



Neuropathology of the intermediolateral nucleus of the spinal cord in sudden unexplained perinatal and infant death

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ABSTRACT

Experimental studies have demonstrated that breathing activity in rats is generated early in embryonic stages in rostral spinal cord, precisely in the intermediolateral nucleus, then establishing a spinal cord–brainstem network.

In this study we aimed to individuate and to define the developmental steps of the intermediolateral nucleus, still inadequately known in humans, in the thoracic spinal cord of a large series of perinatal and infant death victims, aged from 17 gestational weeks to 10 months of life. Besides we investigated a possible link between alterations of this nucleus and sudden unexplained perinatal and infant death.

The normal developmental pattern of the human intermediolateral nucleus consists of a progressive maturation of its neurons, that change from a round to a polygonal shape with long axons and significantly decrease in number.

Various degrees of intermediolateral nucleus hypodevelopment (neuronal immaturity in a normal structure/hypoplasia/agenesis) were found almost exclusively in unexplained fetal and infant death victims. Besides, a significant correlation was found between maternal smoking in pregnancy and the neuropathological results.

In conclusion this work underlines the negative effects of prenatal nicotine exposure on the development of autonomic nervous centers checking the vital functions, already in early gestational stages, when the integrity of the intermediolateral nucleus is indispensable for the first breathing bursts.

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1. Introduction

It is essential to have a clear understanding of the ontogeny of the neural networks in the human nervous system that are critical for survival at birth, particularly for respiratory system control. Maturation of the breathing neural circuit, making it capable of generating a robust rhythm-driving ventilation that can adjust to homeostatic needs, is essential for postnatal life. Moreover, episodic respiratory movements appear already *in utero*, that are necessary above all for normal lung development in the human fetus (Boddy and Dawes, 1975; Jansen and Chernick, 1991).

Given the inaccessibility of the human fetal nervous system for investigations *in vivo*, an advanced knowledge of normal development of the respiratory pattern has been obtained from experimental animal studies, prevalently on prenatal rodents. These have demonstrated that spontaneous rhythmic bursts are

generated early in embryonic stages in rostral spinal segments (Hanson and Landmesser, 2003; Ren and Greer, 2003).

In particular, the intermediolateral nucleus (ILN), located in the thoracic spinal cord, is able to transmit robust, spontaneous discharge patterns that spread into the rostral ventral medulla (RVM). In turn, the distribution of axonal labelling shows a descending RVM pathway that directly innervates the ILN of the thoracic spinal cord (Hanson and Landmesser, 2003; Pyner and Coote, 1998; Spanswick and Logan, 1990).

We have previously highlighted the developmental vulnerability of structures of the brainstem controlling the vital functions in sudden unexplained fetal death and the SIDS (Lavezzi et al., 2004, 2005b; Lavezzi and Maturri, 2008a,b). In this study, assuming the existence of a brainstem–spinal cord respiratory network also in man, we extended our research to the ILN in the thoracic spinal cord in a large series of perinatal and infant death victims, aged from 17 gestational weeks to 10 months of life. The first aim was to gain a detailed picture of the localization, morphology and developmental steps of this nucleus, that are still inadequately known in humans, and the second, to investigate a possible link between alterations of the ILN and sudden fetal and infant death.

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2. Materials and methods

A total of 65 brains were collected from 29 fresh ante-partum stillbirths (17–40 gestational weeks, with a peak from 36 to 40 weeks), 7 newborns who died within the first 2 days of life and 29 infants aged between 1 and 10 months (mean age: 3.5 months).

For each case, a complete clinical history, with particular reference to the maternal history and lifestyle, and including the death scene examination in infant victims, was collected. None of the mothers had any significant pathology. While taking the medical history, the mothers were asked for information about any smoking habit before, during and after pregnancy. Characteristics of the mothers by smoking category were: 11% ($n = 7$) reported smoking only in pregnancy, 23% ($n = 15$) smoking before, during and after pregnancy and 55% ($n = 36$) no smoking. In 7 cases (11%) no information about smoking was available.

Consent. Parents of all the victims of the study provided written informed consent to autopsy, with the Milan University L. Rossi Research Center institutional review board approval.

The victims were subjected to a complete autopsy, including examination of the placental disk, umbilical cord and membranes in perinatal deaths. In all cases an in-depth histological examination of the autonomic nervous system was made, according to the protocol routinely followed by the "Lino Rossi Research Center for The Study and Prevention of Unexpected Perinatal Death and the SIDS" of Milan University (Matturri et al., 2008).

