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Adverse effects of prenatal tobacco smoke exposure on biological parameters of the developing brainstem

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We aimed to study the consequences of chronic exposure to tobacco smoke in utero on the morphological and functional maturation of the brainstem by comparing stillbirths of smoker mothers versus nonsmoker mothers. A total of 42 stillbirths, aged 25–40 gestational weeks, underwent autopsy according to our guidelines (http://users.unimi.it/~pathol/sids_e.html). The brainstem was studied on serial sections and by immunohistochemistry to assay the expression of the *EN2* gene, somatostatin (SS) and the tyrosine hydroxylase enzyme (TH). We observed a significant correlation between maternal smoking and sudden intrauterine unexplained death (SIUD), hypoplasia of the ArcN, no immunostaining of the EN2 in the arcuate nucleus (ArcN), and of TH in the locus coeruleus (LC) ($P < 0.05$). An increased incidence of maternal smoking was also observed in fetuses with SS negativity in the hypoglossus nucleus (HypoglN). Exposure in utero to maternal smoking may strongly interfere with brain biological parameters, giving rise not only to structural developmental abnormalities of the arcuate nucleus, but also to a decrease of noradrenergic activity in the LC, of *EN2* gene expression in the ArcN and of SS in the HypoglN.

Keywords: Smoke exposure; EN2; Somatostatin; TH; Brainstem development; Sudden intrauterine unexplained death; SIUD

Introduction

The smoking habit in pregnant women continues to be a major health concern. In particular, exposure to maternal tobacco smoke during pregnancy is associated with intrauterine growth retardation, abruptio placentae, low birth weight and a significantly higher risk of perinatal and infant mortality (Cnattingius and Lambe, 2002; Cnattingius et al., 1985; Cole et al., 1972; Fortier et al., 1994; Kyrklund-Blomberg et al., 2001; Wisborg et al., 2001).

The carbon monoxide coming from tobacco smoke easily crosses the placental barrier by passive diffusion, causing a 4-fold increase in the level of carboxyhemoglobin in umbilical cord blood; this has the property of inhibiting the release of oxygen into fetal tissues (Benowitz et al., 1990; Luck et al., 1985). The consequent chronic hypoxia may alter the physiological development of organs and tissues, especially those most susceptible to hypoxic damage, including the brain (Levin and Slotkin, 1998; Lichtensteiger et al., 1988).

Little is yet known about the effects on the central nervous system of prenatal exposure to cigarette smoke. Up to now, the studies performed on brain damage related to nicotine absorption in utero have been conducted in animal models, chiefly rats or mice (Gospe et al., 1996; Lichtensteiger et al., 1988; Navarro et al., 1989; Slotkin et al., 1987). In these species, birth occurs at a neurodevelopmental stage analogous to that of the end of the second trimester of a human pregnancy (24th week), so the findings are not generalizable to humans (Slotkin, 1992; Thomas et al., 2000). In fact, the main significant maturational processes of the human brain occur in the third gestational trimester. Nevertheless, many studies using animal model systems extend the nicotine exposure into first postnatal periods in rodents and monkey, mimicking exposure in third human gestational trimester (Slotkin et al., 2002, 2005; Thomas et al., 2000).

Herein, we aimed to assess whether prenatal absorption of nicotine could affect the developmental biology of the fetal human brain. By comparing a large group of prenatal stillbirths of smoker mothers versus nonsmoker mothers, we were able to study the consequences of chronic exposure to nicotine in utero on the morphological and functional maturation of the brainstem, the structure where the main cardiorespiratory centers are localized, evaluating a number of different aspects.

The study protocol, the same one as we routinely apply in all cases of sudden perinatal and infant death, according to the Lombardy Region Decree n. 11693 (Lombardy Region, 2002), included: (1) morphological examination in serial histological sections of the principal nuclei of the brainstem (the parabrachial/

Abbreviations: SIUD, sudden intrauterine unexplained death; IED, intrauterine explained death; SS, somatostatin; TH, tyrosine hydroxylase; ArcN, arcuate nucleus; HypoglN, hypoglossus nucleus; LC, locus coeruleus.

