Effect of Propofol Fresensius 1% on nervous system ultrastructure of *Biomphalaria alexandrina*.

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ABSTRACT

This study was aimed to evaluate the effect of propofol fresensius 1% on the nervous system of *Biomphalaria alexandrina* snails, which represent the intermediate host of *schistosoma mansoni* after acute exposure (5ml/L) for 24 hours and chronic exposure (1ml/L) for 72 h, using scanning and transmission electron microscope. After chronic exposure, visceral ganglion was observed compressed and elongated, not paired with pedal ganglia and its nerves did not contact with it. 1ml/L propofol completely blocked synaptogenesis between visceral and pedal neurons. After acute exposure, the varicosities have few granular vesicles, whereas in the chronic exposure, the varicosities were empty and no granular vesicles were found inside it. Poorly developed endoplasmic reticulum, few Golgi bodies, elongated mitochondria, and more intercellular connective space were observed.

INTRODUCTION

Propofol's main action appears to be as a GABA-A receptor modulator, enhancing GABA-mediated inhibition (Hara *et al.,* 1993). Ketamine is primarily a non-competitive NMDA receptor antagonist, blocking the open receptor at a site within the pore (Orser *et al*., 1997). In addition, other cellular actions, including effects on nicotinic acetylcholine and5-HT3 receptors (Violet *et al.*, 1997; Browning and Lukowiak, 2008) and ion channels and conductances (Yamakage *et al*., 1995; Wu *et al*., 1997) have been reported for both agents, but these do not explain the excitatory effects described above. Molluscan neurons have been widely used for investigating and screening the effects of pharmacological agents (Girdlestone *et al*., 1989a,b; Mills *et al.,* 1992; Woodall and McCrohan 2000; Woodall *et al.,* 2003; Nacsa *et al*., 2015; Bogodvid *et al.*, 2017; Elekes *et al*.,2018; Wyeth, 2019). This is largely due to their highly accessible central nervous systems (CNS), with 'giant' neurons which can be individually identified from one preparation to the next (Kyriakides *et al.*, 1989). This means that the same neuron with known characteristics as well as identified synaptic connections,can be used to study and compare responses to different agents within an intact nervous system in vitro. *Lymnaea stagnalis* has been exploited in studies of the actions of inhalation anaesthetics such as halothane, isoflurane and enflurane. All of these agents induce

full and reversible anaesthesia in the whole animal, measured as a loss of the whole body withdrawal reflex, at clinically relevant doses, with an ED50 close to the anaesthetic requirement of mammals (Girdlestone *et al.,* 1989a). Woodall and McCrohan (2000) showed that the actions of the intravenous anaesthetics propofol and ketamine on animal behavior and neuronal activity in the snail *L. stagnalis*, particularly in relation to excitatory effects observed clinically. When injected into the whole animal, neither agent induced total anaesthesia. Rather, behavioural activity was enhanced by propofol (10_5 M) and ketamine (10_7 M), indicating excitatory effects. When superfused over the isolated central nervous system (CNS), differential effects were produced in two identified neurons, right pedal dorsal 1 (RpeD1) and visceral dorsal 4 (VD4). Resting membrane properties were largely unaffected. However, spike after hyperpolarisation was significantly reduced in RpeD1, but not VD4, with some evidence of increased excitability. In addition, an intrinsic bursting property (post-stimulus burst) in VD4 was altered by propofol (10_7 M). The results suggest significant excitatory components in the actions of some intravenous anaesthetics, as well as a potential role in modifying excitation and bursting mechanisms in the CNS (Woodall and McCrohan, 2000).