In particular, after fixation in 10% phosphate-buffered formalin, the brainstem, where the main structures controlling the vital functions are located, and the spinal cord were processed and embedded in paraffin. Transverse serial sections of the midbrain, pons, medulla oblongata and spinal cord (cervico-thoracic tract) were made at intervals of 60 μm . For each level, five 5 μm sections were obtained, three of which were stained for histological examination using hematoxylin–eosin, Klüver–Barrera stains and Bielchowsky's silver impregnation technique. The remaining sections were saved for further investigations and stained as deemed necessary.

The routine histological evaluation of the brainstem was focused on the locus coeruleus, parafacial/facial complex, superior olivary complex, retrotapezoid nucleus, parabrachial/Kölliker–Fuse complex in the pons/mesencephalon, and on the hypoglossus, the dorsal motor vagal, the tractus solitarius, the ambiguus, the pre-Bötzinger, the inferior olivary and the arcuate nuclei in the medulla oblongata.

The in-depth examination of the ILN, the target of this study, was performed in all cases at the same spinal cord levels (from T1 to T5).

All the histological analyses were carried out by two independent and blinded observers and comparison among the results was performed to evaluate the inter-observer reproducibility.

In 35 cases, even after the in-depth autopsy examination, the death remained totally unexplained. A diagnosis of "unexplained stillbirth" was therefore made for 16 fetuses, who died suddenly after the 17th gestational week before complete expulsion or retraction from the mother, a diagnosis of "unexplained early neonatal death" for 4 newborns who died between birth and the first 2 postnatal days, and of SIDS for 15 infants who died within the first 10 months of life. In particular, ordinary myocardium lesions (such as muscle fibre necrosis), cardiac conduction system alterations (accessory atrio-ventricular communications, cartilaginous metaplasia of the fibrous body), positive microbiological findings, decreased heart weight, genetic variants in LQTS genes (long-QT syndrome), all features frequently reported in SIDS (Kariks, 1988; Matturri et al., 2000; Thiene, 1988; Goldwater, 2009; Kelmanson, 1996; Arnestad et al., 2007), were not found in the 15 cases of sudden infant death of the study. Regarding the infant sleeping position at death, 8 victims were noticed in prone position and 7 in supine position. In the remaining 30 cases, 13 stillbirths, 3 newborns and 14 infants, a precise cause of death was formulated at autopsy. These cases were regarded as "controls".

Table 1 summarizes the case profiles in this study, indicating the sex distribution, range of ages and death diagnosis.

2.1. Statistical analysis

The statistical significance of direct comparisons between the groups of victims was determined using analysis of variance (ANOVA). Statistical calculations were

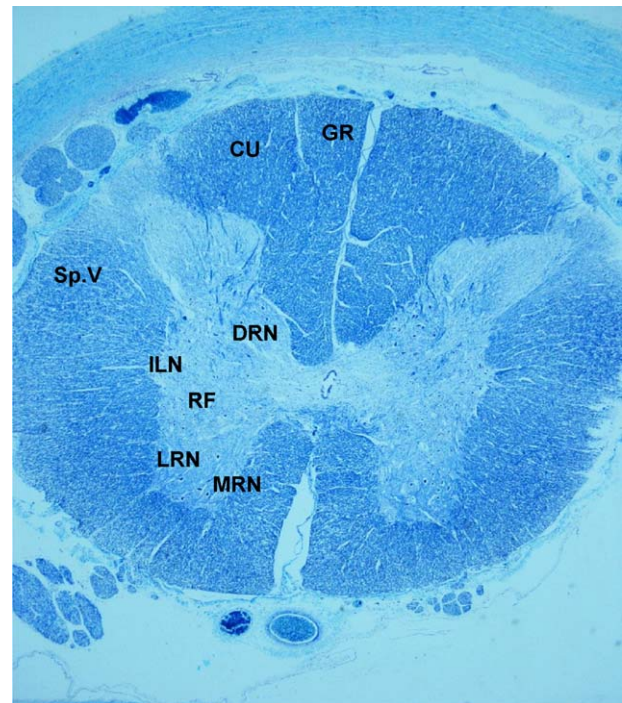


Fig. 1. Photomicrograph of a coronal section of thoracic spinal cord (T5) of a control newborn aged 1 month. Klüver–Barrera stain. Magnification: 4 \times . CU = cuneatus fasciculus; GR = gracilis fasciculus; ILN = intermediolateral nucleus; LRN = lateral reticular nucleus; MRN = medial reticular nucleus; RF = reticular formation; Sp.V = nucleus tractus spinalis trigemini.

carried out on a personal computer with SPSS statistical software. Statistical significance was set at a value of $p < 0.05$.