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Kölliker–Fuse complex and the locus coeruleus in the pons and the hypoglossus, the dorsal motor vagal, the tractus solitarius, the ambiguus, the inferior olivary, the arcuate nuclei and the ventrolateral respiratory reticular formation in the medulla oblongata) (Lavezzi et al., 2004c; Matturri et al., 2004); (2) immunohistochemical evaluation of the expression of the EN2 gene that is involved in brainstem embryogenesis and development (Davis and Joyner, 1988; Vollmer and Clerc, 1998) and (3) immunohistochemical analysis of the neurotransmitters (catecholamines and somatostatin) that control important physiological functions, in particular cardiorespiratory activity, already from the first weeks of gestation (Bacopoulos and Bhatnagar, 1977; Carpentier et al., 1996; Lavezzi et al., 2004a, 2005; Pickel et al., 1975).

Material and methods

We studied 42 fresh stillbirths. Stillbirth is defined as delivery of a dead fetus occurring at 25 completed weeks of gestation or later (Kalousek and Gilbert-Barness, 1997). Our collected cases, aged from 25 to 40 gestational weeks, were subjected to complete autopsy, including examination of the placental disk, umbilical cord and membranes and an in-depth histological examination of the cardiorespiratory autonomic nervous system, according to the protocol routinely followed by the Institute of Pathology, University of Milan and available on the web site: http://users.unimi.it/~pathol/sids_e.html (Matturri et al., 2004).

In particular, after fixation in 10% phosphate-buffered formalin, the brainstems, target of this study, were processed and embedded in paraffin. Transverse serial sections were made through the entire extension of the pons and medulla oblongata, and the blocks were cut at intervals of 30 μm. For each level, twelve 5 μm sections were obtained, two of which were routinely stained for histological examination using alternately hematoxylin–eosin and Klüver–Barrera stains. Three additional sections at each level were subjected to immunohistochemistry for the study of: (a) the EN2 gene expression; (b) the neurotransmitter somatostatin; and (c) the tyrosine hydroxylase enzyme (TH), involved in catecholamine (adrenaline, noradrenaline and dopamine) synthesis. The remaining sections were saved and stained as deemed necessary for further investigations.

At the histological examination, the principal nuclei of the brainstem were identified and analyzed, namely the parabrachial/Kölliker–Fuse complex, the locus coeruleus in the pons and the hypoglossus, the dorsal motor vagal nucleus, the tractus solitarius, the ambiguus, the inferior olivary, the arcuate nuclei and the ventrolateral respiratory reticular formation in the medulla oblongata. The identification of these structures was performed with the back-up of the Plates by Olszewski and Baxter (1982).

A diagnosis of "Sudden Intrauterine Unexplained Death (SIUD)" (Froen et al., 2001; Lavezzi et al., 2004c) was established for 30 fetuses aged 34–38 gestational weeks, who died suddenly for no apparent reason and with no definition of the cause of death despite postmortem examination.

In the remaining 12 victims, a precise cause of death was diagnosed at autopsy, and they were classified as "Intrauterine Explained Death (IED)". The causes included: a malpositioned umbilical cord in 2 cases, dilated cardiomyopathy in 1 case, hypertrophic cardiomyopathy in 3 cases, pneumonia in 1 case, severe chorioamnionitis in 3 cases, septicemia in 1 case and Potter's syndrome in 1 case.

While taking the medical history, the mother was asked for information about any smoking habit during pregnancy. Sixteen mothers (38%) declared they were active smokers (all of more than 3 cigarettes/day and all before becoming pregnant) and 26 (62%) were nonsmokers.

Table 1 summarizes the case profiles in this study, with their relative death diagnosis and mother's smoking status.

Immunohistochemical methods

EN2 immunohistochemistry

EN2 gene expression was detected by means of the monoclonal antibody Mab 4D9 that specifically recognizes engrailed EN-2 proteins (Davis et al., 1988).

Tissue sections (4 μm) were washed in 0.01 M PBS at pH 7.4 for 5 min and then rinsed in 3% hydrogen peroxide in PBS for 15 min in order to inactivate endogenous peroxidase. After peroxide treatment, tissue sections were washed three times for 5 min each in PBS and then incubated in 10% normal rabbit serum in PBS for 1 h followed by overnight incubation at 4°C with the monoclonal primary antibody 4D9 directed against the EN2 protein, diluted 1:75 in PBS.

