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Original Article

Hypoplasia of the arcuate nucleus and maternal smoking during pregnancy in sudden unexplained perinatal and infant death

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Maternal smoking during pregnancy is the most important risk factor for sudden perinatal and infant death in more industrialized countries. The frequent observation of hypoplasia of the arcuate nucleus in the brainstem of these victims prompted the verification of whether maternal cigarette smoking could be related to defective development of this nucleus during intrauterine life, by affecting the expression of specific genes involved in its developmental process. In serial sections of the brainstem of 54 cases of sudden and unexplained fetal and infant deaths (13 stillbirths, 7 neonatal deaths and 34 sudden infant death syndrome (SIDS) victims), morphological and morphometrical analysis was used to observe the different structural alterations of the arcuate nucleus (bilateral hypoplasia, monolateral hypoplasia, partial hypoplasia, delayed neuronal maturation and decreased neuronal density) detected in 24 cases (44%). Correlating this finding with smoking in pregnancy, a significantly increased incidence of cytoarchitectural alterations of the arcuate nucleus was found in stillborns and SIDS victims with smoker mothers compared to victims with non-smoker mothers. Moreover, the observation of a wide range of developing morphological defects of the arcuate nucleus related to maternal smoking led to the hypothesis that the constituents of the gas phase in cigarette smoke could directly affect the expression of genes involved in the development of this nucleus, such as the homeobox En-2 gene.

Key words: arcuate nucleus, brainstem, maternal smoking, sudden infant death syndrome, sudden perinatal death.

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INTRODUCTION

Our anatomo-pathologic investigations on the autonomic nervous system in fetal and infant deaths have indicated that hypoplasia of the medullary arcuate nucleus, which is located at the ventral surface of the medulla oblongata and is implicated in central chemoreception, cardiopulmonary and blood pressure response, is often present both in sudden infant death syndrome (SIDS) and in unexplained perinatal death (stillbirth after the 25th week of gestation, as well as neonatal death within the first month of life).[1–4] This has made it possible to establish the congenital and probably genetically based nature of this developmental defect, showing a link between SIDS and unexplained fetal death.

Stillbirth is defined as delivery of a dead fetus occurring at or after 25 completed weeks of gestation. Neonatal death refers to death of liveborn within the first month of life and SIDS, to postneonatal infant mortality under 1 years of age.

The present study focused on morphological and morphometrical evaluation of the arcuate nucleus and the search for any correlation between its cytoarchitectural pattern and maternal smoking during pregnancy. Structural anomalies of the arcuate nucleus can feature a wide range of patterns. In particular, from our studies it has emerged that hypoplasia of the arcuate nucleus can be bilateral, monolateral (generally in the right side) or partial (limited to a tract of its extension, mostly to the lower two thirds). In addition, we have observed in some cases complete agenesis of the arcuate nucleus and in others, even in cases of normal structures, delayed neuronal maturation and/or decreased neuronal density.[5]

Many epidemiological studies have reported that parental cigarette smoking, particularly by the mother during pregnancy, is the most important risk factor for both SIDS and late fetal death in more industrialized countries.[6–10] In this work we aimed to verify, in a large number of cases of

death between the 25th gestational week and the first year of life, whether maternal cigarette smoking could be related to a developmental abnormality of the arcuate nucleus induced during intrauterine life.

MATERIALS AND METHODS

We analyzed a total of 54 cases of sudden fetal and infant deaths (13 stillbirths, 7 neonatal deaths and 34 SIDS victims, aged from 25 gestational weeks to 12 postnatal months) that remained unexplained after thorough case investigations and complete autopsies, including examination of the placental disks, umbilical cords and membranes in fetuses.

The medulla oblongata was submitted to a detailed examination. All samples were fixed in 10% phosphate-buffered formalin, processed and embedded in paraffin. Transverse serial sections were made through the entire extension. The block was cut at intervals of 30 µm.

For each level, twelve 5-µm sections were obtained, three of which were routinely stained using alternately HE, Bielschowsky and KB stains for histological examination, while the remaining nine were saved and stained as deemed necessary for further investigations. The number of levels and, consequently, of serial sections throughout the entire medulla varied in relation to the age of the subject analyzed. In the fetus, from the 25th week of gestation, the average number of sections was 360 (corresponding to 30 groups of 12 serial sections), while in the fetus at term and/or in the newborn it was 600 (50 groups). In SIDS victims of 3–4 months or over 6 months a total of 900 and 1440 sections were obtained from 75 and 120 levels, respectively.

