Magnesium sulphate protect against propofol-induced neurotoxicity in developing rats’ cerebral cortex

Ahmed S. Sadek¹, Akram M. Amer², Rehab T. Khattab¹, Azza M. Youssef²

¹Anatomy and Embryology Department, Faculty of Medicine, Ain Shams University, Egypt, ²Anesthesia Department, Faculty of Medicine, Ain Shams University, Egypt

SUMMARY

Propofol-induced neurotoxicity in developing brain is an evolving problem. Magnesium is proved to be neuro-protective when administered antenatally in case of pre-eclampsia and eclampsia. The aim of this work is to investigate the protective role of magnesium sulphate (MgSO₄) against propofol-induced neurotoxicity in developing rats’ cerebral cortex (CC). Forty albino rats of the Sprague-Dawley strain aged 5 to 7 days were divided into four groups; Control group (Group A); MgSO₄ group (Group B) receiving MgSO₄ at a dose of 270 mg/kg; Propofol group (Group C) receiving propofol at a dose of 20 mg/kg; and Propofol-MgSO₄ group (Group D) receiving both drugs simultaneously. All drugs were given by intraperitoneal (IP) injection. 48 hours following exposure, blood samples were collected from rats for cytochrome-c plasma assay. Also, Specimens from rats’ CC were processed for light microscopic examination. An immune-histo-chemical study was conducted using Glial Fibrillary Acidic Protein (GFAP) and Bcl-2-associated X protein (BAX).

Propofol administration led to disruption of the cortical laminar architecture, with a significant decrease in the number of pyramidal cells. Also, GFAP and BAX immune-stained sections revealed a significant increase in the percentage of their immune-reaction as compared to the control group, together with a significant increase in the serum level of cytochrome-c. Administration of MgSO₄ with propofol could improve the histological picture of the cortex, including its laminar appearance and neuronal density. Also MgSO₄ could decrease GFAP and BAX immune-reaction, with no change that could be noticed in the serum level of cytochrome-c. MgSO₄ could be used as neuroprotective agent against propofol-induced cortical injury in infant rats.

Key words: Magnesium sulphate – Propofol – Neuro-protective – Cytochrome-c

INTRODUCTION

Propofol is an intra-venous anaesthetic agent that is widely used in the paediatric population in different aspects ranging from procedural sedation to total intravenous anaesthesia for surgical procedures (Cravero, 2012). Also, it gained popularity in paediatric sedation due to its favourable pharmacological profile with rapid onset, short duration and clear recovery even after prolonged infusion (Kruessell et al., 2012).

Recently, concerns about anaesthetic-induced neurotoxicity are raised dramatically. Anaesthetics with gamma-aminobutyric acid-A (GABA-A) may have an agonist effect as propofol might lead to neuroapoptosis and degeneration in developing rats’ brain (Pesic et al., 2009; Flick et al., 2011). Long term neurobehavioral disturbances and learning difficulties were studied in children after exposure to different anaesthetic agents with high positive correlations (Bong et al., 2013). Recent studies showed that propofol might impair children’s learning memory and cognitive functions...
Magnesium sulphate protect against propofol-induced neurotoxicity
during the period of brain growth [6]. This effect was attributed to hippocampal neuro-apoptosis, together with the over-expression of pro-inflammatory cytokines in the brain (Han et al., 2015).

Apoptosis and neuronal toxicity mediated by propofol may occur through stimulation of intrinsic mitochondrial apoptosis cascade with the release of cytochrome-c and activation of caspase-3, 8 and 9 (Zou et al., 2013). These effects commonly occur during the period of rapid synaptogenesis, which extends from the intrauterine period at mid gestation till 2 to 4 years after birth in humans, but in rats it occurs during the first 2 weeks after birth especially in the 5th to 7th day (Straiko et al., 2009).

Magnesium is used in different aspects of medicine with few adverse effects and low cost (Dubé and Granny, 2003). The neuro-protective effects of magnesium were investigated in several studies that revealed its useful effects after cerebral ischemia (Pearce et al., 2015). Also, mothers who received MgSO₄ for pre-eclampsia or eclampsia gave birth to infants with fewer incidences of adverse neurological outcome and less incidence of developing cerebral palsy (Conde-Agudelo and Romero, 2009).