3. Results

Firstly, we proceeded to define the localization and the morphology of the ILN, the target of this study, in the spinal cord of the control cases. The classic text by Testut (1923) was used as reference.

Fig. 1 shows a histological transverse section of the thoracic spinal cord at level T5 in a control newborn (aged 1 month). The ILN can be seen as a cluster of neurons between the dorsal and ventral horns (intermediate tract of the horns), lateral to the central canal (the cerebrospinal fluid-filled space that runs longitudinally through the entire length of the spinal cord, contiguous with the ventricular system of the brain). In the ventral horn two nuclei of the reticular formation, the medial reticular nucleus (MRN) and the lateral reticular nucleus (LRN), are clearly visible.

Then we tried to trace the morphological developmental steps of the human ILN. At the earliest observation (17th to 18th

Table 1
Case profiles in the study.

Victims	Age (range)	Sex (n.)		Death diagnosis	
		M	F	Explained death (n. 30)	Unexplained death (n. 35)
Fetuses (n. 29)	17–40 gw	14	15	Necrotizing chorioamnionitis (n. 7) Congenital heart disease (n. 5) Potter's syndrome (n. 1)	Unexplained stillbirth (n. 16)
Newborns (n. 7)	1–4 pd	4	3	Congenital heart disease (n. 3)	Unexplained early neonatal death (n. 4)
Infants (n. 29)	1–10 m	16	13	Pneumonia (n. 6) Congenital heart disease (n. 7) Pericarditis (n. 1)	SIDS (n. 15)

gw = gestational week; pd = postnatal day; m = month; SIDS = sudden infant death syndrome.



Fig. 2. Normal structure of the intermediolateral nucleus at either side of the central canal in a histological section of thoracic spinal cord of a fetus aged 22 gws. Klüver–Barrera stain. Magnification: 20 \times .

gestational week – gw) the spinal cord clearly shows only one indistinct reticular nucleus in the ventral horn; the ILN is not visible. This histological aspect persists for several weeks, with the only difference that the two reticular nuclei (MRN and LRN, respectively) start to be discernible.

The ILN appears well recognizable around the 21st to 22nd gw as a group of numerous, round neurons assembled in the intermedial region of the horns, near and on either side of the central canal. They have a large eccentric nucleus, an evident nucleolus, strongly stained Nissl bodies at the periphery of a clear cytoplasm, and sketchy processes (Fig. 2). The same ILN morphology can be observed in fetuses who died at 26–28 gws.

Successively, the ILN neurons progressively decrease in number and take on a polygonal shape. At 32–34 gws the ILN consists of a limited number of large, elongated multipolar neurons with a dense Nissl substance widespread in the cytoplasm, a central nucleus and very long axons and dendrites (Fig. 3). These are the definitive features and remain constant after birth.