Following incubation with the primary antibody, the sections were washed three times in PBS. For visualization of the EN2 protein, the sections were incubated for 1 h in biotinylated goat anti-rabbit immunoglobulin G diluted 1:400 in PBS. After another three rinses for 5 min each in PBS, the site of the antigen–antibody reaction was revealed with anti-mouse immunoglobulin followed by peroxidase–antiperoxidase complex. The unlabeled antiserum was placed on the sections for 2 h, diluted 1:200 at room temperature. The sections were then exposed to the peroxidase–antiperoxidase complex for 2 h at 1:200 dilution. The sites of peroxidase activity were visualized with 0.3% hydrogen peroxide in buffer containing 0.04% diaminobenzidine tetrahydrochloride and 0.5 g nickel ammonium sulfate. Slides were rinsed, dehydrated, mounted and examined by light microscopy.

Somatostatin (SS) immunohistochemistry

Lyophilized rabbit serum diluted in PBS was used in this study. This antiserum recognizes the N-terminal part of SS-28, composed of 28 amino acids.

SS immunoreactivity was visualized by the peroxidase–antiperoxidase method. In order to neutralize endogenous peroxidase, sections were pretreated with a solution of 0.3% hydrogenase peroxide for 20 min. After rinsing in buffer, sections were exposed for 48 h to the specific primary antiserum diluted 1:150 at

Table 1
Case profiles of the study

	Death diagnosis	Maternal smoking habit	
		Smokers	Nonsmokers
STILLBIRTHS (n = 42)	SIUD (n = 30)	15	15
	IED[a] (n = 12)	1	10

[a] Malpositioned umbilical cord (2 cases); congenital heart diseases: dilated cardiomyopathy (1 case); hyperthrophic cardiomyopathy (3 cases); pneumonia (1 case); severe chorioamnionitis (3 cases); septicemia (1 case); Potter's syndrome (1 case). SIUD = sudden intrauterine unexplained death, IED = intrauterine explained death.

25°C. After 10 min in buffer, the site of antigen–antibody reaction was revealed with anti-rabbit immunoglobulin followed by peroxidase–antiperoxidase complex. The unlabeled antiserum was placed on the sections for 2 h, diluted 1:200 at room temperature. The sections were then exposed to peroxidase–antiperoxidase complex for 2 h at a dilution of 1:200. The sites of peroxidase activity were visualized with 0.3% hydrogen peroxide in buffer containing 0.04% diaminobenzidine tetrahydrochloride and 0.5 g nickel ammonium sulfate. Slides were rinsed, dehydrated, mounted and examined by light microscopy.

Tyrosine hydroxylase (TH) immunohistochemistry

For TH immunostaining, the sections were rinsed three times in 0.1 M Trizma-buffered saline (TBS) followed by a 48-h incubation at 4°C with a 1/500 dilution of primary rabbit antiserum to TH. The dilutions were prepared with a solution of 1% normal goat serum (NGS) and 0.25% Triton X-100 in 0.1 M Tris–saline. This was followed by a 2.5-h incubation with biotinylated goat anti-rabbit immunoglobulin G diluted 1/200 with 1% NGS in Tris–saline. The tissue was then incubated for 2 h with the avidin–biotin complex diluted 1/100 with 1% NGS in Tris–saline (Vector). Between each incubation, the sections were rinsed three times with 1% NGS in Tris–saline. The sections were then treated for 6 min with a 0.05% solution of 3,3′ diaminobenzidine and 0.01% hydrogen peroxide, rinsed in phosphate buffer, mounted on gel-coated slides, cleared in xylene and coverslipped with Depex mounting medium.

For all the immunohistochemical methods, negative controls were prepared by replacing the respective primary antibodies with PBS in the incubation. In these procedures, staining always failed to occur.

Chemicals used	Source
Rabbit serum	Novocastlab—Newcastle,UK
Antibody 4D9	Vinci-Biochem, Florence, Italy
Goat anti-rabbit immunoglobulin G	Novocastra Laboratories—Newcastle, UK
Peroxidase–antiperoxidase complex	Vector Laboratories, Burlingame, CA, USA
Rabbit antiserum to TH	Novocastra Laboratories—Newcastle, UK

Immunohistochemistry evaluation

To evaluate the immunoreactivity for TH, SS and EN2 proteins, we applied a rating system for each method, based on the examination of all the immunostained sections along the caudorostral extension of the brainstems. Only neurons with intense brown staining were considered to be positive.

We quantified the scoring for each brainstem nucleus as follows:

− = no positive neuron, or presence of weakly immunostained neurons;
+ = a number of positive neurons ≤ 30% (moderate positivity);
++ = a number of positive neurons >30% (strong positivity).

