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Role of Sedative-Hypnotic Agents in Neurodegeneration: Effects of Midazolam and Thiopental on Apoptosis and Oxidative Stress Expression in Neonatal and Adult Rat Brains

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ABSTRACT

AIM: To investigate the effects of midazolam (MDZ) and thiopental on neonatal and adult rat brains.

MATERIAL and METHODS: The study included adult and 7-day-old rats that were administered 9 mg/kg of MDZ, 60 mg/kg of thiopental, or both. The Bax, procaspase-3, and caspase-3 levels were assessed using Western Blot analysis and the total oxidative stress index (OSI) values were measured spectrophotometrically.

RESULTS: The procaspase-3 and caspase-3 levels were 12% and 6% lower in the neonatal MDZ group when compared to the control group. The Bax, procaspase-3, and caspase-3 levels were higher in the neonatal thiopental group by 25%, 4%, and 34%, and in the MDZ group by 16%, 19%, and 43% when compared to the neonatal control group. In the adult rats, the caspase-3 levels were 10 times higher in the MDZ group when compared to the control and thiopental groups. Moreover, the caspase-3 levels were 7 times higher in the adult thiopental group when compared to the control group. The OSI values in the neonatal rats were significantly higher in the neonatal MDZ and neonatal thiopental groups when compared to the control group ($p<0.05$). Similarly, the OSI values in the adult rats were significantly higher in the neonatal MDZ and neonatal thiopental groups when compared to the control group ($p<0.05$).

CONCLUSION: MDZ and thiopental may promote apoptosis and oxidative stress, and thereby result in neurotoxicity, with MDZ showing a greater effect in adults and thiopental showing a greater effect in neonates.

KEYWORDS: Midazolam, Thiopental, Neurodegeneration, Oxidative stress, Neonatal rats

INTRODUCTION

Midazolam (MDZ) is a short-acting benzodiazepine sedative-hypnotic agent that binds to selective binding sites on the GABAA (γ-Aminobutyric acid type A) receptor, thereby potentiating the effects of GABA by increasing the frequency of the chloride channel opening and facilitating the binding of GABA to its own binding site. In addition to its sedative and hypnotic effects, MDZ also exerts anxiolytic and anticonvulsant effects (10). In addition, MDZ is commonly used in clinical practice, predominantly as a premedication drug prior to anesthesia, a sedative agent prior to diagnostic or surgical procedures performed under local anesthesia, for anesthetic induction and maintenance, and long-term sedation in intensive care unit (ICU) patients (23).

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MDZ has also been reported to have anticancer, neurotoxic, and, on the contrary, neuroprotective effects (26). In several in vitro studies, MDZ has been shown to promote apoptosis and to reduce proliferation in neuroblastoma and mouse Leydig cells (6,16,24). Some other studies have also suggested that MDZ and other anesthetic agents have neurodegenerative effects on brain cells, particularly in the developing human brain (3). Similarly, some animal studies have shown that MDZ leads to learning and memory impairment along with neurodegeneration in the developing rat brain (9). In contrast to these studies, however, some other studies have indicated that MDZ prevents motor neuronal death from oxidative stress attack mediated by the c-JUN N-terminal kinase/extracellular signal-regulated kinase signaling pathway and that MDZ can be useful in diseases that cause neuronal degeneration (12).

Thiopental is a short-acting sedative-hypnotic barbiturate with a similar mechanism of action to that of MDZ. Thiopental is also widely used for anesthetic induction, similar to MDZ. Thiopental, just like MDZ, has been shown to have various effects. In rats, for instance, thiopental has been shown to decrease the expression of glutamic acid decarboxylase (GAD), which plays a role in the synthesis of GABA from glutamic acid, and cause neuronal degeneration (17). Moreover, thiopental has been shown to cause neurotoxicity by increasing oxidative stress in the adult rat brain (1). In contrast, thiopental, just like MDZ, has also been reported to have favorable effects, such as inhibiting apoptosis in neuroblastoma cells (20).

In the present study, it was aimed to investigate the effects of MDZ and thiopental on adult and neonatal rat brains and also examine the effects of these 2 drugs on apoptosis and reactive oxygen species (ROS) generation in the rat brain in order to determine which of these two drugs is more reliable in each age group.