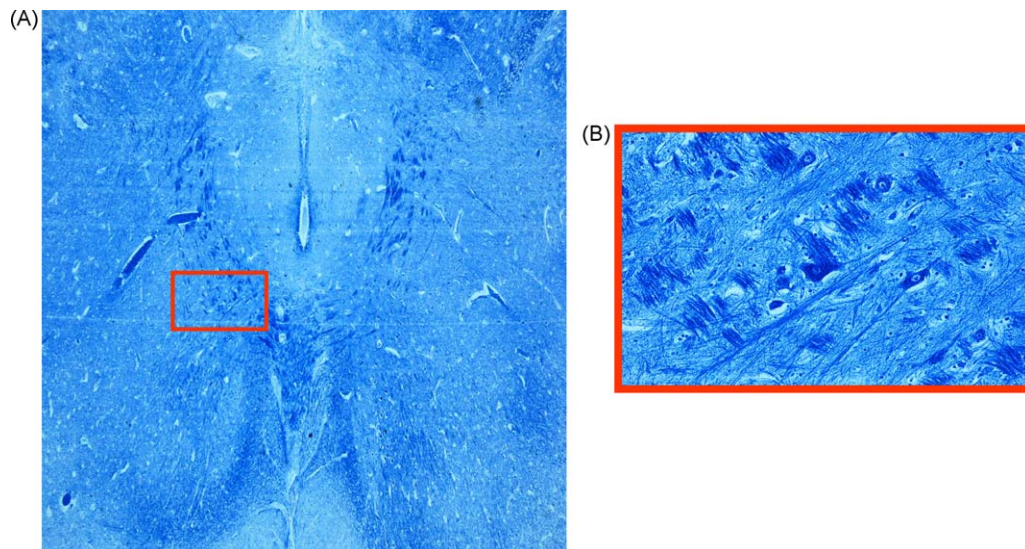


Fig. 3. Normal structure of the intermediolateral nucleus in a histological section of thoracic spinal cord of a fetus aged 32 gws. The nucleus (at greater magnification in B) consists of a limited number of large, elongated multipolar neurons with a central nucleus, a dense Nissl substance widespread in the cytoplasm, and very long axons and dendrites. Klüver–Barrera stain. Magnification: A = 10 \times ; B = 20 \times .

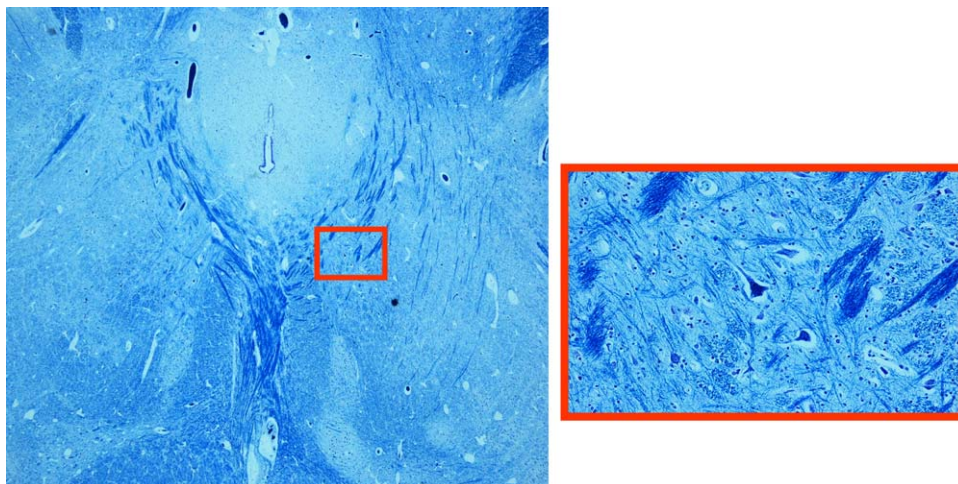


Fig. 4. Hypoplasia of the intermediolateral nucleus. Histological section of thoracic spinal cord of a SIDS victim aged 1 month. The intermediolateral nucleus (at greater magnification in B) shows a much lower number of well-differentiated neurons. Klüver–Barrera stain. Magnification: A = 10 \times ; B = 20 \times .

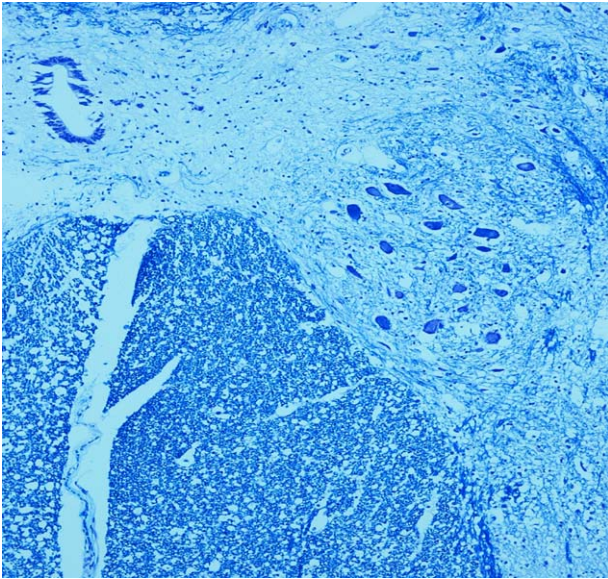


Fig. 5. Immaturity of the intermediolateral nucleus. Histological section of thoracic spinal cord of a SIDS victim aged 2 months. The intermediolateral nucleus shows a very immature structure with many round neurons, a marginal Nissl substance and short axons and dendrites. Klüver–Barrera stain. Magnification: 20 \times .

3.1. Neuropathology of the ILN in unexplained perinatal death and SIDS

In 21 victims of unexplained death (60%) we observed various alterations of the ILN as compared with age-matched subjects.

