Tissue and Cell 47 (2015) 506–514

Contents lists available at ScienceDirect

Tissue and Cell

journal homepage: www.elsevier.com/locate/tice

Neurotoxicological effects of nicotine on the embryonic development of cerebellar cortex of chick embryo during various stages of incubation

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ARTICLE INFO

Article history:
Received 28 March 2015
Received in revised form 3 July 2015
Accepted 3 July 2015
Available online 13 July 2015

Keywords:
Chick embryo
Nicotine
Cerebellum
Histopathology
TEM
Purkinje cells

ABSTRACT

Long-acting nicotine is known to exert pathological effects on almost all tissues including the cerebellar cortex. The present work was designed to elucidate the effect of nicotine on the development of cerebellar cortex of chick embryo during incubation period.

The fertilized eggs of hen (*Gallus gallus domesticus*) were injected into the air space by a single dose of long acting nicotine (1.6 mg/kg/egg) at the 4th day of incubation. The embryos were taken out of the eggs on days 8, 12 and 16 of incubation. The cerebellum of the control and treated embryos at above ages were processed for histopathological examination. The TEM were examined at 16th day of incubation.

The results of the present study revealed that, exposure to long-acting nicotine markedly influence the histogenesis of cerebellar cortex of chick embryo during the incubation period. At 8th day of incubation, nicotine delayed the differentiation of the cerebellar analge; especially the external granular layer (EGL) and inner cortical layer (ICL). Furthermore, at 12th day of incubation, the cerebellar foliation was irregular and the Purkinje cells not recognized. By 16th day of incubation, the cerebellar foliations were irregular with interrupted cerebellar cortex and irregular arrangement of Purkinje cells.

Immunohistochemical analysis for antibody P53 protein revealed that the cerebellar cortex in all stages of nicotine treated groups possessed a moderate to weak reaction for P53 protein however; this reaction was markedly stronger in the cerebellar cortex of control groups. Moreover, the flow cytometric analysis confirmed that the percentage of apoptosis in control group was significantly higher compared with that of nicotine treated group.

At the TEM level, the cerebellar Purkinje cells of 16th day of treated groups showed multiple subcellular alterations in compared with those of the corresponding control group. Such changes represented by appearing of vacuolated mitochondria, cisternal fragmentation of RER, irregular grooves of Golgi tubules. Also, multiple cytoplasmic vacuoles and aggregation of Nissl granules were recorded around pyknotic nucleus.

1. Introduction

Smoking is strongly addicting, with a nearly 97% retrogression rate for unhelpful quit attempts (Hughes et al., 1992), and may cause one or more of each five deaths each year (CDC, Health effects of smoking, 2008). Tobacco exposure is not only a health concern for adults, however it has also been shown to cause harmful effects on the health of the fetus, newborn, child, and adolescent (Rogers, 2008; Prokhorov et al., 2006; Kum-Nji et al., 2006; Mathers et al., 2006). Indeed, the long-term effects of nicotine exposure may be more thorough at these younger ages, since development of neural pathway is not yet complete and the inherent plasticity of the developing brain makes it particularly vulnerable to drug-induced alteration.

Standard textbooks, databases, and safety cards consistently mentioned that the lethal dose of nicotine for adults is 30–60 mg, leading to safety warnings that smoking of five cigarettes or 10 ml of a dilute nicotine-containing solution could kill an adult. Hayes (1982) stated that, the 60-mg dose would correspond to an oral LD50 of around 0.8 mg/kg, a dose that is considerably smaller than the values determined for laboratory animals, which are ranging from 3.3 (mice) to more than 50 mg/kg (rats).

In spite of the LD50 of 0.8 mg/kg would involve that the toxicity of nicotine is comparable to or even higher than that of cyanide,

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http://dx.doi.org/10.1016/j.tice.2015.07.002
0040-8166/

fatal nicotine intoxications are relatively rare, and there are countless records of subjects who survived consumption of nicotine in amounts far higher than 60 mg (Larson et al., 1961).

Cigarette smoke is rich with many of toxic products, especially nicotine and cyanide (Sayers and Drucker, 1999). Maternal cigarette smoke during gestation period is associated with raised incidence of neural deterioration in offspring (Wielgus et al., 2004). Nicotine is a strongly addictive poisonous and toxic alkaloid compound which primarily, stimulates the brain and then inhibits it. Nicotine enters into the body through different modes of tobacco intake like smoking, snuff or chewing. It has been established that, nicotine is a neuroteratogen which compromises the development of fatal neural pathways during the development of brain (Pauly and Slotkin, 2008; Dwyer et al., 2009).