MATERIALS AND METHODS

Snails

Biomphalaria alexandrina were collected from irrigation schemes at Giza Governorate, Egypt. The snails were then washed thoroughly with dechlorinated tap water and maintained in the Medical Malacology Laboratory, Theodor Bilharz Research Institute, Egypt, at room temperature ($25\pm1°C$), in plastic aquaria (16 x 23 x 9 cm) containing dechlorinated tap water and covered with glass plates. The snails were fed lettuce leaves and blue green algae (*Nostoc muscorum*). Lettuce leaves were given daily and its amount was adjusted to the number and size of the snails and the algae were added weekly. Snails were examined twice weekly for natural infection up to 6 successive weeks. Uninfected, healthy snails were used in the experimental tests.

Experiment

Biomphalaria alexandrina breeding from lab generation 7-8mm in shell diameter are divided into 3-groups. 1st group exposed for 5ml/ L propofol fresensius 1% for 24, 2nd group exposed for 1ml/L for 72 hours of propofol fresensius 1% and 3rd group for control then dissected. After dissection of such specimens, the nervous system was drawn under a binocular dissecting microscope to make electronic microscope examination.

Propofol fresensius 1% is anaesthetic agent for intravenous injection or infusion manufactured by fresensius Kabi Austria GmbHA. 8055 Graz, Austria on behalf of Anesthetic agent.

Scanning Electron Microscope

Ultrastructure study of central nervous system was fixed by processeding according to Glauert, (1974). They were fixed for 30 min in 2.5% glutharaldehyde in PBS buffer at room temperature. All samples were centrifuged gently, washed 3 times in PBS, postfixed for min in 2% osmium tetroxide in PBS buffer at 4^0C, and dehydrated in 4 changes of graded alcohol (50, 70, 90, and 100%) at 5 min gap. Finally pellet was examined on fornvar coating grids by Scanning Electron Microscope (Inspect S; FEI, Holland) illustrating the shape of C.N.S in treated and

control samples at Electron Microscopy Unite of Theodor Bilharz Research Institute (TBRI).

Transmittion Electron Microscope

Central nervous system was fixed by immersion in iced 2.5% glutaraldehyde in 0.2 M phosphate buffered (pH 7.2) for 2 hr and post-fixed in 0.05 M sodium cacodylate buffer and 1% osmium tetroxide in the same buffer and dehydrated in acetone for embedding in Araldine. Ultra-thin sections from selected blocks were obtained with a Reichert OM-43 Ultratome, doubly-stained with lead citrate and uranyl acetate and examined with a Jeol 100 C Electron Microscope at 50 Kv. Semithin sections, approximately 1-2 um in thickness, stained with alkaline toluidine blue, were used for selecting the area of interest.

RESULTS

1- Scan electron microscope:

A- Nervous system in control snail:

The present study revealed that the nervous system of *B. alexandrina* snails consists of a double ring of 9 ganglia and an anteriorly situated pair of buccal ganglia (Fig. 1a). The upper ring consists of the paired cerebral ganglia, paired pleural ganglia, paired parietal ganglia (though left and right ganglia are of unequal size) and the single median visceral ganglion. The paired pedal ganglia lie ventral to this upper ring and are connected to it via the cerebra-pedal connectives and pedal pleural connectives. The pedal ganglia are connected to one another by the dorsal pedal commissure and the ventral pedal commissure. Pleural ganglia have no commissure, but they receive three connectives from cerebral, pedal ganglia and from the visceral loop.

B- Effect of Propofol on morphology of ganglia:

In control snail, when visceral ganglion (V.G) was soma-soma paired with pedal ganglia (Pe.G), excitatory synapses between V.G and Pe.G develop reliably (100%). Neurons were located and they projected thick axons. In the neuropil varicose processes were found juxtaposed thick axons suggesting a close contact with them (Fig. 1b). To investigate whether propofol affects cholinergic synaptic transmission between V.G and Pe.G, recordings were made from the paired cells in the presence of anesthetic. After acute exposure 24 h in 5ml/L propofol, Pedal and visceral ganglia were slightly shrunken and visceral nerves between the cells ending both at the axonal contact point and around the pedal somata (Fig. 1c). In the neuropil varicose processes were found juxtaposed the thin axons. 5ml propofol reduced synaptic transmission. After chronic exposure 72 h in low concentration 1ml/L propofol, visceral ganglion was compressed and elongated, not paired with pedal ganglia and its nerve did not contact with pedal ganglia (Fig. 1d). Presynaptic action potentials in V.G failed to generate excitatory postsynaptic potentials in Pe.G. 1ml/L propofol completely blocked synaptogenesis between visceral and pedal neurons.
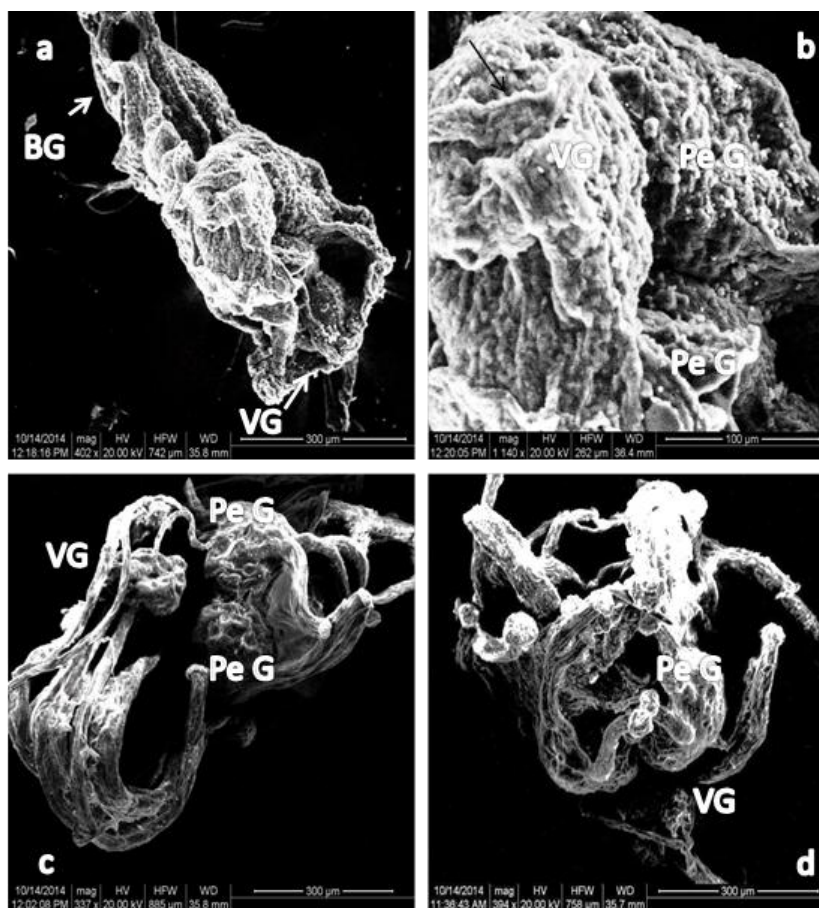
Fig. 1: Scanning electron micrographs of control *Biomphalaria alexandrina* central nervous system a,b showing normal structure and morphology of ganglia and axons (arrow), buccal ganglia (BG), median visceral ganglion (VG), pedal ganglia (Pe G) . (c) After 24 h, 5ml/L propofol Pedal and visceral ganglia were slightly shrunken, presynaptic processes ending both at the axonal contact point and around the Pedal somata. (d) After 72 h in low concentration 1ml/L propofol, visceral ganglion was compressed and elongated, not paired with pedal ganglia and its nerve did not contact with pedal ganglia.

2- Transmission Electron microscope:
A- Ultrastructure of central nervous system:

The ultrastructure of central nervous system control sections of these ganglia was investigated. Generally, it showed oval nucleus, mitochondria and smooth endoplasmic reticulum sER (Fig. 2a). Active Golgi body and rough endoplasmic reticulum often organized in regular arrays and a great number of free ribosomes. The electron dense neurosecretory granules were observed. These were attributed to the activity of both Golgi apparatus and rER that have the main role in their formation. Varicosities filled with granular vesicles of variable electron density were also observed (Fig. 2b).