The scores, referred to bilateral sides of every nucleus, were assigned by two independent and blinded observers, and comparison among the data was performed to evaluate the interobserver reproducibility. In cases of discordance among the investigators, the case was reviewed and discussed until a unanimous result was obtained.

Statistical analysis

The associations between maternal cigarette smoking habit and sudden intrauterine death, ArcnN hypoplasia, EN2, SS and TH expression were determined using Fisher's Exact Test. The selected level of significance was $P < 0.05$.

Results

Morphological study

Histological examination performed on brainstem serial sections demonstrated, in most of the 42 cases, a normal morphology of the principal nuclei throughout their extension. Nevertheless, developmental abnormalities of the arcuate nucleus (ArcN) emerged in 13 of the 30 sudden death victims (SIUD) (43%) and in none of the stillbirths who had died of known causes (IED) (Table 2).

Different degrees of hypodevelopment of the ArcN were observed: bilateral hypoplasia in 5 cases (Fig. 1), partial hypoplasia in 6 cases and monolateral hypoplasia in 2 cases. Hypoplasia of the respiratory reticular formation was also present in concomitance with ArcN hypodevelopment in 7 SIUD cases.

Immunohistochemical study

EN2 protein

In 30 cases, immunohistochemical analysis of the EN2 protein expression showed an intense positivity (score: ++) in all the neurons of the ArcN and a moderate expression (score: +) in the inferior olivary nucleus. In all the other structures, the *EN-2* gene resulted unexpressed. In 12 sudden prenatal deaths (SIUD), we found negativity for the EN-2 protein specifically related to the ArcN neurons (Table 2).

Table 2
Distribution of the morphologic, genetic and physiologic brainstem alterations in the analyzed cases

	Death diagnosis	Hypoplasia of ArcN	EN2-negative neurons in ArcN	SS-negative neurons in HypoglN	TH-negative neurons in LC
Stillbirths (n = 42)	SIUD (n = 30)	13	12	10	17
	IED (n = 12)	–	–	2	2

SIUD = sudden intrauterine unexplained death; IED = intrauterine explained death; ArcN = arcuate nucleus; SS = somatostatin; HypoglN = hypoglossus nucleus; LC = locus coeruleus.

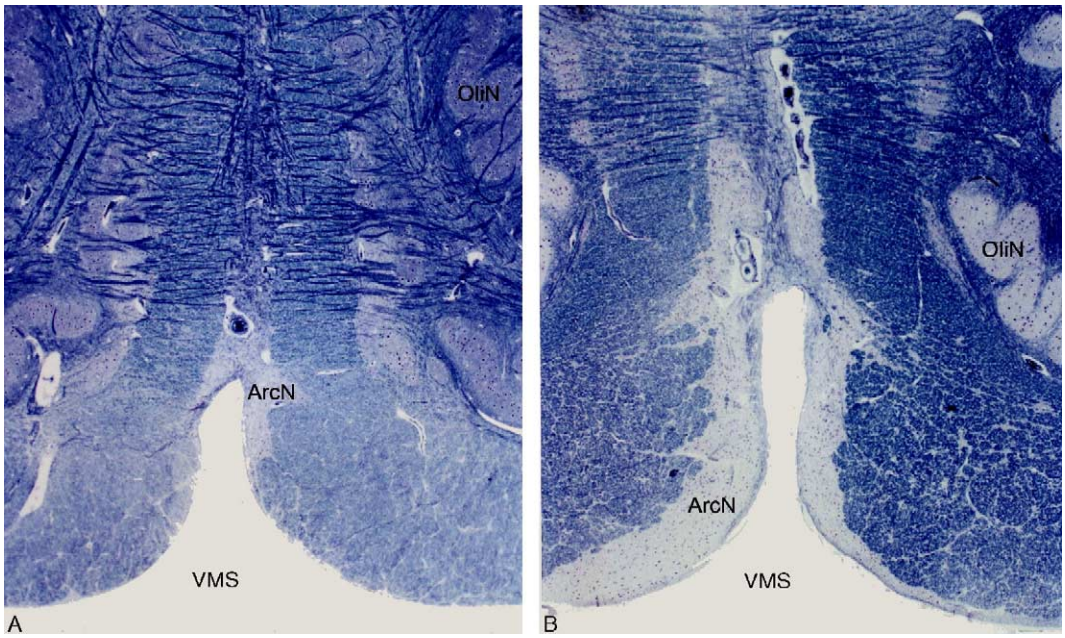

Fig. 1. Hypoplasia of the arcuate nucleus in a sudden intrauterine unexpected death at the 40th gestational week (A) compared to a normal arcuate nucleus in an age-matched stillbirth dead of known cause (B). ArcN = arcuate nucleus; VMS = ventral medullary surface; OliN = olivary nucleus. Klüver–Barrera, 2.5×.

