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Neuronal nuclear antigen (NeuN): A useful marker of neuronal immaturity in sudden unexplained perinatal death

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ABSTRACT

Introduction: In the developing brain neuronal differentiation is associated with permanent exit from the mitotic cycle. Neuronal nuclear antigen (NeuN) is a nuclear protein widely expressed in the mature postmitotic neurons.
Methods: We applied NeuN immunocytochemistry in 65 cases of perinatal death (16 victims of sudden intrauterine unexplained death syndrome/SIUDS, 19 of sudden infant death syndrome/SIDS and 30 controls) to test the physiological status of the brain neurons. In addition we applied both TUNEL and Caspase 3 immunohistochemical methods in order to highlight a possible relation between decreased NeuN expression and apoptotic outcome. We also attempted to see whether or not NeuN pathological changes can be related to cigarette smoke absorption in pregnancy.
Results: NeuN staining was considerably reduced or lost in SIUDS/SIDS compared to controls. However neurons with decreased NeuN-labeling showed no sign of apoptosis. A significant association was found between NeuN depletion and maternal smoking.
Conclusion: Altered NeuN expression can be a marker of immature and/or suffering neurons. The exclusive presence of this pattern of expression in SIUDS/SIDS victims, leads us to recommend the NeuN immunohistochemistry as a routine method in neuropathological protocols to convalidate a diagnosis of sudden perinatal death.

1. Introduction

The neuronal nuclear antigen (NeuN) is a specific protein expressed in post-mitotic neurons. The corresponding antibody, developed by Mullen in 1992 [1], primarily stains the neuronal nucleus, but the cytoplasm and dendrites are also immunoreactive, though to a lesser extent. Noteworthy it does not stain the immature nerve cells until they exit from the cell cycle and achieve a stage of development that at least approaches mature function. This is coincident with the migration of the neuroblasts from their birthplace in the embryonic neural tube to their final position, outgrowth of axons and generation of synapses [2–5].

With the inexplicable exception of several neuronal cell types, such as the Purkinje cells and the dentate nucleus neurons in the cerebellum, the neurons of the inferior olivary nucleus in the medulla oblongata and the glial cells, that generally are not recognized by the NeuN antibody, the vast majority of neurons is strongly NeuN positive already in fetal life [1,2].

In addition to representing a marker of maturing neurons, the NeuN immunohistochemistry can be applied in neuropathologic studies to highlight their physiological status. Precisely, while intense NeuN expression is shown by healthy neurons, a decreased NeuN positivity in postembryonic life can be indicative of degeneration of differentiated neurons. In particular, immunoreactivity is significantly weakened after a severe injury, such as cerebral hypoxia/ischemia [6–8]. Infants who have suffered fetal distress or perinatal asphyxia may show less brain NeuN immunostaining than infants who have not experienced such insults [2].

Stressors, such as hypoxia, hypercarbia and asphyxia are known as pathogenetic factors in SIDS [9–12], resulting in functional and/or morphological developmental alterations of brain neurons. Nevertheless, among the countless existing works in the literature on this field, our previous contributions included, there are no reports taking into consideration the NeuN expression as index of neuronal distress in SIDS.

Insofar, we aimed to evaluate the immunoexpression of NeuN in a group of victims already object of our prior studies but not formerly investigated in this regard. We reconsidered a total of 65 subjects aged from 17 gestational weeks to 10 postnatal months, who had died of known or unknown causes. Our aims were firstly to obtain basic information about the manifestation of NeuN in the study groups and to evaluate a possible wrong expression in sudden perinatal and infant death, in addition to specific morpho-functional alterations of the autonomic

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nervous system we had already reported [13–19]. Then, in order to evaluate if the NeuN depletion can be indicative of neuronal degeneration, we applied the TUNEL method as hallmark of apoptosis [20,21] and Caspase3 immunohistochemistry as a signal of dying cells [22].

Finally, we considered a possible correlation between NeuN pathological changes and hypoxic injuries related to cigarette smoke absorption in pregnancy.

2. Methods

A total of 65 brains were considered for this study, from 29 fresh ante-partum stillbirths (17–40 gestational weeks—gw, mean age: 37 gw), and 36 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 lifestyle, and including the death scene examination for infant victims, was collected. None of the mothers had any significant pathology. The mothers were also asked for information about the smoking habit particularly before and during pregnancy. Thirty mothers (46%) were active smokers before and during pregnancy while 35 (54%) declared no history of cigarette smoking. Since the retrospective assessment of the smoking habit of a mother, mainly after the death of his son, is sometimes unavoidable, the negative self-reports were verified by the urinary measuring of cotinine, the main metabolite of nicotine.

