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Effects of spermine and the passive avoidance learning (PAL) following cerebral ischemia in chicks: Association with neuroprotection of pyramidal cells

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ABSTRACT

The aim of this study is to investigate the effects of spermine and the passive avoidance learning on hippocampus following transient cerebral ischemia in the chicks. The study is composed of the pure control (CG), sham (SG) and experimental groups (n = 20). Experimental groups (ischemia group, IG and ischemia-spermine group, ISG) were exposed to ischemia for 20 min whereas the SG was exposed to sham operation and CG group was not exposed to any operation. Passive avoidance learning (PAL) was applied to the half number of the subjects in each group. Both before and after 7 days from the ischemia, operated animals were taken to PAL and then they were sacrificed. Total numbers of neurons in the hippocampus were stereologically estimated using Cresyl violet stained sections. We detected that number of neurons was increased following PAL and especially spermine treatment. According to our results, we suggested that spermine may reduce the deleterious effects of the ischemia by causing to increase in the neuronal number and so, it may be slightly supportive to the PAL.

1. Introduction

Natural polyamines, e.g. spermine and putrescine, which is their precursor, carry great significance for the nervous system development and maturation (Adibhatla et al., 2002). Several neurophysiological and metabolic effects and functions are linked to them including the calcium-dependent transmitter release, modulation of ionic channels, control of nucleic acid and protein synthesis (Coert et al., 2000). Polyamines have complex biological effects as both neurotoxic (Conway, 1998) and neuroprotective (Coert et al., 2000) roles are associated with them. These conflicting finding may be caused by the biphasic effect exerted by the polyamines on the N-methyl-D-aspartate (NMDA) receptor (Rock and Macdonald, 1995). It was indicated by the electrophysiological studies that, NMDA receptor are enhanced by the polyamines when they are in low concentrations. On the other hand, elevated concentrations of these amines may lead to low degree enhancements or the inhibition of the currents of NMDA receptors (Rock and Macdonald, 1992).

A significant risk factor for the brain dysfunction and damage is cerebral hypoperfusion. Dysfunction and damage of neurons may be occurring in the brain as a result of the ischemic condition (Kaplan et al., 1991). Ischemia of the brain very easily and quickly affects the cells of it, especially neurons. The differences occurring after ischemia depends on ischemia time, location of occluded vessel and neuron types (Otori et al., 2003). After performing that all types of experimental ischemia models, non-appeared nucleolus and clear nuclear morphology, appearance of cytoplasmic or nuclear vacuoles and eosinophilic changes could be respectively occurred. Then, irreversible degeneration may be developed in the neurons that including mitochondrial damage, pycnotic nucleus. Finally the degenerated neurons may be removed by means of the apoptotic or necrotic pathway (Wachirayah and Knokwan, 2014). Also an increased loss in the number of the neurons may be occurs after ischemia (Liu et al., 2013). So we used an ischemia model to detect protective or inductive

Abbreviations: CG, pure control; SG, sham; IG, ischemia group; ISG, ischemia-spermine group; PAL, passive avoidance learning; NMDA, N-methyl-D-aspartate; IMM, intermediate medial mesopalium; LPO, lobus *para*-olfactorus; TO, tuberculum olfactorum; Hp, hippocampus; CNS, central nervous system; PBS, phosphate buffered solution; MeA, methylanthranilate; CA, cornu ammonis; CE, coefficient of error; CV, coefficient of variation

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effects of spermine and PAL from neuronal death or increase of the neuron number, respectively.

Neuro-protective treatments aim to shorten the time needed for reperfusion and to minimize the tissue injury with shortening the reperfusion damage (Yan et al., 2013). The severity of the tissue injury occurring after ischemia depends on ischemia time, location of occluded vessel and neuron types (Otori et al., 2003). Following to perform that all types of experimental ischemia models, non-appeared nucleolus and clear nuclear morphology, appearance of cytoplasmic or nuclear vacuoles and eosinophilic changes could be respectively occur. Then, irreversible degeneration may be developed in the neurons that including mitochondrial damage, pycnotic nucleus. Finally the degenerated neurons die by means of the apoptotic or necrotic pathway (Wachirayah and Knokwan, 2014). Ischemia-reperfusion injury also may cause to significant change of polyamine metabolism (Han et al., 2009). At this point, spermine treatment was evaluated as useful following ischemia due to their possible roles on angiogenesis and upregulating autophagy (Dong et al., 2014; Han et al., 2013). But, the effect of spermine on hippocampus and ischemia caused brain damage is not yet well known (Li et al., 2007). On the other hand, while passive avoidance learning (PAL) leads to increasing of the number of cells in the intermediate medial mesopalium (IMM), lobus para-olfactorus (LPO) and tuberculum olfactorum (TO). Even numerical density of synapses increased in IMM, LPO and dorsal hippocampus (Hp) following the PAL (Dermon et al., 2002; Unal et al., 2002; Verew, 1991). Chicks have a similar organization of central nervous system (CNS) with mammalians. Also their CNS' topography, its developmental origin is well known. Apart from this chick hypothalamus, they have cornu ammonis, dentate gyrus, subicular, entorhinal cortex homologue to mammalians. They have well developed CNS when they are hatched. For the feeding, they need to determine quickly difference between the dangerous or non-dangerous items (Stern, 2005). So, PAL is easily applicable to chicks, and also surgical procedures for example ischemia models can easily perform in the chicks due to having well developed and not fully-calcified skull (Unal et al., 2002).