A depth morphometric analysis of the arcuate nucleus, object of the present study, was performed with an Image-Pro Plus Analyzer (Media Cybernetics, Silver Spring, MD, USA) at the three main levels of the brainstem: (i) rostral (at the border between medulla oblongata and pons); (ii) intermediate (at obex level); and (iii) caudal (in correspondence to lower pole of the olivary nucleus and of the area postrema).

The following parameters of the arcuate nucleus were evaluated: (i) section area (expressed in mm^2), (ii) neuronal density (expressed as number of neurons per mm^2), and (iii) neuronal size (cytoplasmic and nuclear area, expressed in μm^2). All the morphometric measurements were indicated as mean values and standard deviation.

During the anamnesis, the mother was asked for information on her smoking habit before and during pregnancy. Women were defined as smokers if they smoked one or more cigarettes a day. Smoking was assigned to two categories (smokers versus non-smokers), and subdivided in relation to the number of cigarettes smoked daily (0, 1–5, and 6 or more cigarettes a day).

Statistical analysis

The association between smoking during pregnancy and alterations of the arcuate nucleus was evaluated by Cox regression analysis. The statistical value of the correlation was determined using the Fisher exact test. The selected level of significance was $P < 0.05$.

RESULTS

Histological examination performed on serial sections of the brainstem demonstrated a normal structure of the arcuate nucleus in 30 of the 54 victims (Fig. 1). In these cases the nucleus was easily recognizable throughout its extension on either side of the ventral surface of the medulla between the caudal pole of the inferior olive and the caudal border of the pons.

In the cranial sections the arcuate nucleus was identifiable both medially and in the initial lateral portions. At the intermediate levels, in correspondence to the obex it appeared clearly in the lateral portions, while it was reduced in the medial area. Down toward the area postrema, particularly in the fetuses, the arcuate nucleus was detectable in the lateral portions but absent medially. The mean section areas at these three levels were 1.80 ± 0.70, 1.28 ± 0.52 and 1.15 ± 0.35 mm^2, respectively. The neurons generally presented a polygonal, bipolar aspect, with a large vesicular nucleus, loose chromatin and evident nucleolus. Their mean density was 122 ± 14 neurons/mm^2, the mean neuronal size corresponded to 110.8 ± 15.6 μm^2 relative to nuclear areas and 208.2 ± 26.4 μm^2 for the cytoplasmic areas.

Structural abnormalities of the arcuate nucleus emerged in 24 cases (44%) (eight perinatal deaths, includ-

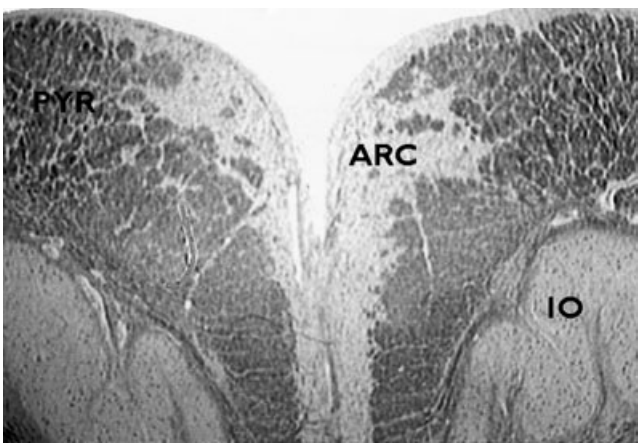

Fig. 1 Normal structure of the arcuate nucleus in a sudden infant death syndrome victim (male, aged 3 months) (KB stain; magnification: ×25). ARC, arcuate nucleus; IO, inferior olive; PYR, pyramid.