B- Effects of propofol on central nervous system ultrastructure:

After acute exposure 24 h, 5ml/L propofol effect on ultrastructure elements of the cell where the nucleus became irregular in shape with several indentations in the nuclear envelope and heterochromatin granules against this membrane (Fig. 2c). Figures (2 d,e) showed a shrunken process filled with lipid droplets and an increased intercellular connective space. The lipid droplets (L) surrounded by sER. The varicosities have few granular vesicles. Whereas chronic exposure at low concentration 1ml/L propofol for 72 h, the varicosities were empty and no granular

vesicles inside it. Poorly developed endoplasmic reticulum, few Golgi bodies, elongated mitochondria, and more intercellular connective space were observed (Fig. 2f).
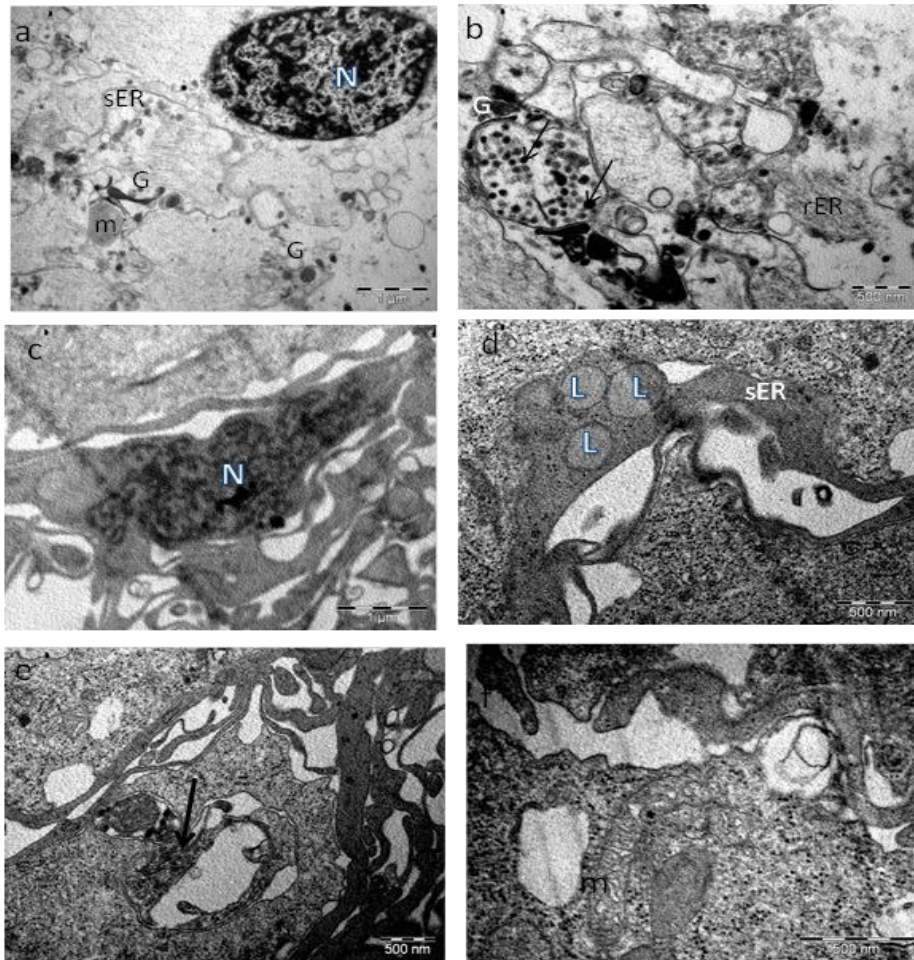

Fig. 2: Transmission electron micrographs of control *Biomphalaria alexandrina* central nervous system a,b showing (a) oval nucleus (N), mitochondria (m), well-developed Golgi complex (G) and smooth endoplasmic reticulum (sER). (b) A large varicosity is densely packed with large granular vesicles and rough endoplasmic reticulum (rER). After 24 h in 5ml/L propofol (c,d,e). (c) Irregular nucleus. (d) Lipid droplets (L) surrounded by (sER). (e) Shrunken process, varicosities have few granular vesicles (arrow). After 72 h in low concentration 1ml/L propofol (f) Empty varicosities and elongated mitochondria (m).