We found different developmental pathological patterns, namely:

- **Agenesis:** In 5 stillbirths who died between the 24th and the 26th gw the ILN was not observable.
- **Hypoplasia:** In 3 late fetal deaths (39–40 gws) and in 5 SIDS victims the ILN showed a much lower number of neurons. Nevertheless, these cells were well differentiated (Fig. 4).
- **Neuronal immaturity:** In 4 fetuses who died after the 35th gw of pregnancy and in 4 SIDS victims (all of whom died within the 3rd month of life), the ILN showed the same immature structure that may be found at 25 gws. The neurons were still numerous, round in shape, with a marginal Nissl substance and short axons and dendrites (Fig. 5).

Only two victims of the infant control group, who died of pneumonia, showed ILN hypoplasia. Thus, the incidence of the ILN alterations was significantly higher in sudden death victims than in infant controls ($p < 0.01$).

In 7 of the 9 SIDS victims with developmental abnormalities of the ILN, we also observed alterations of various brainstem and cerebellum structures (hypoplasia/agenesis of the arcuate, the pre-Böttinger and the raphé nuclei; delayed cerebellar cortex maturation). The most frequent association was with the hypodevelopment of the medullary arcuate nucleus and of the obscurus raphé nucleus.

With regard to fetal death victims, 7 of the 12 cases with delayed development of the ILN showed hypoplasia of the pontine facial/parafacial complex. In the remaining 5 cases, hypoplasia/agenesis of the ILN was the only alteration observed.

Finally, we correlated all the neuropathological findings with the mothers' smoking habit. A significant correlation was evident between maternal smoking, alterations of the ILN, and sudden death. In fact, 8 of the 12 unexplained stillbirths and 6 of the 9 SIDS victims with ILN hypodevelopment had smoker mothers ($p < 0.05$).

4. Discussion

While most mammalian brain structures do not become fully functional until significant postnatal development has occurred, the neuronal components of the respiratory system must already be mature and functional during fetal life and above all at birth, to generate a rhythm that allows for gas exchange and to integrate swallowing and other behaviour with breathing (Greer et al., 2006).

This statement has been borne out in our studies that have shown, in late fetal deaths, a very high incidence of alterations of the nervous centers appointed to control breathing. We hypothesize that in the last weeks of pregnancy, towards the time of birth, a general check occurs of all the essential functions for extra-uterine life, particularly of the respiratory activity. Sudden unexpected and unexplained fetal death could therefore be ascribed to a selective process of suppression in presence of developmental alterations of the respiratory rhythm-generating neuronal centers, to prevent the more serious event of neonatal death (Lavezzi and Matturri, 2008b; Lavezzi et al., 2005a).

In experimental studies on rats, Ren and Greer (2003) demonstrated that prior to the inception of an organized respiratory pattern, robust rhythmic discharges are generated within the rostral spinal cord during the formative embryonic period.

Examining the spinal rhythmic activity from the time of the initial axonal outgrowth to the inception of organized respiratory rhythmogenesis in late gestation, the same authors (Ren et al., 2006) demonstrated the ability of rat thoracic segments in the first developmental stages to transmit these spontaneous bursts into the spinomedullary axis, in the absence of synaptic activity. They suggest that non-synaptically mediated conductances, potentially by extracellular ionic flux, act in concert with neurochemical transmission to promote the spread of initial rhythmic breathing patterns in the developing nervous system.

This rhythmic pattern generated in spinal cord involves not only the respiratory but also the locomotor neuronal function, necessary for the fetal movements in utero and the normal development of fetuses. Greer et al. (1992), in brainstem–spinal cord in vitro preparations isolated from fetal rats, demonstrated that the respiratory and locomotor rhythmogenesis essentially start early in fetal life at the same developmental period, in the absence of input from higher centers. The central pattern generator of the locomotor rhythm, that produces the coordinated activation of flexor and extensor motoneurons during fetal movements, has been identified in lumbar spinal cord segments (Cazalets et al., 1995; McCrea and Rybak, 2008).

The main nucleus that initiates the respiratory function is located in upper thoracic segments of the spinal cord and is likely the ILN (Spanswick and Logan, 1990). This nucleus, when completely differentiated, contains the majority of the sympathetic preganglionic neurons (Cabot, 1996) involved in the autonomic activities, under the control of the rostral medulla respiratory neurons that provide both excitatory and inhibitory inputs (Deuchars et al., 1997). Thus the ILN has an important function as an effector of orthosympathetic reflexes.