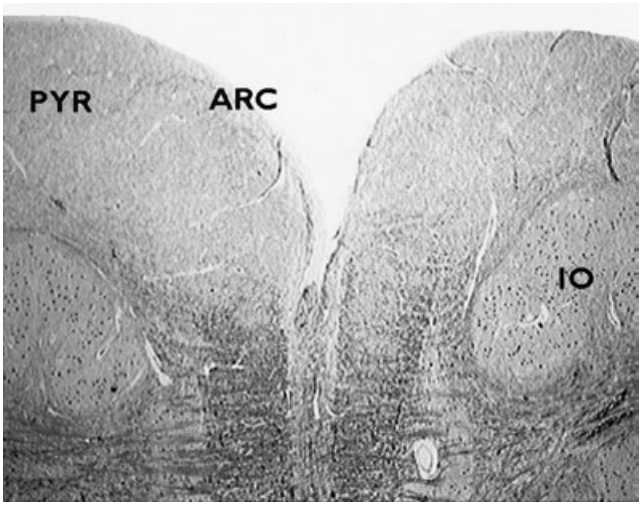

Fig. 2 Hypoplasia of the arcuate nucleus in a sudden infant death syndrome victim (male, aged 2 months) (KB stain; magnification: ×25). ARC, arcuate nucleus; IO, inferior olive; PYR, pyramid.

ing five fetuses and three newborns; and 16 infants who died of SIDS).

Different degrees of this developmental defect were observed (Fig. 2). Bilateral hypoplasia was found in nine cases, monolateral (on the right side) in four cases, partial (limited to the lower two thirds of its extension) in four cases and agenesis in three cases. In total, the mean area values in the cases of hypoplasia were: 0.80 ± 0.18 mm^2 at the rostral level, 0.48 ± 0.10 mm^2 at the intermediate and 0.51 ± 0.12 mm^2 at the caudal level.

The morphometric parameters of the neurons (density and size) in the cases of arcuate nucleus hypoplasia were superimposable to those obtained in cases with normally structured arcuate nucleus (mean number of neurons per mm^2, 118 ± 16; mean nuclear area, 110.9 ± 22.3 μm^2; mean cytoplasmic area, 203 ± 15.1 μm^2).

In four further cases, all SIDS victims, we observed normal architecture of the arcuate nucleus but neuronal depletion (mean neuronal density per mm^2, 48 ± 27) and/or delayed neuronal maturation (mean nuclear area, 67.3 ± 11.7 μm^2; mean cytoplasmic area, 106 ± 25.5 μm^2). These immature neurons were lengthened in shape with a flattened nucleus, compact chromatin and poorly evident nucleolus (Fig. 3).

Thirty-three (61%) of the mothers were non-smokers and 21 (39%) were smokers during pregnancy. Among the smokers, eight smoked one to five cigarettes a day, and 13 smoked more than five cigarettes daily. Table 1 shows the association between smoking habit during pregnancy and structural alterations of the arcuate nucleus.

The frequency of arcuate nucleus defects in stillborns was seven of seven cases with smoking mother versus one of 13 cases with non-smoking mother and the former was significantly higher than the latter ($P < 0.05$). Likewise, the frequency of arcuate nucleus defects in infants was 13 of 15 cases with smoking mother during pregnancy versus three of 19 cases with non-smoking mother ($P < 0.05$).

The severity of the alterations was increased when the mothers smoked more than five cigarettes a day, but this correlation was not statistically significant.

DISCUSSION

Exposure to tobacco smoke before birth is a leading cause of intrauterine growth retardation, abruptio placentae, lower birth weight,[11–14] and increased risk of stillbirth and death in the first year of life.[6–10] Cnattingius *et al.* found that maternal smoking increased the risk of intrauterine fetal death by 40% during the third trimester.[10] Klenman *et al.* showed that the risk of death before and during delivery, of neonatal mortality and death between the second and the twelfth month of life was related to the number of cigarettes smoked by the mother during pregnancy.[7] Moreover, in a large epidemiological study re-examining the factors generally thought to pose a risk of SIDS (black race, birthweight less than 1600 g, gestational age at birth less than 37 weeks, 5-minute Apgar score less than 7, male gender, more than two previous pregnancies, maternal age less than 20 years, maternal education level less than 12 years, maternal smoking during pregnancy), Taylor *et al.* concluded that only maternal smoking during pregnancy was independently associated with SIDS.[15]

Prenatal smoke exposure reduces fetal oxygenation caused by increased blood levels of carboxyhemoglobin.[16] In fact, carbon monoxide (CO), one of the agents primarily responsible for the adverse effects of cigarette smoke, readily crosses the placenta where it binds to hemoglobin causing fetal hypoxia. Exposure to smoking *in utero* might increase the infant's vulnerability to diseases, interfere with the immune system predisposing to infections, as well as cause damage to the developing organs.

