of large somata and start to extend elaborate dendritic arbor synapses.

The granule cells precursors are added later than the Purkinje cells, towards the end of the embryonic period, deriving from the upper rhombic lip, that is the dorsolateral portion of the alar plate, from where the arcuate nucleus of the medulla oblongata also arises [24,32,33]. These cells reach the superficial zone of the cerebellum and organize themselves into a pluristratified transient structure: the external granular layer.

Successively, due to proliferation and inward migration through the molecular layer, the external granular layer cells give rise to the deeper, definitive internal granular layer, situated below the layer of Purkinje cells. After birth the external granular layer progressively reduces in thickness and disappears, after a time interval that varies according to the species.

The deep cerebellar nuclei, such as the Purkinje cells, arise from the ventricular zone of the metencephalic alar plates.

Numerous experimental studies using electrophysiological and anatomical investigation techniques have demonstrated a close relation of the cerebellar deep nuclei with the inferior olivary nucleus (ION) located on the ventrolateral surface of the medulla, that derives from the caudal part of the rhombic lip, i.e. the lower rhombic lip [22,34-38]. In fact, it has been demonstrated that the ION is the sole source of climbing fibers that innervate, besides the Purkinje cells in the cerebellar cortex, a specific part of the cerebellar deep nuclei. A projection from the cerebellar medullary zone to the ION completes this olivo-cortico- nuclear organization.

To date, little is known about the morphological and functional development of the cerebellum in man (the main research articles reported in the literature are presented in the Discussion). Therefore, we recently investigated the structural and biological patterns of human cerebellum in a wide group of fetal and infant death victims, aged from the 17th gestational week to one year of life. Besides the morphological aspects of the cerebellar structures, we evaluated in both sudden unexplained deaths and control cases with explained death, the expression of several biomarkers implicated in proliferative processes (c-fos, PCNA and apoptosis), as well as of somatostatin, a neurotransmitter strongly involved in central nervous system differentiation [39,40], and of EN2, a homeobox engrailed gene that seems to govern the anatomic organization of structures derived from the rhombic lip [41,42]. Finally, we analyzed the presence of reactive astrocytic gliosis, a non-specific response to brain injuries [43,44].

We evaluated whether morphofunctional disorders of the cerebellum are present in SUPD and SIDS victims, that we have previously demonstrated to be associated to specific alterations of the brainstem [7-18].

Finally, the suggestion by some authors that the protracted development of the cerebellum should make this structure particularly vulnerable to a broad spectrum of extrinsic environmental injuries [45-47] and the observation in our previous studies of a very high incidence of structural and/or functional alterations of different brainstem nuclei in stillborns and SIDS victims with smoker mothers [14,17,18], prompted us to verify whether maternal cigarette smoking could also be related to morphological and/or physiological developmental abnormalities of the cerebellum.

MATERIAL AND METHODS

The study was performed on 35 cases of sudden unexplained death (12 SIUD, 5 SNUD and 18 SIDS) and 20 control cases.

Here is briefly described the autopsy protocol routinely followed by our Institute [48-50]. This protocol provides an in-depth histological examination of the cardiorespiratory autonomic nervous system and, in particular, a complete examination on serial sections of the medulla oblongata, the pons, the lower third of the mesencephalon, and the cerebellum.

The cerebellum is excised from the brainstem by cutting through the cerebellar peduncles before proceeding to the horizontal sections. The cerebellar hemispheres are cut in the sagittal plane in serial parallel sections at 0.5 cm intervals, beginning at the vermis and then proceeding to the right and left of the midline.

In fetuses the brainstem, from the lower third of the midbrain to the lower pole of the olive, is

processed entire, whereas in infants it is cut into four blocks. The first, cranial block extends from the border between the medulla oblongata and pons up to the upper pole of the olivary nucleus. The second, intermediate block, corresponding to the sub-median area of the inferior olivary nucleus, tooks the obex as the reference point and extends 2-3 mm above and below the obex itself. The third, caudal block, includes the lower pole of the inferior olivary nucleus and the lower adjacent area of the medulla oblongata. A fourth block is cut of the brainstem including the ponto-mesencephalic portion, sectioned in a caudo-rostral direction.

The brainstem and cerebellum specimens are subsequently fixed in 10% phosphate-buffered formalin and embedded in paraffin. Transverse serial sections are made at intervals of 30 µm (levels). For each level, twelve 5 µm sections are obtained, two of which routinely stained for histological examination using alternately hematoxylin-eosin, and Klüver-Barrera stains.