The aim of the present study is to examine the neurotoxic effect of propofol on developing rats’ cerebral cortex (CC) and to investigate whether MgSO₄ could protect against this deleterious effect or not.

MATERIALS AND METHODS

Experimental animals
Forty albino rats of the Sprague Dawely strain aged 5 to 7 days, with average body weight 6-8 gm were used in this study. Rat pups were placed in a temperature controlled incubator set to an ambient temperature of 35-36°C. Animals were allowed to recover in the incubator for 1 hour and then returned to their mothers to feed. An experiment was executed in conformity with National Institutes of Health (NIH) guidelines for the maintenance and use of science lab animals; NIH Publication 1986 (86/609/EEC) and in accordance with local laws and ordinances.

Drugs
Propofol manufactured for intravenous human use (Diprivan®; AstraZeneca Ltd, Macclesfield, UK), Propofol was administered by intra-peritoneal (IP) injection at a dose of 20 mg/kg (Milanovic et al., 2010).
MgSO₄ ampoule 25 ml 10% (Egypt Otsuka pharm. Co.). MgSO₄ was administered by IP injection at a dose of 270 mg/kg (Sameshima et al., 1999).

Experimental design
Animals were divided into four groups: control group (Group A; N=5) receiving saline at a volume equivalent to that given to experimental rats by IP injection, MgSO₄ group (Group B; N=5) receiving only MgSO₄ at a dose of 270 mg/kg by IP injection, Propofol group (Group C; N=15) and Propofol+MgSO₄ group (Group D; N=15) receiving both drugs simultaneously. Loss of the righting reflex was used as a sign of anesthetic-induced unconsciousness and sleeping. The administered dose of propofol could impair the righting reflex for 45±5 minutes. Animals were closely monitored throughout the experiment. The pups appeared pink with no visible signs of cyanosis. Apical cardiac pulsation and respiratory rate were also closely monitored throughout the experiment, and it was in the range of variation less than 20% from the baseline value.

Forty eight hours following termination of propofol exposure, arterial blood samples were obtained from all rats for cytochrome-c plasma assay. Then, animals from all groups were decapitated by a curved sharp scissor and their brains were exposed and carefully extracted from the cranial cavity. Specimens from the CC were placed in 10% neutral-buffered formalin solution for one week, and then processed to paraffin blocks. 5 µm paraffin sections were stained with Haematoxylin and Eosin (Drury and Wallington, 1980) and Glees silver stain (Bancroft and Gamble, 2002). An immune-histo-chemical study was conducted using Glial Fibrillary Acidic Protein (GFAP) and Bcl-2-associated X protein (BAX).

Plasma cytochrome-c assay
50 µl arterial blood samples were obtained from the left cardiac ventricle of each rat by cardiac puncture. Serum samples were diluted 1:2 in assay buffer. Anti-rat/mouse cytochrome-c monoclonal antibody (catalog no, MCTC0, R&D Systems, Germany) was added to the sample. A biotin-conjugated monoclonal anti-cytochrome-c antibody was then added and bounded to the cytochrome-c captured by the first antibody, Streptavidin-horseradish peroxidase (HRP) was added and bounded to biotin-conjugated anti-cytochrome-c. A substrate solution reactivated with HRP was added, and a colored product formed was proportional to the amount of cytochrome-c presented in the sample. A standard curve was prepared from seven cytochrome-c standard dilutions, and the cytochrome-c sample concentration was determined. The minimum detectable dose (MDD) of rat/mouse cytochrome-c was typically less than 0.5 µg/ml. The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration (Ben-Ari et al., 2003).

Immunohistochemical staining for GFAP
GFAP immune-staining is the most commonly used method to examine the distribution of astro-
cytes and their response to neural degeneration or injury. The modified Avidin-biotin immune peroxidase technique for GFAP was applied to demonstrate astrocytes (Martin and O’Callaghan, 1995). Primary anti-GFAP antibody goat polyclonal IgG, anti-rat, produced by Dako Cytomation, was put on 10 μm paraffin sections of the CC. Working dilution was 1:1000. An immune-histo-chemical reaction was carried out using the Avidin-biotin-peroxidase system, followed by incubation with 1/100 normal rabbit serum for 20 minutes in order to exclude non-specific background. GFAP-containing cells (Astrocytes) appeared brown and nuclei appeared blue because of counterstaining with haematoxylin (Bancroft and Gamble, 2008).