However, the mechanism according to which CO might affect fetal brain development has not been elucidated. Few studies have been made, prevalently in experimental animals, on alterations in neurotransmitter levels.[17–19] Tolcos *et al.* demonstrated, in the medulla of CO-exposed fetuses of guinea pigs, a significant decrease in tyrosine hydroxylase-immunoreactivity in the tractus solitarius nucleus, dorsal vagal motor nucleus, area postrema and hypoglossal nucleus compared with controls.[17] Structural alterations have also been described, especially in the cerebellum, in cerebral white matter and basal ganglia in cats after prenatal CO exposure.[20]

In humans, Storn *et al.* found an increase in brainstem reactive astrogliosis, mainly in the inferior olivary nucleus,

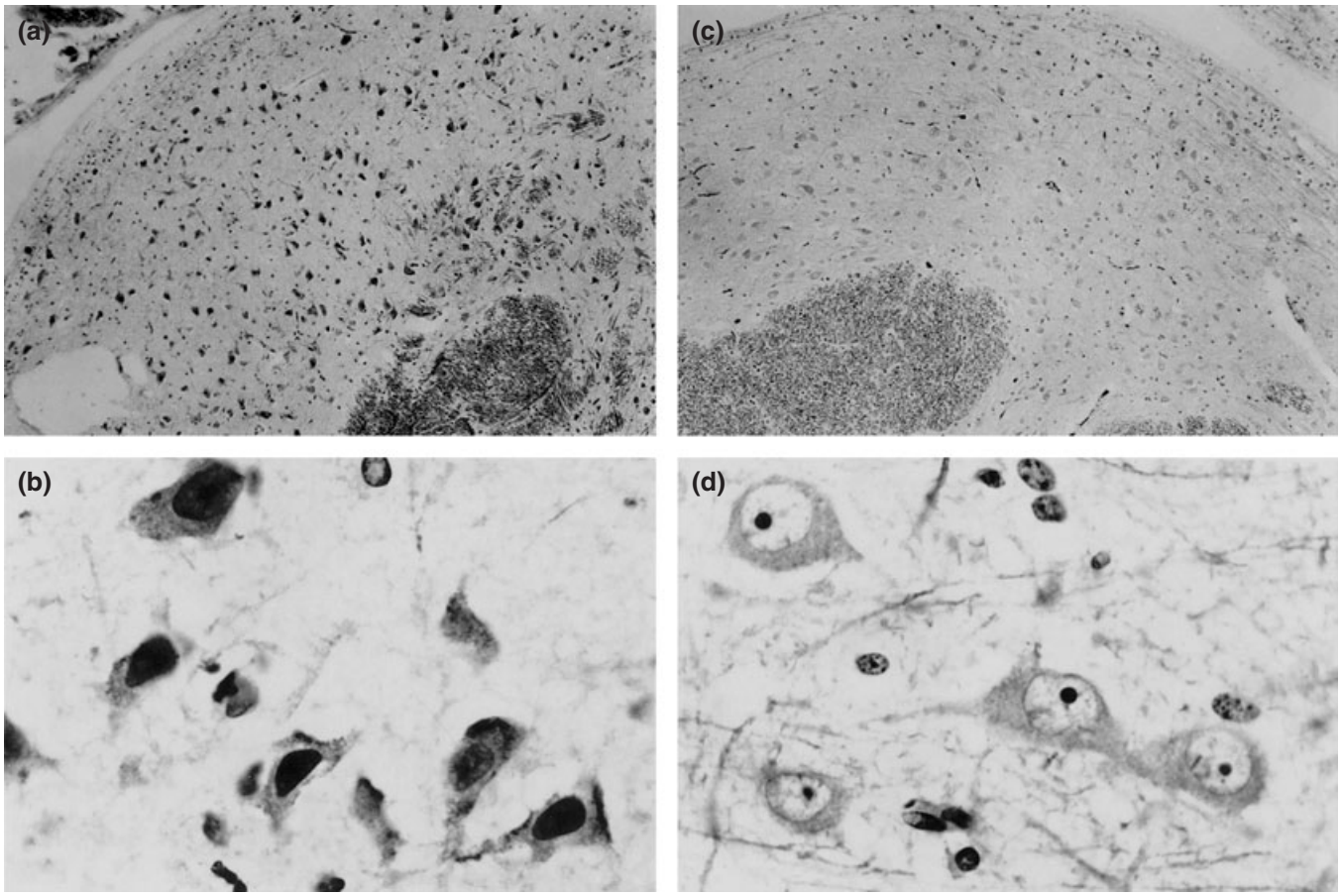

Fig. 3 (a,b) Neuronal immaturity in a normal structured arcuate nucleus. Brainstem intermediate section of a sudden infant death syndrome victim (female, aged 3 months). (c,d) Well-developed neurons in a normal structured arcuate nucleus of an infant of the same age (KB stain; magnifications: (a,c) ×10; (b,d) ×100).

Table 1 Histological diagnosis of the arcuate nucleus in 20 cases of perinatal death (13 stillbirths and seven neonatal deaths) and in 34 SIDS victims in relation to smoking habit of the mothers during pregnancy

Histology of the arcuate nucleus	Perinatal death (no. 20) Cigarettes/day			SIDS (no. 34) Cigarettes/day		
	0	1–5	≥5	0	1–5	≥5
Normal structure	12 (60%)	–	–	16 (47%)	1 (3%)	1 (3%)
Bilateral hypoplasia	1 (5%)	–	2 (10%)	1 (3%)	1 (3%)	4 (12%)
Monolateral hypoplasia	–	1 (5%)	–	1 (3%)	–	2 (6%)
Partial hypoplasia	–	1 (5%)	1 (5%)	–	–	2 (6%)
Agenesis	–	–	2 (10%)	–	–	1 (3%)
Normal structure with neuronal defects (DNM and/or DND)	–	–	–	1 (3%)	1 (3%)	2 (6%)

DND, decreased neuronal density; DNM, delayed neuronal maturation; SIDS, sudden infant death syndrome. Level of significance $P < 0.05$.

in perinatal and SIDS victims prenatally exposed to cigarette smoke.[21] However, morphological alterations arising in the development of the human autonomic nervous system, associated with maternal smoking, have not so far been recognized.