The cerebellum is the part of brain that responsible for balance. At the 4th day of incubation in chicken embryos, the cerebellum develops from the neuroepithelium of the dorsal part of the fourth ventricle that formed between mesencephalon and rhombencephalon (Feirabend, 1990). The cerebellar mantle layer becomes subdivided into an inner and outer mantle layer at the early embryonic period. The outer mantle layer (OML) differentiates into, the deep outer mantle layer and superficial mantle layer parts. The deep OML develops into the central cerebellar nuclei whereas the superficial OML develops into the external granular layer. The inner mantle layer differentiates into inner cortical layer, which subsequently gives rise to the future Purkinje cells (Feirabend et al., 1985). Lee et al. (2001) had been described the Purkinje cells as the biggest neuronal cell kind in the cerebellar cortex which develop from the ventricular germinal zone (VZ). They also added that, these cells have the most accurate synaptic interactions among the different neurons of cerebellum. Long-term nicotine treatment did not change the cerebellum weight or the size (volume) of the cerebellar vermis. The long-term nicotine treatment regimen did result in a significant loss of mature Purkinje cells in the cerebellum, however, such a loss of Purkinje cells was not nicotine dose-related (Chen et al., 2003). Moreover, Wielgus et al. (2004) assured that the nicotine caused a significant reduction in brain weight of chick embryo. Chandrakar et al. (2011) recorded that, the chick embryos which treated with nicotine has a significant decrease in their head size and trunk diameter.

Apoptosis specifically refers to the meant and beneficial death of cells within the body (Lavrik et al., 2005). Many intrinsic factors are required for completion of apoptosis, from of these factors are the P53 gene and caspases. P53 is a tumor suppressor gene that in an inactivated form tends to be associated with a high risk of certain cancers and inhibition of apoptosis (Bai and Zhu, 2006). Caspases are highly essential proteins (cysteine proteases that cleave polypeptides) that are necessary for the fulfillment of apoptosis, in most of organisms (Gown and Willingham, 2002). There are several different kinds of caspases that are useful to perform apoptosis. Initiator caspases, for example, activates effector caspases. These activated effector caspases go on to cleave certain cellular components that are necessary for apoptosis. Other caspases are known as inhibitors which can effectively suppress or stop apoptosis (Lavrik et al., 2005).

The present work was aimed to clarify the neuro-toxicological effects of nicotine on the development of cerebellar cortex of chick embryo during various periods of incubation.

2. Materials and methods

2.1. 2.1Chemicals and instruments

2.1.1. Full list of chemicals

Nicotine (pure, 99% from Sigma Chemical Comp.), saline solution (nicotine solvent), formaldehyde, glutraldehyde, osmium tetraoxide.

2.1.2. The dose

The test chemical (99% pure nicotine, Sigma Chemical Co.), dissolved in saline solution and injected into air space of eggs at a single dose of 1.6 mg/kg/egg. The dose was chosen in average according to Hayes (1982) and CDC (2005).

2.1.3. Instruments

Egg incubator (Brand: WQ-88 egg incubator), 1 cc syringe fitted with a very short fine needle (5 mm long). FACS (flow activated cell sorter) Calibur Flow Cytometer (Becton Dickinson, Sunnyvale, CA, USA).

2.2. Experimental design

For the present work, 30 freshly fertilized eggs of hens (*Gallus gallus domesticus*) were obtained from a White Leghorn stock supplied by a local breeder were used. After an incubation (WQ-88 egg incubator) period of 24 h at 37 °C, and 65% humidity, the eggs were randomly divided into two groups. Each group consisted of 15 fertilized eggs and returned back to the incubator.

I. The first group was kept normal as a control and injected by saline solution into air space at 4th day of incubation.
II. The second group was treated with a single dose of nicotine solution (1.6 mg/kg/egg). This solution was injected gently (to avoid any damage for the inner egg membrane) into the air space of 4th day incubated eggs using a 1cc syringe. Such dose of nicotine has a prolonged duration of action due to its slow absorption through the egg membranes. The incubated eggs were tested daily by candling technique to confirm the normal development of the embryo.

Specimens of cerebellum were taken at 8, 12 and16th days of incubation for histopathological investigations and other specimens were kept frozen for flow cytometric detection of caspase-3. On day 16 of incubation, embryos from control and nicotine treated groups were dissected and the cerebellum was removed and processed for ultrastructural studies.

2.3. Histological investigations

The whole chick embryos from the control and treated groups were removed on day8, 12, and 16th day of incubation, and then dissected to remove the whole brain. The cerebellum was taken and consistent tissue samples of each stage were fixed in buffered 10% neutral formalin for one week. The tissue was dehydrated in ascending series of alcohols, cleared in xylol and embedded in paraffin wax. For routine histological examinations, tissue sections of 4–6 μm were stained with hematoxylin and eosin to demonstrate the histopathological alterations in the cerebellum.

2.4. Immunohistochemical staining for P53 expression

For immuno-histochemical detection of P53 protein, the Paraffin-embedded tissue sections of cerebellum for the two groups were incubated with primary antibodies (monoclonal antibody RM-2103-R7, 7.0 ml of antibody pre-diluted in 0.05 mol/l tris–HCl, pH 7.6 containing stabilizing protein and 0.015 mol/l sodium azide, Produced by Epitomics, Inc. Using Technology Licensed Under Patent no. 5,675,063, Thermo Fisher Scientific, Anatomical Pathology, 46360 Fremont Blvd., Fremont, CA 94538, USA) for 16 min at 37 °C. Sections were stained with p53-immunoperoxidase stain (Cuello, 1993). The intensity of nuclear staining for P53 protein was recorded as weak, moderate, or strong reaction.