Additional sections are subjected to immuno-histochemistry for the study of: a) Proliferating Cell Nuclear Antigen (PCNA); b) c-fos gene; c) apoptosis d) neurotransmitter somatostatin; e) EN2 gene; f) astrogliosis (glial fibrillary acidic protein). The remaining sections are saved and stained as deemed necessary for further investigations.

At the histological examination the pertinent nuclei are outlined, namely the arcuate nucleus, the hypoglossus nucleus, the dorsal vagus motor nucleus, the tractus solitarii nucleus, the ambiguus nucleus, the inferior olivary nucleus, the trigeminal tractus and nucleus, and the ventrolateral reticular formation in the medulla oblongata; the locus coeruleus, the parabrachial/Kölliker-Fuse complex in the pons; the cortex layers (external granular layer; molecular layer, Purkinje cell layer and internal granular layer) and the medullary deep nuclei (the dentate nucleus, the fastigial nucleus, the globose nucleus and the emboliform nucleus) in the cerebellum.

Immunohistochemical Methods

For the PCNA, c-fos, EN2 and GFAP immuno-histochemistry, sections are air dried at room temperature overnight, then deparaffinized and brought to TRIS-HCl-buffered saline solution (TBS-pH=7.6). After blocking the endogenous peroxidase with 3% hydrogen peroxide sections are immuno-stained with the specific monoclonal or polyclonal antibody using the avidin biotin complex method with overnight incubation. Diaminobenzidine is used as a chromogen with a light hematoxylin counterstain. Biotinylated rabbit anti-mouse IgM is added as secondary antibody.

For the apoptotic detection, after blocking the endogenous peroxidase, deoxynucleotidyl trans-ferase (TdT 0.3 U/ml) is used to incorporate dig-oxigenin-conjugated deoxyuridine (dUTP 0.01mM/ml) into the ends of DNA fragments. The signal of TdT-mediated dUTP Nick End Labeling (TUNEL) is then detected by an anti-digoxigenin antibody conjugated with peroxidase. Counterstaining is performed by immersing the slides in methyl green for 10 minutes.

Immunohistochemistry Evaluation

All the immunostained sections are examined by two independent and blinded observers at the light microscope. Only the cells with intense immunos-taining are considered to be positive.

DISCUSSION

From the literature, there are only few reports on the morphological and functional development of the human cerebellum, particularly focused on the cerebellar cortex.

Gadson and Emery in 1976 [51] have established neuronal density and DNA content of the cortex layers from birth to 14 years. This study demonstrated that aneuploid cells can be detected during normal cerebellar development and that cortex lobules reach their maturity starting 12 to 24 months after birth.

Succesively, Laquerrière ***et al***. [52] have studied the ontogeny of somatostatin receptors in the human cerebellar cortex from mid-gestation to the 15^{th} postnatal month. In short, these receptors, detectable at high concentrations in the fetal cerebellum, showed a progressive decline after birth.

Other Authors [53,54] have investigated the spatial and temporal distribution of apoptotic cells from the embryonic stage to 4 postnatal months. They agree that apoptosis is a physiologic phenomenon in cerebellar cortex development, associated with cell differentiation, migration and synaptic connection establishement.

Donkelaar ***et al***. [55], more recently, analyzed the morphogenesis and histogenesis of the cerebellum and the main cerebellar malformations affecting both the cerebellar vermis and the hemispheres.

Several works showed interest to cerebellar cortex with regard to SIDS victims. In particular, a cytological investigation, performed by Oehmichen ***et al***. [56], indicated increased density of Purkinje cells among the youngest SIDS infants, but a statistically significant difference compared with matched controls could not be established. Other authors observed a greater thickness and cell density of the EGL in SIDS cases than in controls [57,58].

In stillborns and infants, for which there was no explanation of death despite postmortem examina-tion, we previously demonstrated morpho-functional developmental alterations of specific nuclei of the brainstem that are classically implicated in respiratory control (the parabrachial/Kölliker-Fuse complex and locus coeruleus in the pons, the dorsal motor vagal nerve, the tractus solitarius, the ambiguus, the

hypoglossus, the arcuate nuclei in the medulla oblongata) [7-18]. In addition, in stillbirth victims the hypoplasia of the respiratory reticular formation has been observed to be often associated with pulmonary hypoplasia [10].