MATERIAL and METHODS

Experimental Procedure

Approval was obtained from the Van Yuzuncu Yil University Laboratory Animals Ethics Board (Approval No: 2018/02). The study included a total of 64 rats, comprising 32 adult male Wistar albino rats, weighing 200–250 g, and 32 7-day-old male and female rats, weighing 10–15 g. The animals were kept under a 12 h/12 h light/dark cycle with ad libitum access to standard rat chow and tap water. The rats were randomly divided into 8 groups with 8 rats in each:

1. Neonatal control group (NC): Dimethyl sulphoxide (DMSO) only
2. Adult control group (AC): DMSO only
3. Neonatal MDZ group (NM): MDZ 9 mg/kg i.p. (Deva I.V)
4. Neonatal thiopental group (NT): thiopental 60 mg/kg i.p. (I.E Ulagay I.V).
5. Neonatal MDZ + thiopental group (NMT): MDZ 9 mg/kg i.p.+ thiopental 60 mg/kg i.p.
6. Adult MDZ group (AM): MDZ 9 mg/kg i.p.
7. Adult thiopental group (AT): thiopental 60 mg/kg i.p.
8. Adult MDZ + thiopental group (AMT): MDZ 9 mg/kg i.p. + thiopental 60 mg/kg i.p.

Six hours after drug administration, the rats were euthanized and the brain tissues were removed. The tissues were divided into two portions; one portion was stored at –80 °C and was later used for the assessment of apoptosis on Western Blot analysis and the other portion was stored at –20 °C and was used for the measurement of the total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI).

Western Blot Analysis

The tissues were homogenized with sonication and lysed in Ripa Buffer that contained protease inhibitors. For the analysis, 75 mcg of the proteins were fractionated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes. The membranes were blocked in 5% skim milk powder in phosphate buffered saline with Tween. The blots were labeled with caspase-3, Bax, and cyclin B antibodies. Actin was used as the loading control. Signal intensity on the blots was determined using the enhanced chemiluminescent detection system.

Assessment of Oxidative Stress

The serum TAS and TOS levels were measured spectrophotometrically using a commercially available kit (8). The OSI is an indicator of oxidative stress, which is defined as the ratio of TOS to TAS. The OSI was calculated based on the following formula: (initially, the TAS levels were converted to μmol/L); OSI (arbitrary unit) = [TOS (μmol H2O2 Eq/L)/TAS (μmol Trolox Eq/L)] × 100 nm.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics for Windows 24.0 (IBM SPSS Inc. Co., Armonk, NY, USA). Continuous variables were expressed as median, mean, standard deviation (SD), minimum, and maximum. The and TOS levels were compared among the groups using the Kruskal-Wallis H test followed by the post-hoc Bonferroni multiple comparisons correction test. $p<0.05$ was considered statistically significant.

RESULTS

Apoptosis and Cell Cycle

In order to understand the effects of MDZ and thiopental on apoptosis and proliferation, the expression/activation levels of Bax and caspase 3 were determined, as apoptotic markers, in addition to cyclin B1 as a proliferation marker. In the neonatal rats, the Bax levels were 8% higher and the procaspase-3 and caspase-3 levels were 12% and 6% lower in the NM group when compared to the NC group, respectively. Similarly, the cyclin B levels were also 11% lower in the NM group when compared to the NC group (Figure 1A-C).

The Bax, procaspase-3, and caspase-3 levels were higher in the NT group when compared to the NM group by 25%, 4%, and 34% when compared to the NC group by 16%, 19%, and 43%, respectively. The cyclin B levels were 13% higher in NT

group when compared to the NM group, while no significant difference was found between the NT and NC groups (Figure 1A-C).

The Bax levels in the NMT group were 20% and 11% higher when compared to those of the NM and NC groups, respectively, and were 4% lower than those of the NT group. Additionally, the procaspase-3, caspase-3, and cyclin B levels were 17%, 72%, and 16% higher in the NMT group when compared to the NC group, respectively (Figure 1A-C).

In the adult rats, although the Bax and procaspase-3 levels were 11% and 15% lower in the AM group when compared to the AC group, respectively, the caspase-3 levels in the same group were 10 times higher than those in the AT and AC groups. Additionally, the cyclin B levels in the AM group were 17% higher than those of the AC group (Figure 2A, B).