**Immunohistochemical study for BAX**

7 μm paraffin sections were deparaffinized in xylene, rehydrated in descending grades of alcohol and immersed in 0.1% hydrogen peroxide to block the endogenous peroxidase activity. Mouse monoclonal antibody (Ab-14 Golden, Lab Vision Clone B-9, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) for BAX was put on each section. The dilution used was 1:50. The antibody was detected by using a biotin-streptavidin system with diaminobenzide (DAB) that applied for 20 min at room temp as a chromogen (Dako Carpentaria California USA) for BAX (Dako Corp., code no, k0673, lot 07110). Slides were counterstained with Mayer’s haematoxylin (Park Scientific Limited, Northampton, UK), dehydrated and covered by cover slips. In negative control slides, the same system was applied but the primary antibody was not added. The cytoplasmic site of reaction was stained brown and nuclei stained blue (El-Ghonaimey, 2015).

**Morphometric study**

The photomicrographs of each section were taken using an Olympus E-330 Live View Digital SLR Camera. The image was displayed on a computer monitor. A guard was used, with guard depths at the top of the section set at 10 μm. The dimensions of the counting box were 39 μm × 31 μm × 25 μm, height being determined using a digital gauge attached to the microscope stage. All neurons whose nucleoli were completely within the counting box were counted (Cullen et al., 2005). The same area was selected in the CC in all sections. The number of pyramidal cells in lamina III was counted by two independent observers blinded to the specimen details to perform an unbiased assessment (Fazeli et al., 2007).

**Quantitative image analysis**

The image analyzer computer system Leica Qwin 500 (England) was used to evaluate the area percentage of immune-stained areas. It was measured from five fields from five serial sections from five randomly chosen animals of each group. The measurements were made using magnification 400 with a measuring frame area 7286.78 μm² in the control and experimental groups.

**Statistical analysis**

The collected data were analyzed using statistical analysis system. Data were presented by mean and standard deviation. One way ANOVA was used to compare the studied four groups of rats according to the number of pyramidal cells in lamina III, the intensity of GFAP and BAX immunoreactions and the serum level of cytochrome-c. The level of statistical significance was defined as P ≤ 0.05 where Scheffe’s multiple comparison procedure was used to indicate the significant differences among all the studied groups.

**RESULTS**

**Histological findings**

Examination of sections from the control and MgSO₄ groups (Groups A & B) looked similar, and showed that the cortex was covered by pia matter and formed of six layers that could not be sharply demarcated. These layers were identified as: (1) Outer molecular layer (L-I); appeared paler than other layers, and contained few deeply stained nuclei of neuroglia cells and few blood capillaries. (2) External granular layer (L-II); contained round-ed granular cells with rounded nuclei. (3) External pyramidal layer (L-III); contained immature medium-sized pyramidal cells with central rounded nuclei and apical dendrites appeared as cytoplasmic caps. (4) Internal granular layer (L-IV); contained small rounded and polygonal cells with rounded nuclei. (5) Internal pyramidal layer (L-V); contained large-sized pyramidal cells, associated with many neuroglia and blood capillaries. (6) Polymorphic layer (VI); contained a large variety of cells of different shape and size (Fig. 1.A).

Examination of sections from the propofol group (Group C) showed disruption of the cortical laminar architecture, with a statistically significant decrease in the number of pyramidal cells in L-III. Congested dilated blood vessels were seen with ballooning of their peri-vascular spaces (Figs. 1.B & E). Instead of the pyramidal cells, there were numerous degenerated neurons that appeared with deeply stained acidophilic cytoplasm and pyknotic nuclei. Also, the subpial space appeared congested and dilated (Fig. 1.C).