The ILN receive inputs from the contiguous reticular formation (RF) in the intermediate zone of the spinal cord (Siegel and Sapru, 2006; Wang, 2008). Thus this nucleus can be interpreted as a structure closely related and belonging to RF.

The RF is an important wide network-system resulted by a astronomical number of connections between interneurons scattered over the entire central nervous system without distinct cytoarchitectural boundaries with the core located in the brainstem. Through these multisynaptic connections the RF controls vital functions, breathing included. Thus we would think that the RF, as it develops earlier than ILN (Hammer et al., 1981), represents the main coordinator and modulator of ILN activity.

In the present study, in the light of the data demonstrating the primary role of the ILN as a source of breathing function in embryonic rats, and its involvement in the bulbospinal respiratory pathways, we extended our research on this nucleus in man.

In particular, we investigated the developmental steps of the human ILN in a wide series of victims aged from 17 gws to 10 months of life, and highlighted various different morphological alterations that appear to be significantly related to sudden unexplained fetal and infant death.

We observed that the human ILN is well recognizable from the 21st week of pregnancy as a group of large, round neurons with poor Nissl substance and short processes in the intermedial region of the spinal horns.

Its normal developmental pattern consists of: (1) progressive maturation of the neurons, that change from a round to a polygonal shape provided with long axons and a widespread Nissl substance; (2) significant reduction in the neuronal number, very probably via the process of naturally occurring cell death (Buss et al., 2006).

Various degrees of ILN hypodevelopment (neuronal immaturity in a normal structure/hypoplasia/agenesis) were found almost exclusively in sudden death victims. A particularly interesting result was the observation, as the only nervous system alteration, of ILN agenesis in 5 stillbirths aged from 24 to 26 gw, the time when it should be present. Instead, ILN hypoplasia, with a decreased number of neurons or delayed neuronal maturation, was present in older victims who died in the last weeks of pregnancy. These data underline the great importance of the ILN in humans, too. Total absence of this structure in the second trimester of pregnancy does not allow continued fetal development. Thus, we can ascribe early fetal death without a plausible cause to agenesis of the ILN.

The alterations of this spinal nucleus observed in late fetal death and SIDS victims very probably result in perturbation of fetal breathing movements that are critical for proper lung development, but above all in a defective coordination of the respiratory rhythm after birth, leading to death. However, while the faulty neurodevelopment of the ILN may play a fundamental role in infant death, it is more difficult to explain the fetal death, given that breathing is not considered a “requirement” for intrauterine survival. We propose as a possible explanation the above-mentioned and reported hypothesis (Lavezzi and Maturri, 2008b; Lavezzi et al., 2005a) of a natural attrition of fetal subjects with anomalous maturation of neuronal centers involved in breathing to avoid the tragedy of postnatal death.

Thus, we can suppose that also the human ILN is a neural center that is capable of generating the first spontaneous bursts of respiratory rhythmic activity in early fetal stages, even before the establishment of the synaptic drive, through the release of neurotrophic signals and modifications of the intracellular milieu along the neural axis.

The respiratory control system, including normoxic ventilatory parameters and responses to hypoxia, is clearly influenced by perturbations during the perinatal period. It is becoming increasingly apparent that breathing disorders, including apneas during prematurity, the congenital central hypoventilation syndrome and the Prader Willi syndrome, are the consequence of adverse environmental factors *in utero*, and particularly of the absorption of maternal cigarette smoke (Vinit and Kastner, 2009).

In the case of maternal smoking in pregnancy, carbon monoxide, a gaseous combustion product of nicotine, may readily cross the placenta by passive diffusion, where it binds to hemoglobin. Consequently, carboxyhemoglobin inhibits the release of oxygen into the fetal tissues, causing hypoxia especially in the most susceptible organs, including the brain (Okeda et al., 1986).

Data from our previous studies provide insight into the negative effects of prenatal nicotine exposure on the structure and/or physiology of both medullary and cerebellar centers checking the vital functions (Lavezzi et al., 2005a,b). These findings are confirmed in the present work. In fact, a significantly higher percentage of sudden death victims with ILN hypodevelopment had smoker mothers.

In view of the increasing evidence that maternal smoking is one of the main contributors to neurological developmental alterations in fetuses and newborns, all pregnant women should be warned that smoking places their fetus at serious risk of a variety of morphological, genetic and functional abnormalities of the brain, that can lead to sudden, apparently unexplained death even in the 1st gws.

Conflict of interest

The authors declare that there is no conflict of interest.

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