The Bax levels in the AT group were 3% and 7% higher than those of the AM and AC groups, respectively. The procaspase-3 levels exhibited no significant difference between the AT and AC groups, while they were 21% higher in the AT group when compared to the AM group. Although the caspase-3 levels in the AT group were almost 7 times higher than those of the AC group, they were 29% lower than those of the AM group (Figure 2A, B).

The cyclin B levels in the AT group were 27% higher than those of the AC group. The Bax and procaspase-3 levels in the AMT group were 34% and 24% higher than those of the AC group, while they were 20% and 10% higher than those of the AM group, and 27% and 5% higher than those of the AT group, respectively. Moreover, the caspase-3 levels in the same group were 9 times higher than those of the AC group and 35% higher than those of the AT group. In the same

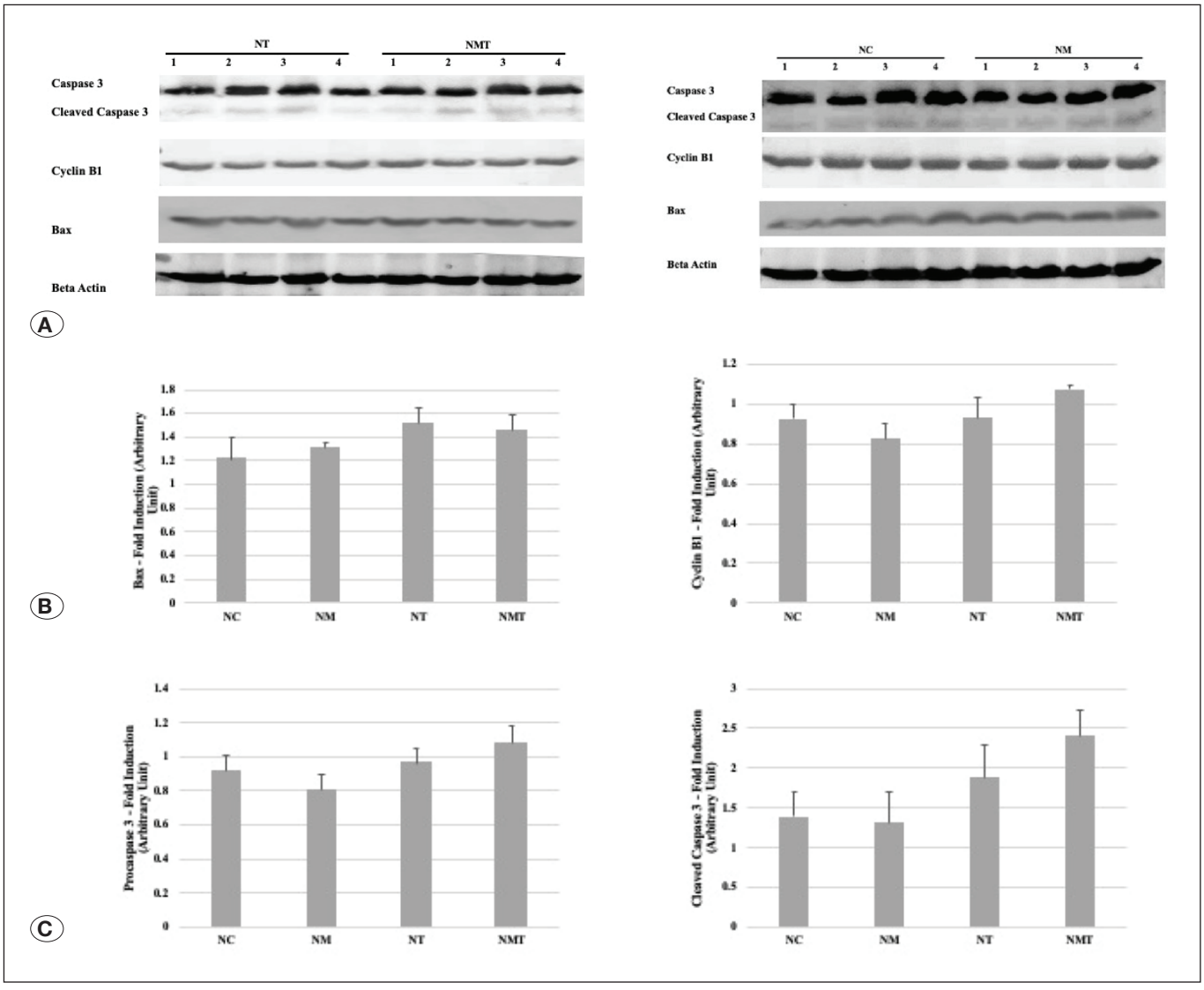

Figure 1: A) Cyclin B, Bax, caspase, and cleaved caspase 3 blots in the NC and MDZ groups. **B)** Cyclin B, Bax, caspase, and cleaved caspase 3 blots in the NT and thiopental + MDZ groups. **C)** Cyclin B, Bax, caspase, and cleaved caspase 3 levels in the neonatal rats.