Examination of sections from the propofol+ MgSO₄ group (Group D) revealed that the CC regained its normal laminar architecture and neuronal density. Also, the congested subpial space, observed in the propofol group, showed marked improvement (Fig. 1.D).
Silver stained sections of the CC from the control & MgSO₄ groups (Groups A & B) revealed dark brown cytoplasm, thick and long apical dendrites and well developed collateral dendrites of the large sized pyramidal cells in L-V. Moreover, many nerve fibers were seen crossing this layer from the previous layers giving the neuropil a homogenous fibrillar pattern (Fig. 2.A). There were many vacuolated neuropilic areas in the cortical sections of propofol group (Group C). Whereas most of the pyramidal cells in L-V lost their apical dendrites, and showed argyrophilic intra-cytoplasmic structures adjacent to their nuclei. Also, most of the nerve fibers appeared more argyrophilic as compared to the control group (Fig. 2.B). While sections from propofol+ MgSO₄ group (Group D) revealed that most of the pyramidal cells in L-V restored their normal appearance. However few cells were still affected and appeared darkly stained. Many nerve fibers still appeared more argyrophilic as compared to the control group (Fig. 2.C).

In the control and MgSO₄ groups (Groups A & B), GFAP immune-stained sections showed negative GFAP immune-reactivity with no astrocytes (Fig. 3.A). There was positive GFAP immune-reactivity in the cortical sections of groups C and D. The reaction was strong positive in propofol group (Group C), as sections showed a statistically significant increase in the area percentage of GFAP immune-reaction, whereas the astrocytes appeared as star-shaped brown cells (Figs. 3.B, C & G).

Immunohistochemical staining for the apoptotic marker BAX indicated a negative reaction in differ-
Fig. 2. Photomicrographs of silver-stained para-sagittal sections of the rat’s CC from different groups; (A) Large-sized pyramidal cells (P) with well developed apical dendrites (↑) within L-V. Also there were many nerve fibers (▲) cross the neuropil (Np) in control group (Group A). (B) The pyramidal cells (P) had no apical dendrites, with argyrophilic intracytoplasmic structures (red arrow head) in Lamina-V. Note multiple vacuolated areas (V) together with argyrophilic nerve fibers (↑) in the neuropil in propofol group (Group C). (C) Most of the pyramidal cells (P) in L-V restored their normal appearance. Note few pyramidal cells still affected and appeared as darkly stained cells (▲). Notice also many nerve fibers (↑) still appeared more argyrophilic in propofol+ MgSO₄ group (Group D).

Fig. 3. Photomicrographs of GFAP & BAX immune-stained para-sagittal sections of the rat’s CC from different groups; A. Negative GFAP immune-reactivity, with no apparent astrocytes in the control group (Group A). B. Increase number of GFAP immune-reactive astrocytes (↑) in the propofol group (Group C). C. Weak positive GFAP immune-reactivity in the Propofol+ MgSO₄ group (Group D). D. Control group (Group A). E. Propofol group (Group C). F. Propofol+ MgSO₄ group (Group D). G. The area percentage of GFAP and BAX immune-reactivity among the 4 studied groups; P<0.001= (*) Significant difference between group C and other groups.
Magnesium sulphate protect against propofol-induced neurotoxicity

**Fig. 4.** The serum level of cytochrome-c among the 4 studied groups. Data presented as mean ± standard deviation. P<0.001 = (*) Significant difference between group C and groups A and B. (**) Non significant difference between group D and group C.

ent layers of the cortex in the cortical sections of the control and MgSO₄ groups (Groups A & B). The reaction was strong and mild positive in the cortical sections of propofol and propofol+ MgSO₄ groups (Groups C & D) respectively indicated by a significant increase in the area percentage of BAX immune-reaction in the propofol group when compared with other groups (Figs. 3.D, E, F & G).

**Cytochrome-c:**

Statistical analysis showed a significant increase in the mean serum level of cytochrome-c in the propofol group when compared to the control and MgSO₄ groups, while there was no significant difference when it was compared to the propofol+ MgSO₄ group (Fig. 4).

**DISCUSSION**

Peri-natal life and early childhood are considered the most intensive periods of brain development, during which there is a rapid neuronal development, differentiation and synaptogenesis in order to establish the complicated networks of the central nervous system (CNS) (Lei et al., 2012).