Somatostatin (SS) protein

SS immunopositive neurons were present in the respiratory nuclei of the brainstem with a variable distribution. In particular, an intense SS expression was seen in the cell bodies and fibers of the parabrachial/Kölliker–Fuse complex and of the locus coeruleus in the pons and in the tractus solitarii nucleus, the dorsal vagus motor nucleus, the ambiguus nucleus, the hypoglossus nucleus, the arcuate nucleus and the respiratory reticular formation in the medulla oblongata of 30 cases. Conversely, in 12 stillbirths (10 SIUD and 2 IED) (17%), the neurons of the hypoglossus nucleus were not immunostained for SS (Table 2).

Tyrosine hydroxylase (TH) protein

In the medulla oblongata of all the analyzed cases, TH immunohistochemistry showed immunoreactive neurons in the dorsal motor vagal nucleus, in the tractus solitarius nucleus and in the ventrolateral reticular formation. The score of positive neurons ranged from + to ++, with no difference between the SIUD and IED victims. On the contrary, in the pons, we found a variable expression of the TH, limited to the locus coeruleus. In fact, in 17 sudden unexplained deaths and in only 2 victims of known diseases, TH was unexpressed in this nucleus, whereas in 25 cases, the locus coeruleus showed a prevalent TH score corresponding to ++ (Table 2).

As stated above, Table 2 shows the distribution of the above specified alterations, namely brainstem arcuate nucleus hypoplasia, negative expression of the *EN2* gene in the arcuate nucleus, of the SS neurotransmitter in the hypoglossus nucleus and of the TH enzyme in the pontine locus coeruleus, among the victims of both SIUD and IED.

Table 3 shows the distribution of the physiologic and genetic aspects analyzed, in cases with both hypoplasia and normal structure of the ArcN. In 6 cases of SIUD, arcuate nucleus hypoplasia was associated to negative expression of the EN2 protein in its residual neurons. Moreover, another 8 victims of sudden death with a normal morphologic and genetic pattern of the ArcN showed negativity for both SS in the hypoglossus nucleus and TH in the locus coeruleus.

Finally, the neurodevelopmental defects observed in the brainstem were correlated to the mother's smoking habit, as shown in Table 4. We observed a significant correlation between maternal smoking and the following parameters: SIUD, hypoplasia of the ArcN, negativity of EN2 immunostaining in the ArcN, negativity of TH in the locus coeruleus ($P < 0.05$). Although the no-staining of SS in the neurons of the HypoglN was statistically related with maternal smoke ($P < 0.05$), it was not significantly related with SIUD ($P > 0.05$) (Fig. 2).

Among 13 SIUD with ArcN hypoplasia, 11 (85%) had smoker mothers and only 2 nonsmokers. The absence of EN2 expression was evident in 11 SIUD with smoker mothers versus only in one case with a nonsmoker mother. In the HypoglN, SS no-staining was observed in 9 stillbirths exposed to smoke (8 SIUD and 1 IED) and in 3 (2 SIUD and 1 IED) not exposed to smoke in utero. Finally, the loss of TH expression in the LC was similarly more

Table 3
Distribution of the genetic and physiologic defects among cases with normal and abnormal morphology of the ArcN

Brainstem alteration	ArcN hypoplasia		ArcN normal structure	
	SIUD ($n = 13$)	IED ($n = 0$)	SIUD ($n = 17$)	IED ($n = 12$)
EN2 negativity in ArcN ($n = 12$)	6	–	6	–
SS negativity in HypoglN ($n = 12$)	–	–	10[a]	2
TH negativity in LC ($n = 19$)	–	–	17[a]	2

SIUD = sudden intrauterine unexplained death; IED = intrauterine explained death ArcN = arcuate nucleus; SS = somatostatin; HypoglN = hypoglossus nucleus; LC = locus coeruleus.

[a] Eight of these SIUD cases showed both SS and TH negativity.