DISCUSSION

This study is the first to demonstrate that anesthetic treatment effect on ultrastructure element of *Biomphalaria alexandrina* central nervous system. The present data demonstrated that the propofol-induced effect on morphology and ultrastructure of visceral and pedal ganglia of *B. alexandrina* which lead to suppression of synaptic transmission. These data are in contrast with previous studies on vertebrate neurons, where propofol did not significantly alter the responsiveness of either glutamate (Perouansky and Antognini, 2003) or glycine receptors (Daniels and Roberts, 1998). In agreement to the present study, Woodall *et al.* (2003) indicated that anesthetic treatment blocks synaptogenesis but not neuronal regeneration of cultured *Lymnaea* neuron.

Browning and Lukowiak (2008) recorded that ketamine causes impairment of procedural memory formation, and that ketamine acts differentially, inhibiting only long-term memory (LTM) formation while having no effect on intermediate-term memory (ITM) formation. Ketamine's ability to inhibit LTM was found not to be due to state dependent learning implying that ketamine's effects are therefore specific to the molecular process involved in procedural LTM formation. This suggests that ketamine may be exerting its differential effects by altering the gene transcription processes necessary and specific for LTM formation. Additionally, ketamine was found to have no effect on retrieval when administered 1 h before testing.

Propofol has been found to inhibit endogenous glutamate release from endosomes (Perouansky and Hemmings, 2003) suggesting that it may also affect transmitter release though a similar action on intact, functional synapses has not yet been demonstrated. Other studies on rat hippocampal neurons in vitro (Orser et al., 1995) argue that propofol-induced suppression of synaptic transmission may be mediated indirectly through an enhancement of GABA receptor function (Bai et al., 2001; Hirota et al., 1998; Wakasugi et al., 1999; Pearce, 2003). This notion is consistent with a recent study, which demonstrates that propofol potentiates GABA ergic synaptic transmission in the   brain and that these effects accompany neuronal activity-mediated, enhancement of *c-fos* expression (Nelson et al., 2002). As propofol and other anesthetics were found to affect GABAA receptors in the sleep pathway (Nelson et al., 2002), the preceding study thus further underscores the importance of such agents in defining normal patterns of neuronal activity in the nervous system. On the other hand, because mutant mice lacking the submit of the GABAA1- receptor still exhibit normal anesthesia in response to propofol, the precise site and the mode of this anesthetic's actions still remain controversial (Homanics and Firestone, 2003).

When action potentials were evoked, spike AHP was reduced in RPeD1, but not in VD4, by both propofol and ketamine. This illustrates the differential effects that pharmacological agents may have on identified neurones with different ion channel complements and also suggests that propofol and ketamine act on voltage dependent components of the neuronal membrane. Ketamine has already been shown to have voltage dependent effects (Parsons et al., 1995); this is unsurprising in view of its effects as an NMDA receptor antagonist. Changes in intracellular calcium are potentially important in the mediation of anaesthesia (Ahmed et al., 1993; Winlow et al., 1993). In one study, propofol was shown to increase intracellular calcium (Mantz et al., 1994), whereas another (Yamakage et al., 1995) reported a reduction in intracellular calcium transients by propofol and ketamine. Halothane and isoflurane have been shown to increase intracellular calcium in hippocampal neurones and also to have an effect on K_ channels involved in the generation of spike AHP (Stapelfeldt and Oleszewski, 1999). Both menthol and inhalation anaesthetics have been shown previously to reduce AHP in RPeD1 (Haydon et al., 1982).