group, the cyclin B levels were 12% higher than those of the AC group, and were 4% and 12% lower than those of the AM and AT groups, respectively (Figure 2A, B).

TAS, TOS, and OSI Levels

In the neonatal rats, the TAS levels were significantly lower in the NT group when compared to the NC group ($p<0.05$). In the adult rats, however, the TAS levels were significantly lower in the AM, AT, and AMT groups when compared to the AC group, with the greatest decrease seen in the AT group ($p<0.05$). The TOS levels in neonatal rats were significantly higher in AM, AT, and AMT groups compared to AC group, with the highest increase seen in the AMT group ($p<0.05$). However, the TOS levels in the adult rats were significantly higher only in the AM group when compared to the AC group ($p<0.05$). The OSI values in the neonatal rats were significantly higher in the NM, NT, and NMT groups when compared to the NC group, with the highest increase seen in the NT group ($p<0.05$). Similarly, the OSI values in the adult rats were significantly higher in the AM, AT, and AMT groups when compared to the AC group, with the highest increase seen in the AM group ($p<0.05$) (Table I).

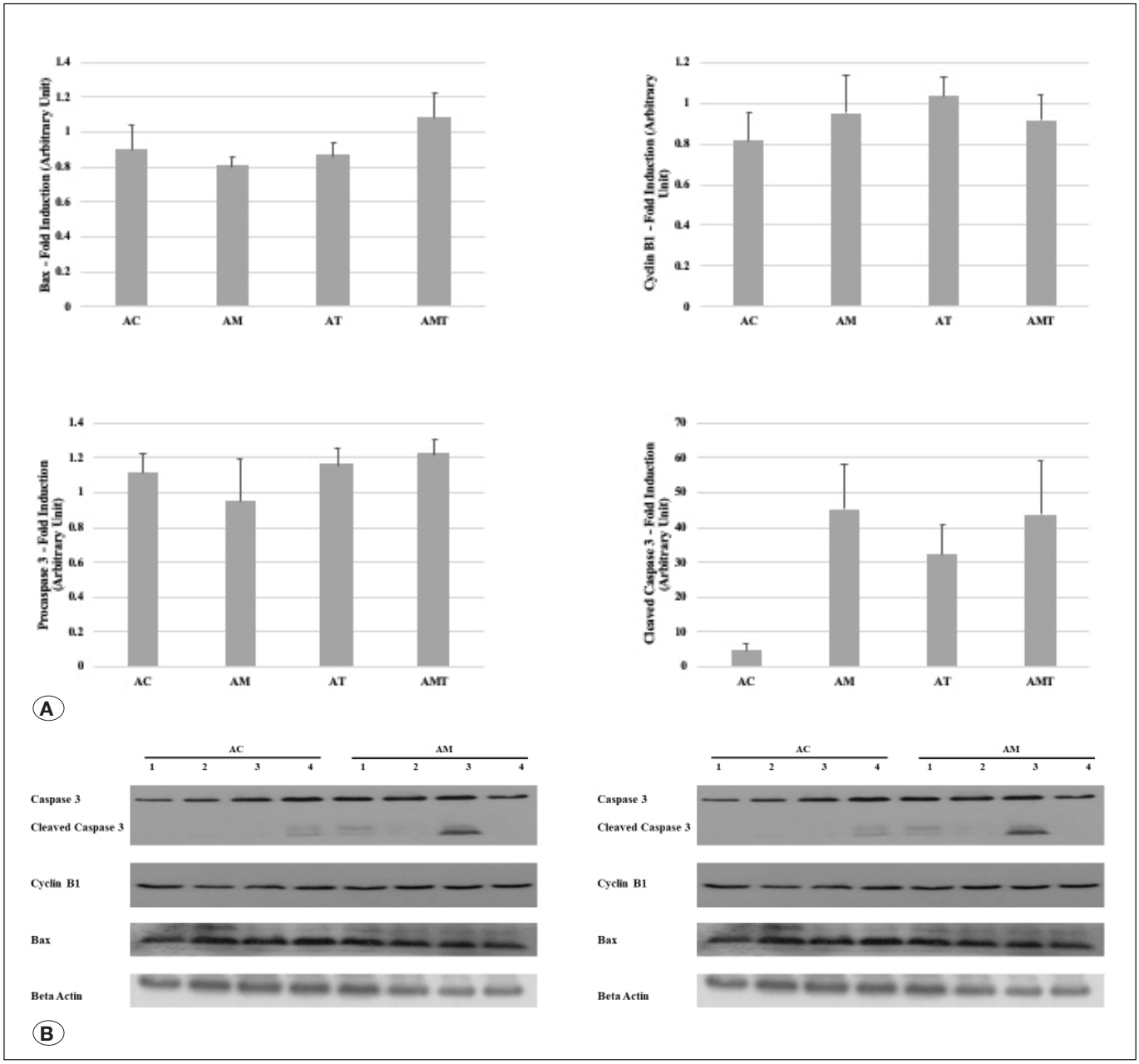

Figure 2: A) Cyclin B, Bax, caspase, and cleaved caspase 3 blots in the AC and MDZ groups. **B)** Cyclin B, Bax, caspase, and cleaved caspase 3 blots in the AT and thiopental + MDZ groups. **C)** Cyclin B, Bax, caspase, and cleaved caspase 3 levels in the adult rats.

Table I: TAS, TOS, and OSI Levels

Groups	TAS	TOS	OSI
NC	1.01 ± 0.14^{c}	4.452 ± 42 ^{e}	0.44 ± 0.08^{f}
AC	2.04 ± 0.16^{a}	13.024 ± 1.67^{c}	0.64 ± 0.12^{f}
NM	0.89 ± 0.13^{c}	8.214 ± 1.07^{d}	0.92 ± 0.20^{e}
NT	0.64 ± 0.12^{d}	13.12 ± 2.15^{c}	2.06 ± 0.36^{a}
NMT	1.02 ± 0.11^{c}	17.143 ± 1.82^{b}	1.67 ± 0.26^{b}
AM	1.41 ± 0.27^{b}	31.881 ± 2.82^{a}	2.27 ± 0.52^{a}