In this study, albino rats aged 5-7 days were used as an animal model to investigate propofol-induced neurotoxicity. In rodent brain, evidences suggested that the detrimental effects of general anesthesia exposure were age-dependent, peaking at day 7, diminishing by day 14, and absent by day 21 (Gutierrez et al., 2010). This age-dependent effects could be explained by GABA receptors conversion from an excitatory form, early during development, to an inhibitory form in more mature neurons (Henschel et al., 2008). Thus, GABAergic excitation should be considered as a contributor to brain damage (Kahraman et al., 2008).

In this study, propofol was administered by IP injection at a dose of 20 mg/kg which was considered the least anesthetic dose that could induce unconsciousness in rat pups. Although continuous infusion is the clinically relevant way of propofol administration in humans, it was avoided in small rodents as venous puncture was technically difficult (Fredriksson et al., 2007). Pesic et al. (2009) reported that propofol administration at a dose of 25 mg/kg could induce cell death in the CC and the thalamus of developing rat brain [4]. On the other hand, Cattano et al. (2008) assumed that 50 mg/kg propofol was the minimal effective dose that could cause significant neuro-apoptosis in infant mice.

Previous studies suggested that propofol could induce apoptosis of neurons and oligodendrocytes during brain development in mice and rats (Cattano et al, 2008; Tu et al., 2011). In this study, propofol administration led to disruption of the cortical laminar architecture, with a significant decrease in the number of pyramidal cells in L-III compared to the control group. Moreover, pyramidal cells were replaced by degenerated neurons with deeply stained acidophilic cytoplasm and pyknotic nuclei. These findings were in coincidence with Yan (2009), who found that propofol might lead to significant structural changes in the pyramidal cell layer of the rat hippocampus. Also, propofol could induce reorganization of the neuronal cytoskeleton and neurite retraction in cortical neuronal cultures of the rat brain (Jensen et al., 1994; Al-Jahdari et al., 2006). Furthermore, Yu et al. (2013) reported a significant reduction in cortical and hippocampal neuronal density, as well as apparent morphological changes in the pyramidal cells of rats.

In this study, silver-stained sections of the propofol group revealed that pyramidal cells had argyrophilic intra-cytoplasmic structures adjacent to their nuclei. Intra-cytoplasmic inclusions are important indicators of neuronal injury. They occurred in viral, metabolic and degenerative disease (Nelson et al., 2003). Cytoplasmic inclusions are a consequence of drug toxicity when microtubules or microfilament are the primary site of injury (Cheville, 2009). The mechanisms underlying inclusion body formation remained not well understood (Papp and Lantos, 1992). Hatakeyama et al. (1988) attributed the presence of intra-cytoplasmic inclusion bodies within the pyramidal cells of Mongolian gerbils to ischemia and reperfusion process.
following bilateral carotid artery occlusion. Recently, an abnormally insoluble tubular form of asynuclein protein was considered to be the major component of neuronal cytoplasmic inclusions (Arima et al., 1998).

In an attempt to understand the mechanism of propofol-induced neurotoxicity, many authors approved that propofol could acutely depress endogenous neurotrophins in the developing brain that were crucial for neuronal development and synaptic plasticity (Hengartner, 2000; Patapoutian and Reichardt, 2001). Another study revealed that anesthetics with GABA-A agonist effect, like propofol, could induce cell damage by disrupting the intracellular calcium homeostasis, and that their toxicity was attributed to increased calcium level (Yang et al., 2008).

MgSO₄ was proved to have neuro-protective effects in many clinical studies, especially following induction of experimental cerebral ischemia (Kaplan et al., 2004; McDonald et al., 1990); Meller et al., 2015; Safer et al., 2015). In this study, administration of MgSO₄ simultaneously with propofol led to improvement of the histological picture of the CC, including its laminar appearance and neuronal density. Although its exact mechanism as a neuro-protector in the developing brain was still unknown, different theories were proposed, including cerebral vasodilatation, decreased inflammatory cytokines and oxygen free radicals (Costantine and Drever, 2011; Magee et al., 2011). Moreover, MgSO₄ was necessary for the pre-synaptic release of acetylcholine from nerve endings and might produce inhibition of calcium influx into cells (Marinov et al., 1996).