In the present study, we demonstrated a significantly increased incidence of cytoarchitectural alterations of the arcuate nucleus in stillborns and in SIDS victims with smoker mothers compared to victims with non-smoker mothers. We observed, in both stillborns and infants, a wide variety of structural defects of this nucleus, ranging from neuronal immaturity in a well-shaped structure to total agenesis. These observations demonstrate that hypoplasia of the arcuate nucleus is already detectable, with a wide range of morphological variability during fetal life and is therefore a congenital anomaly, probably determined by structural and/or functional abnormalities of genes involved in CNS development.

Our knowledge of neural-specific genes expressed in neurogenesis is still very poor, particularly in humans. Experimental studies point out that neuronal development involves several steps that can be monitored by marker genes.[22,23] In particular, the homeobox-containing gene *En-2* has been shown by mutational analyses to be a candidate genetic marker for rhombic lip-derived structures, such as the arcuate nucleus.[24,25]

Therefore, we can assume that mutational or functional alterations of this gene are implicated in hypoplasia of the arcuate nucleus. Furthermore, the observation in the present study of a wide range of developing morphological defects of the arcuate nucleus related to maternal smoking allows us to postulate that the constituents of the gas phase in cigarette smoke might directly affect the *En-2* gene and modulate its expression, or even determine its complete deletion and hence agenesis of the nucleus.

In conclusion, we believe it would be possible to reduce the frequency of hypodevelopment of the arcuate nucleus, and consequently the frequency of fetus and infant deaths, by reducing the number of pregnant smokers. Our studies will continue to focus on chromosome 7, particularly on the terminal bands of its long arm where the gene *En-2* is mapped,[26] to confirm the involvement of this gene in developmental defects of the arcuate nucleus.

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