Thus, we believed that neuronal mechanisms responsible for defective compensatory responses to hypoxia were associated to substantial functional and/or structural changes of these nuclei.

Now, on the basis of the new results, we assert that even subtle alterations of the cerebellum can be involved in SUPD and SIDS. In fact, this study allows us to point out, besides the ontogenesis of the human cerebellar cortex, so far never underlined, the presence of different cerebellar biological features in unexplained death victims.

Development of the Cerebellar Cortex in Man

Here in we indicate, on the basis of our results, the dynamic sequence of morphological and biological steps that occur in human cerebellar cortex development, from the second trimester of gestation to the first postnatal year.

Around the 17^{th}-18^{th} weeks of fetal life, the external granular layer is the only recognizable layer, with a thickness of 10-15 small cells. Successively, a pluristratified Purkinje cell layer is also identifiable and, after 30 weeks, a 4-layered structure is evident: external granular layer (EGL), molecular layer (ML), Purkinje cell layer (PCL) and internal granular layer (IGL).

In prenatal life proliferation is the predominant phase, testified by c-fos and PCNA positivity, which is at first widespread throughout the thickness of the cortex, and subsequently restricted to the EGL. In fact, c-fos and PCNA are known to be markers of cell replication that regulate growth and differentiation in a variety of tissues. Firstly, the proto-oncogene c-fos synthesizes the corresponding DNA-binding protein as a signal for cell activation with mitogenic effect [59,60]. Thus, it precedes the PCNA manifestation indicative of DNA-duplication [61].

The biological features of cerebellar cortex development include positivity to the neurotransmitter somatostatin (SS) and the EN2 gene, observed to be more intensely expressed in the second and third gestational trimesters, respectively.

The SS is a neuropeptide with a wide distribution in the central nervous system (CNS), that controls various physiological processes [39,40]. We have previously documented intense positivity of the SS in brainstem nuclei involved in cardiorespiratory activity in stillbirths, and its abrupt reduction in postnatal deaths [12,13]. In the present study, too, we observed high levels of SS-expression, confined to the PCL during prenatal life and declining after birth. This finding diverges from the report by Laquerriére ***et al***. [52]. These authors, although with different methods (precisely, by binding and autoradiographic studies on frozen human cerebellar samples) found in prenatal life and in first postnatal months a high concentration of SS receptors mainly in the granule cells of both the external and internal layers.

However, we believe that, independently of a specific localization, SS plays an important role, rather than in neurotransmitter activity, in the development and maturation of the cerebellar cortex, as of other structures of the CNS.

Moreover, the EN are homeobox-engrailed genes that have been implicated in the development of the CNS. In particular, the EN2 gene seems to have an essential role in the anatomic organization of structures derived from the rhombic lip, in particular of the arcuate nucleus in the ventral surface of the brainstem and of the EGL in the cerebellar cortex [55].

We recently observed that the expression of the EN2 gene is very high in human ArcN neurons from the 17^{th} to 22^{nd} gestational week, decreases thereafter up to the first days after birth and then disappears [16].

The same temporal pattern of EN2 manifestation has been found in this study in the EGL neurons of the cerebellar cortex, confirming the same embryologic origin as that of the arcuate nucleus of the brainstem.

During the postnatal period, the EGL involutes, leading to the definitive 3-layered organization of the cerebellar cortex observed at 12 months of life. The main biological observations in the cerebellar cortex after birth are: 1) an abrupt decline of c-fos, PCNA, EN2 and SS expression and 2) an increasing number of dying cells in the EGL, responsible for its progressive reduction in thickness.

Apoptosis, in particular, seems to be an essential process in cerebellar cortex maturation [62,63]. An increasing number of granule cells, in fact, undergo programmed cell death, at subsequent stages of differentiation during the course of their migration to the IGL and the regressive process.

BIOLOGICAL ALTERATIONS OF THE CEREBELLUM IN SUDDEN UNEXPLAINED DEATH

Overall, in 62% of the sudden deaths, and only in 10% of the controls alterations of the biopathological features of the cerebellar cortex and of the dentate nucleus were present.