2.1. 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.

2.2. Anatomopathological protocol

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 [23,24].

After fixation in 10% phosphate-buffered formalin, the brains were processed and embedded in paraffin. In particular, transverse serial sections of the midbrain, pons, medulla oblongata and spinal cord (cervico-thoracic tract), where the main structures controlling the vital functions are located, were made at intervals of 60 μm. For each level, six–seven 5 μm sections were obtained, two of which were stained for histological examination using hematoxylin–eosin and Klüver–Barrera stains, two sections for immunohistochemical detection of NeuN and apoptosis, respectively. 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, the parafacial/facial complex, the superior olivary complex, the retrotrapezoid nucleus, the superior olivary nucleus, the parabrachial/Kölliker–Fuse complex in the pons/mesencephalon; 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 and the intermediolateral nucleus in the spinal cord.

In 35 cases, after the in-depth autoptic examination, the death remained totally unexplained. A diagnosis of "sudden intrauterine unexplained death syndrome/SIUDS" was therefore made for 16 fetuses, who died suddenly after the 17th gestational week before complete expulsion or retraction from the mother, and a diagnosis of "sudden infant death syndrome/SIDS" for 15 infants who died within the first ten months of life. In the remaining 30 cases, 13 stillbirths, and 17 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, death diagnoses and maternal smoking habit.

2.2.1. Immunohistochemical techniques

2.2.1.1. NeuN immunohistochemistry. Representative sections from paraffin-embedded tissue blocks were stained using commercially supplied mouse monoclonal antibodies against the neuronal nuclear antigen NeuN (Millipore Chemicon International, MAB377). A standard avidin–biotin complex (ABC) technique was used with peroxidase–diaminobenzadine to visualize and develop the antigen–antibody reaction. The antibody dilution at 1:100 was used. Incubating solutions were boiled in boilded in citrate bufferat pH 6.0, in a microwave oven, for 5 min at high power, then 5 min at 50% power, and finally cooled for 20 min. Sections were counterstained lightly with Mayer's hematoxylin.

Evaluation of the NeuN immunohistochemical results. Since in health status the NeuN immunopositivity is diffused in almost the entire brain, for a uniform and representative evaluation of the results we selectively examined at light microscope only the histological sections from the caudal pons given the general presence, above all in the ventral portion, of a wide diffuse population of neurons (the so called "*griseum pontis*").

Only the cells with intense brown immunostaining were considered to be really positive. Moreover, also a weak brown intensity was taken into account.

We quantified the scoring for each case, using a ×40 lens, as follow:

− = no positive cell (*negativity*)
+ = a number of positive cells ≤ 30% per unit area (*moderate positivity*)
−/+ = a number of cells with only weak positivity ≤ 30% per unit area (*weak positivity*)
++ = a number of positive cells > 30% per unit area (*strong positivity*).

The unit area was represented by square millimeteter (mm^2).

2.2.1.2. Apoptosis detection. To detect cells undergoing apoptosis, we applied the TUNEL method and immunohistochemistry for Caspase 3.

TUNEL staining. To detect cells undergoing apoptosis, we used the technique of Terminal-Transferase dUTP Nick End labeling (TUNEL Apoptag plus peroxidase in situ Apoptosis detection kit, S7101, Chemicon). Sections were pretreated with proteinase k (20 μg/ml) for 15 min. Endogenous hydrogen peroxidase activity was quenched

Table 1
Case profiles of the study.

Victims	Age (range)	Sex		Death diagnosis	
		M	F	Explained death Controls (n.30)	Unexplained death (n.35)
Fetuses (n.29)	17–40 gw	12	15	Necrotizing chorioamnionitis (n.7) Congenital heart disease (n.5) Potter's syndrome (n.1) *Smoking mothers (n.3)*	SIUDS (n.16) *Smoking mothers (n.12)*
Infants (n.36)	1–10 m	20	16	Pneumonia (n.6) Congenital heart disease (n.10) Pericarditis (n.1) *Smoking mothers (n.1)*	SIDS (n.19) *Smoking mothers (n.14)*

gw = gestational week; m = month.
SIDS = Sudden Infant Death Syndrome.
SIUDS = Sudden Intrauterine Unexplained Death Syndrome.