Table 4
Distribution of the neurodevelopmental defects of the brainstem in unexplained and explained fetal deaths in relation to maternal smoking habit

Brainstem alteration	Smoking mothers		Nonsmoking mothers	
	SIUD	IED	SIUD	IED
Hypoplasia ArcN (n = 13)	11	–	2	–
EN2 negativity ArcN (n = 12)	11	–	1	–
SS negativity HypoglN (n = 12)	8	2	2	0
TH negativity LC (n = 19)	15	2	1	1

SIUD = sudden intrauterine unexplained death; IED = intrauterine explained death ArcN = arcuate nucleus; SS = somatostatin; HypoglN = hypoglossus nucleus; LC = locus coeruleus.

frequent among stillbirths from smoker mothers (16 cases: 15 SIUD and 1 IED), whereas this finding was present in only 1 SIUD and 1 IED from nonsmoker mothers.

The 6 cases with simultaneous presence of ArcN hypoplasia and negative *EN-2* gene expression and the 8 cases with both TH no-staining in the LC and SS no-staining in the HypoglN were all fetuses of smoker mothers.

Discussion

Alterations in the autonomic nervous system have been reported in infants born of smoker mothers (Cole et al., 1972), whereas the specific substrates of fetal brain damage caused by prenatal smoke absorption require elucidation.

The great part of the knowledge in this field is owed to experimental studies (Gospe et al., 1996; Lichtensteiger et al., 1988; Navarro et al., 1989; Slotkin et al., 1987). In particular, several authors (Gospe et al., 1996; Johns et al., 1982) have demonstrated that perinatal exposure to smoke significantly affects the biochemical composition of the brain directly in fetuses and seems to cause a reduction in DNA concentration, particularly in the hindbrain. On the contrary, the reports on effects of environmental tobacco smoke exposure on human fetuses are rare (Caims and Wonnacott, 1988; Kinney et al., 1993).

On the basis of the results of this study, we can hypothesize that in human fetuses of smoker mothers deep morpho-functional and transcriptional alterations can occur in the brainstem, a structure rising from the hindbrain.

We have reported significantly increased rates of neurodevelopmental defects related to the ArcN structure, *EN2* gene, and TH enzyme expression, in 42 fresh fetuses, victims of unexplained and explained deaths, some of whose mothers smoked 3 or more cigarettes a day before and during pregnancy ($P<0.05$, Fisher's Exact Test).

Hypoplasia of the ArcN was, in fact, significantly more frequent in stillborns with smoker mothers (85%) compared with nonsmoker mothers.

In addition, the *EN2* gene that has been suggested in experimental studies (Davis and Joyner, 1988; Vollmer and Clerc, 1998) to play an important role during differentiation of the nervous structures rising from the rhombic lip, particularly of the ArcN, was strongly expressed in all the neurons of this nucleus, prevalently in fetuses of nonsmoker mothers. On the contrary, the *EN2* gene was unexpressed in 12 SIUD victims, 11 with smoker mothers and only 1 with a nonsmoker mother. Six of these stillborns (50%) were affected by hypoplasia of the ArcN.

With regard to SS, we observed that, during fetal development, this neurotransmitter exhibits a widespread anatomical distribution throughout both cell bodies and fibers of the respiratory nuclei in the brainstem (the tractus solitarii nucleus, the dorsal vagus motor nucleus, the ambiguus nucleus, the hypoglossus nucleus, the arcuate nucleus, the respiratory reticular formation, the parabrachial/Kölliker–Fuse complex and the locus coeruleus). Therefore, we can assume that the high diffusion of SS in brainstem respiratory centers during fetal life is strongly correlated with inhibition of intrauterine breathing.

Moreover, in 9 victims with smoker mothers (8 SIUD and 1 IED) and only in 3 stillbirths (2 SIUD and 1 IED) with nonsmoker mothers, we found negativity for SS in the hypoglossus nucleus (HypoglN), without morphological evidence of cyto-histologic alterations.

The HypoglN, even if it is not considered as a classic respiratory center, contains motoneurons which exert respiratory-related rhythmical discharges after birth. In particular, it controls the extrinsic muscles of the tongue, mainly the genioglossus that is important in maintaining a patent airway, especially during inspiration (Roda et al., 2002; Withington-Wray et al., 1988).

We deduce that the victims who exhibited neurons with no-staining SS in the hypoglossus nucleus in intrauterine life had undergone developmental delay of somatostatinergic transmission maturation in this nucleus, probably determined by smoke absorption in utero. Incorrect differentiation of the HypoglN may consequently have deleterious effects on the inhibition of ventilatory activity, leading to abnormal breathing movements in fetuses that can have a fatal outcome (Lavezzi et al., 2004a).