1) Alterations of the Cerebellar Cortex

Several variations from the above delineated cytoarchitectural and biological steps of cerebellar cortex development were observed prevalently in cases of sudden death. Unexpected findings in SIDS victims were: 1) morphological immaturity of the EGL neurons; 2) anomalous apoptotic models (programmed death of the Purkinje cells (Fig. **1**)

and/or of the IGL cells; lack of physiologic death of the EGL in its involutional process). In perinatal death: 1) negative expression of SS and of the EN2 gene; 2) intense c-fos positivity.

Dissimilar alterations have been reported in literature in the cerebellar cortex as regards SIDS victims [56-58]. We suggest that all the reported developmental changes, including our own observations, are different manifestations of delayed or faulty maturation of the cerebellar cortex in sudden unexpected death victims.

2) Alterations in the Dentate Nucleus

A significant increase of the reactive astrocyte density, besides the neuronal c-fos and apoptotic expression, were present in the dentate nucleus nearly exclusively of unexplained death victims.

Reactive astrocytic gliosis, identifiable by immunoreactivity for the glial fibrillary acidic protein (GFAP), is a non-specific response to brain injuries that can be observed in man after the first gestational trimester [43,44]. In particular, hypoxic events between 30 weeks of gestational age and 2 months of postnatal age usually induce the proliferation of activated astrocytes in the reticular nuclei, the tractus solitarii nucleus, the dorsal vagus motor nucleus, the arcuate nucleus and in the inferior olivary nucleus [22,43,44]. These areas, where activated astrocytes are found to be increased in number, are important in the physiology of breathing and arousal. Reduced respiratory and arousal control after hypoxia has been suggested to be the frequent final mechanism inducing death in SIDS [64,65].

We can add that in SUPD and SIDS the neuronal loss and increased reactive astrocyte density are frequent pathological findings also in the cerebellar dentate nucleus.

INVOLVEMENT OF THE CEREBELLUM IN THE RESPIRATORY CONTROL

The extensive reactive gliosis and the high frequency of c-fos and apoptotic neuronal positivity that we have found in the cerebellar cortex and in the dentate nucleus can be explained as reaction of the cerebellar structures to hypoxia.

Different studies have, in fact, demonstrated that the proto-oncogene c-fos, one of the immediate early genes, so defined because it does not require