Syed and Winlow (1991) suggested underlying excitatory actions of propofol, particularly at lower concentrations, which may be specifically directed towards intrinsic 'bursting' mechanisms within the cell, with potential consequences for the emergent rhythmical output from the network as a whole. Similarly, the output of rhythm-generating neuronal networks may be an important target for anaesthetics in the mammalian CNS. For example, respiratory sinus arrhythmia, a manifestation of rhythmic output from the brainstem, has been shown to be significantly affected by

propofol, providing a reliable indicator of depth of anaesthesia (Pomfrett *et al.*, 1993).

The present data suggest that the vesicles are synthesized and filled at Golgi complexes, which are frequently observed in close association with vesicle groups, and often contained electron-dense material. This result was agreed with Turner *et al.,* (1980).

In conclusion propofol has strong effect on ultrastructure feature of neuron cells. The nucleus became irregular in shape, the mitochondria changed from oval to elongated and poorly developed endoplasmic reticulum, few Golgi bodies which affecting on the presence of granular vesicles in varicosities in axon.

ACKNOWLEDGMENT

Compliance with Ethical Standards:
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.
Conflict of interest:
The authors declare that no conflict of interest.

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ARABIC SUMMARY

تأثير البروبوفول فريسينسيس ١% على التركيب الدقيق للجهاز العصبى لقواقع *Biomphalaria alexandrina* كمؤشر حيوى.

حنان شحات مسلم و شيرين محفوظ منصور

قسم بحوث البيئه والرخويات الطبيه ـ معهد تيودور بلهارس للابحاث- جيزه- مصر

يجب تطوير نتائج تقدم الرعاية الصحية للكائنات الحية ، سواء الفقاريات أو اللافقاريات ، من خلال دراسة الجهاز العصبي الذي يمثل مركز جميع الأنشطة والخصائص ، لذا استخدمت هذه الدراسة القواقع من الرخويات تحت تأثير التخدير لمعرفة التغيرات في الشكل الخارجى للجهاز العصبي عند الاسترخاء باستخدام الميكروسكوب الالكترونى ، في المستقبل من الممكن ليس فقط تحسين صفات القواقع الناقلة للطفيل لتكون اكثر مقاومة لاى غزو خارجى ولكن ايضا تغيير الكيمياء بين الطفيل وانسجة القوقع . تهدف هذه الدراسه الى تقييم تأثير البروبوفول فريسينسيس ١% على الجهاز العصبى لقواقع *بيومفلاريا الكسندرينا* كمؤشر حيوى للافقاريات والذى يمثل العائل الوسيط لطفيل البلهارسيا المعوية. بعد التعرض الحاد لتركيز ٥مل /ل لمدة ٢٤ ساعة و التعرض المزمن لتركيز١مل /ل لمدة ٧٢ ساعة بالمقارنة مع المجموعة الضابطة بواسطة المجهر الالكترونى الماسح والنافذ . التعرض المزمن عند تركيز منخفض ١ مل / لتر من البروبوفول لمدة ٧٢ ساعة ،العقده العصبية الحشوية كانت منضغطة ومطولة وغير مقترنه بالعقدة العصبية القدمية واعصابها كانت غير متصلة معها. ١مل / لتر من البروبوفول تمنع تماما مراحل التشابك العصبى بين الاعصاب الحشوية والقدميه. ٥مل /لتر من البروبوفول لمدة ٢٤ ساعة، الدوالات كانت تحتوى على القليل من الحويصلات الحبيبية، في حين أن التعرض المزمن عند تركيز أقل ١ مل / لتر من البروبوفول لمدة ٧٢ ساعة ، كانت الدواليات فارغة ولا توجد حويصلات حبيبية بداخلها. لوحظ هناك نمو ضعيف للشبكة الإندوبلازمية، عدد قليل من أجسام جولجى، ميتوكوندريا مطوله و مساحات كبيرة بين الخلايا.