In this study, there was a strong positive GFAP immune-reaction in the propofol group, which became weak following MgSO₄ administration in the propofol+ MgSO₄ group. Astrogliosis assessed by enhanced GFAP immune-reaction was a dominant response to brain damage (Moumdjian et al., 1991; Massaro, 2002). Astrogliosis could increase diffusion barriers in the CNS due to the hypertrophy of astrocytic processes which might impair diffusion of ions, neurotransmitters, and other neuroactive substances in the brain, and thus could influence the extent of CNS injury (Roitback and Sykova, 1999). Also, Hamby et al. (2012) mentioned that gene transcription was increased for numerous proteins that controlled immune-reactive astrocyte formation and function after CNS injury (Hamby et al., 2012). A possible explanation for faster increase in GFAP immune-reaction in neonatal rats following cortical injury in comparison with adults could be that after lesion of the developing brain, as there was a more rapid resolution of cellular debris (Vasic et al., 1998). MgSO₄ was proved to have both direct neuroprotective and glio-protective effects when used in ischemic stroke (Muir, 2001). In this study, there was no GFAP immune-reaction in the control group. Similarly, Balasingam et al. (1994) assumed that the CC of neonatal mice showed no GFAP immune-reactive astrocytes, although it contained this intermediate filament protein (Balasingam et al., 1994). Also, in an attempt to investigate the early phase of astrocyte response to neonatal cortical lesion, Vasic et al. (1998) found that the CC of control rats and the contra-lateral CC of operated animals showed no GFAP immune-reaction in astrocytes. This finding could be attributed to lack of myelin sheath together with the relatively immature immune system in neonatal animals (Maxwell et al., 1990).

BAX is a protein encoded by the BAX gene. It acts as an anti-apoptotic regulator that is involved in many cellular activities (www.ncbi.nlm.nih.gov/gene/581. Entrez Gene: BCL2-associated X protein). In this study, there was a significant increase in the area percentage of BAX immune-reaction in the propofol group when compared to other groups. This result was in agreement with Creeley et al. (2013) who detected a significant number of apoptotic neurons in the fetal monkey brain after prenatal exposure to propofol. Zhang et al. (2010) explained that anesthesia exposure could increase BAX levels, facilitate cytochrome-c release from the mitochondria to the cytosol, induce activation of caspase-9 and caspase-3, and finally cause apoptosis in vitro and in vivo (Zhang et al., 2010).

Cytochrome-c is a 14-kDa hemoprotein that normally presents in the outer surface of the inner mitochondrial membrane (Ott et al., 2002). Yon et al. (2005) reported that mitochondrial integrity impairment was one of the first signs of neuronal dysfunction following anesthesia exposure. Similarly, the present study demonstrated a significant increase in the mean serum level of cytochrome-c in the propofol group when compared to the control group. In patients, an elevated level of circulating cytochrome-c was always associated with mitochondrial injury (Renz et al., 2001). The inner mitochondrial membrane undergone an increase in permeability, called the mitochondrial permeability transition (MPT), which led to opening of a membranal high-conductance channel called permeability transition pore (PTP). PTP was accompanied by cytochrome-c release in vitro. This process was regulated by Bcl-2 proteins including BAX and BAK (Kluck et al., 1997; Shimizu et al., 2000). In the present study, there was no significant difference in the mean serum level of cytochrome-c in the propofol group when it was compared to propofol+ MgSO₄ group. In an attempt to explain this finding, Hsu et al. (1982) found that addition of magnesium to the rat hepatic microsomal preparation showed an increased activity of NADPH-cytochrome-c. Also, Lemasters and Nieminen (2002) added that Bax-induced cytochrome-c release is facilitated by MgSO₄ and could not be blocked by transition inhibitors. On the other hand, Piana et al. (2008) mentioned that magnesium
could protect against the increase in the permeability of mitochondrial outer membrane, thus modulating the amount of cytochrome-c molecules transferred from the mitochondrial inter-membrane space into the cytosol.

In conclusion, cautions must be taken when using propofol in newborns and young children. More targeted and focused clinical research is needed to examine the potential neuro-protective effects of MgSO4 on propofol exposure during early CNS development.

REFERENCES


Magnesium sulphate protect against propofol-induced neurotoxicity