The purpose of the current study was to research the effects of spermine and the PAL on the hippocampus after the occurrence of transient cerebral ischemia in the chicks by using stereological techniques. So, it is hoped that findings of present study would encourage investigations, which will promote development in the treatment of cerebrovascular diseases.

2. Methods

Domestic chicks (n = 80; *Gallus domesticus*) were bought from a commercial breeder. One-day-old chicks were put in cardboard pens (35 × 40 × 40 cm height) at 2 h after from hatching and checked for learning capacity. For testing the learning performance, the imprinting stimulus was given by suspending a red table tennis ball (4 cm diameter) with a string in the pen's centre. It was accepted by the chick as its 'mother'. But, the chicks which were not able to adapt, were removed from the study. To promote eating and pecking, the floor of the pen was coated with white paper and chick starter crumbs, tapped periodically using a small round rod, were sprinkled to the base. Water was accessible *ad libitum*. All procedures of this study were approved by Animal Ethical Committee of Yuzuncu Yil University, 2004/04-10, Van Turkey. On day 7, all animals were randomly divided into four main groups. Then, sham and ischemia operations were made on the relevant groups at the same day (the first day of the study):

- **Pure control group (CG)**: This group composed of 20 healthy chicks without any application.
  - ○ PAL was not applied to 10 chicks of the group **(CGA)**
  - ○ PAL was applied to other 10 chicks of the group **(CGB)**
- **Sham group (SG)**: This group composed of 20 healthy chicks. The bilateral internal carotid arteries were sutured with 4-0 type surgical silk but not clumped in these chicks.
  - PAL was not applied to 10 chicks of the group **(SGA)**
  - PAL was applied to 10 chicks of the group **(SGB)**
- **Ischemia group (IG)**: The bilateral internal carotid arteries were clumped with Bulldog clump during 5 min.
  - PAL was not applied to 10 chicks of the group **(IGA)**
  - PALwas applied to 10 chicks of the group **(IGB)**
- **Ischemia and spermine group (ISG)**: In this group, 20 min before the ischemia process intraperitoneally 10 mg/kg/day spermine was given.
  - PAL was not applied to 10 chicks of the group **(ISGA)**
  - PAL was applied to 10 chicks of the group **(ISGB)**

After 7 days from the ischemia-reperfusion, PAL procedure was applied; and anesthesia was induced in all chicks by inhalation of sevoflurane vapour in oxygen mask (3%) (Sevorane, Abbott, Istanbul, Turkey). Then, the brains of the chicks were fixed by perfusion with intracardially given fixatives (Firstly: 0.9% serum physiologic, secondly: 4% paraformaldehyde in Phosphate Buffered Solution (PBS). Fixed tissues either stored in the fridge one night in the sucrose containing PBS and sectioned with a cryostat; or processed after being passed in alcohol, xylene and embedded in paraffin. The paraffin blocks were sectioned at 20 μm thicknesses with a conventional microtome for routine histological evaluation and stereological analyses, respectively.

2.1. Ischemia procedure

Bilateral common carotid arteries were clumped under sevoflurane anesthesia. After that, bilateral common carotid arteries were exposed using a cervical incision and with a ventral cervical incision, they were carefully separated from the carotid sheath and the cervical sympathetic and vagal nerves. The bilateral common carotid arteries were clumped with Bulldog clump during 5 min. Following ischemia procedure, the clump was relaxed and reperfusion occurred. Finally, skin was closed with 4.0 silk sutures. The operation was done on a heating pad to keep the body temperature was at 37.5 ± 0.5 °C as the operation was carried out on a heating pad and the animals were not removed from the pad until the time that they were free from the effects of anesthesia.

2.2. Learning of passive avoidance

This procedure was applied at the 7 days after from the ischemia operation (i.e at the 8th day of the study). A white, plastic bead, which was slicked to a bottle tip, was shown to chicks from 5 cm distance 3 times with 5 min intervals. Chicks were accepted to 'peck' or 'do not peck' the bead as a reaction. The chicks, which didn't peck the bead, were excluded from the study and other animals put instead of them. After 10 min of this pre teaching exercise the same bead, which was put in water, was showed to PA (–) chicks and the bead, which was put in the methylanthranilate (MeA), was showed to PA (+) chicks (Unal et al., 2002; Verew, 1991). The chicks, which pecked the methylanthranilated beads, were accepted to shake their necks and roam their peck to the floor. The chicks that learnt the passive avoidance later did not come near to the bead.

2.3. Number of the hippocampal neurons

A pilot study was done for the determination of the sampling and counting schedule. The first section to be analyzed was determined randomly among the first five sections. Every fifth section was chosen from the series, which corresponds to a one-fifth section-sampling fraction (SSF). Optical fractionator method was used for estimation of the total neuron number, about 15–20 sections from each brain are enough (Gundersen and Jensen, 1987; Tunc et al., 2007; West et al., 1991). Each sampled section at 20 μm thicknesses was put on slides coated with gelatin–formaldehyde mixture and cresyl violet (Sigma, St