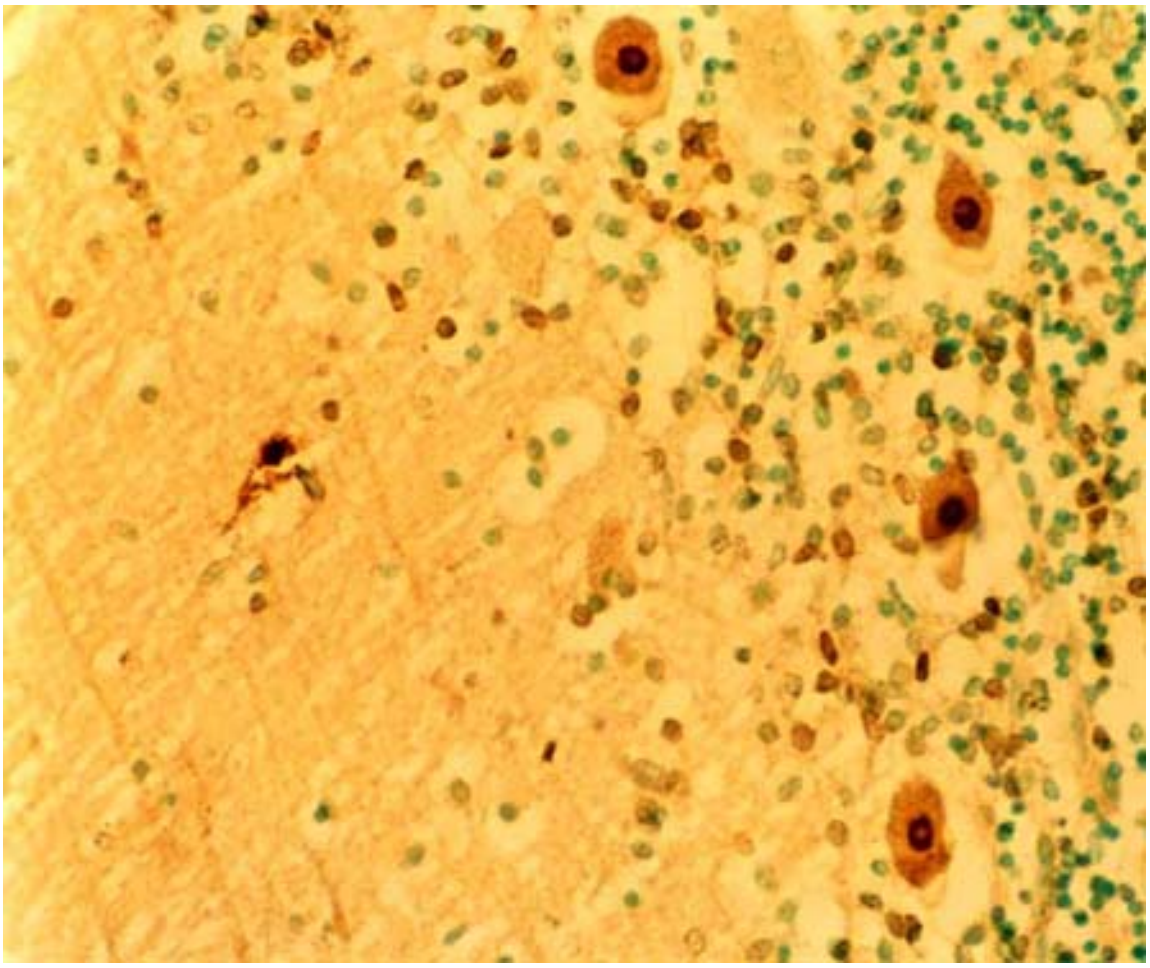

Fig. (1). Programmed cell death of Purkinje cells in the cerebellar cortex of a 7-month-old male infant deceased suddenly and unexpectedly. Magnification: 20x.

de novo synthesis of proteins, is the first gene activated by noxious signals, particularly in presence of hypoxia [59,60]. Therefore, immunohistochemical labeling of the fos-corresponding protein in activated neurons has been recognized as an important component of the neuronal response to injuries to the brain. Experimental works have demonstrated in particular that exposure to hypoxia markedly increases fos-immunoreactivity within the rat cerebellar deep nuclei [66,67].

Likewise, several Authors have provided evidence, on the basis of TUNEL-staining studies in rodents, that apoptosis is implicated in serious DNA fragmentation in the central nervous system neurons after hypoxic insults, particularly in the cerebellum [62,63].

The possible involvement of the deep nuclei in the respiratory modulation has been proposed in different experimental works [68-71]. In particular, altered ventilatory activity, predominantly expiratory, has been found in dentate nucleus of cats [44] after electrical stimulation of this region. Xu ***et al***. [71] found that the rat cerebellar deep nuclei contain respiratory-modulated neurons that receive inputs from peripheral and central chemoreceptors within the respiratory central network, and are important in increasing respiration during hypoxia. In fact, these nuclei, in particular the dentate nucleus, are critical structures for somatic muscle contraction control, including muscle tone of the upper airways muscles [69,71].

In addition, the cerebellar cortex contributes to the control of muscle activity, working as a clock determining the time intervals between two successive contractions. This activity also extend to respiratory muscles that are implicated in the restoration of blood pressure and breathing rhythm in cases of hypoxia [72].

The major risk factor for hypoxic events in the fetal and perinatal period is cigarette maternal smoking [73,74]. In cases of maternal smoking in pregnancy, carbon monoxide, a gaseous combustion product of nicotine, may readily cross the placenta, where it binds to hemoglobin, by passive diffusion. Consequently, carboxyhemoglobin inhibits oxygen release into the fetal tissues causing hypoxia, especially in the most susceptible organs, including the encephalon. Nicotine can thus give rise to long-term postmitotic DNA damage in neuronal cells, particularly in those areas of the brain regarded as selectively vulnerable, such as the cerebellum, because of the long duration of its development.

In our study, a significant correlation was evident between maternal smoking, SUPD/SIDS and alterations of biological features of the cerebellum.

We postulate that smoke can exert adverse influences on the successful deployment of the genetic programs committed to the protracted process of pre- and postnatal cerebellar development. The morpho-biological defects of the cerebellum observed in this study could have contributed to the failure of regulatory reactions to breathing challenges, with loss of coordination of upper airway muscle activity leading to sudden death in vulnerable periods.

This study provides further confirmation of the conclusions of our last papers [14,17,18], whereby maternal smoking is one of the main contributors to developmental neurological alterations in the offspring and can be responsible of sudden unexplained death in stillbirths, newborns and infants.

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