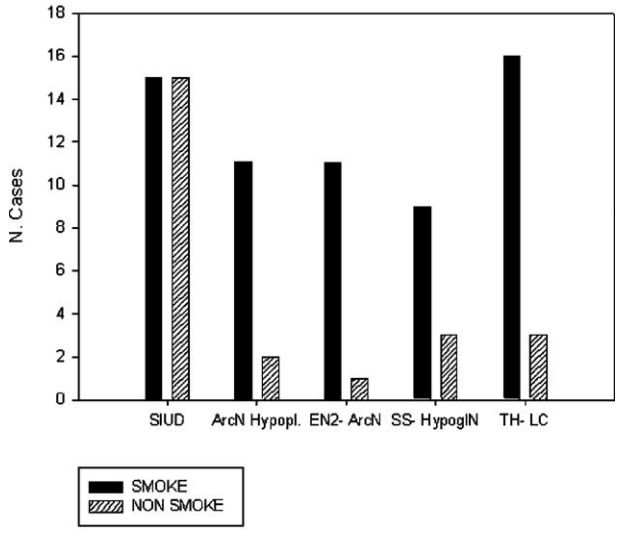

Fig. 2. Comparison of fetuses of smoking mothers versus fetuses of nonsmoking mothers. We observed a significant correlation between maternal smoking and the following parameters: sudden intrauterine unexplained death (SIUD), hypoplasia of the arcuate nucleus (ArcN Hypopl.), negativity of EN2 (EN2−) immunostaining in the ArcN, negativity of tyrosine hydroxylase (TH−) in the locus coeruleus (LC) ($P < 0.05$). Although the negativity of somatostatin (SS−) in the neurons of the hypoglossus nucleus (HypoglN) was statistically related with maternal smoke ($P < 0.05$), it was not significantly related with SIUD ($P > 0.05$).

We postulate that other neurochemical abnormalities observed in the brainstem, relative to TH, a rate-limiting enzyme in catecholamine biosynthesis, could also be ascribed to smoking in pregnancy (Bacopoulos and Bhatnagar, 1977; Pickel et al., 1975). In fact, we detected physiological defects of TH, confined to the pontine locus coeruleus (LC), the major producer of noradrenaline, above all in a wide subset of sudden unexplained fetal deaths of smoker mothers. Despite a normal LC morphology, these cases (15 SIUD and 1 IED with exposure and only 1 SIUD and 1 IED without exposure to smoke in utero) presented neurons with no TH immunostaining or decreased TH immunostaining. High concentrations of the TH protein were, instead, detectable in the LC neurons of all the other stillborns.

On the basis of the results of this study, we support the hypothesis of a close relation between maternal cigarette smoking during pregnancy and many abnormal processes in human brain development. Indeed, smoke exposure in utero may strongly interfere with biological parameters of the brain, giving rise not only to the structural developmental abnormalities of the arcuate nucleus we have previously documented (Lavezzi et al., 2004b), but also to a decrease of noradrenergic activity in the LC, of *EN2* gene expression in the ArcN neurons and possibly of SS control in the HypoglN.

All these abnormalities can have serious consequences, such as pulmonary hypodevelopment, that we have already reported to be significantly associated to ArcN hypoplasia (Matturri et al., 2003), even leading to apparently unexplained sudden death of the fetus.

Our observations of brainstem alterations in some victims of nonsmoker mothers could be attributable to the fact that many women in pregnancy are exposed to nicotine as a result of passive smoking, through the spouse or in the work environment. Furthermore, it should be considered that retrospective assessment of the maternal smoking habit, particularly if performed after the fatal event, is sometimes unreliable (Heath et al., 2003; Walsh et al., 1996) due to the fact that smoker mothers are reluctant to admit tobacco use, possibly because of feelings of guilt.

In conclusion, all pregnant women should be warned that smoking places their unborn children at serious risk of a variety of morphological, genetic and functional abnormalities of the brainstem that can cause stillbirth.

Moreover, the low success rate of smoking cessation among pregnant women suggests that efforts to reduce tobacco use in pregnancy should focus on preventing cigarette smoking among teenaged girls. In fact, most women begin smoking as teenagers, becoming addicted to nicotine early in life and finding it very difficult to quit, even during pregnancy.

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