AT	0.97 ± 0.14^{c}	14.613 ± 2.01^{c}	1.47 ± 0.40^{bc}
AMT	1.27 ± 0.14^{b}	16.19 ± 1.89^{b}	1.28 ± 0.11 ^{cd}

NC: *Neonatal control,* ***AC:*** *Adult control,* ***NM:*** *Neonatal midazolam,* ***NT:*** *Neonatal thiopental,* ***NMT:*** *Neonatal midazolam + thiopental,* ***AM:*** *Adult midazolam,* ***AT:*** *Adult thiopental,* ***AMT:*** *Adult midazolam + thiopental.*

DISCUSSION

MDZ has been shown to promote apoptosis in numerous in vitro and in vivo studies investigating cell lines (16). MDZ induces apoptosis through various mechanisms. A previous study suggested that MDZ activates GABAA and peripheral-type benzodiazepine receptors, thereby leading to increased cytochrome C levels and ultimately, to increased apoptosis through the intrinsic pathway. The same study, however, advocated that MDZ has no effect on proapoptotic proteins, such as Bcl-2, procaspase-9, and procaspase-3 (10,16,21). Study conducted of embryonic neuronal cell culture of rats was shown midazolam GABA and glutamate receptor altered and increase the apoptosis (22). The study proposed that MDZ activates the intrinsic pathway of apoptosis independent of benzodiazepine (25). Another study conducted hepatocellular carcinoma cell was shown midazolam accelerates apoptosis by elevated microRNA-124-3p and suppressing PIM-1 (19). However, it has also been suggested that although MDZ results in no changes in the Bcl and Bax proteins in mouse Leydig cells, it activates caspase-3 and -9, ultimately leading to apoptosis through the intrinsic pathway (10). On the contrary, there are some other studies proposing that MDZ has no effect on apoptosis. For instance, MDZ has been shown to have no effect on caspases or apoptosis in glioblastoma and astrocyte cell lines, despite leading to increased necrotic cell injury (14,18). On the other hand, MDZ has also been suggested to have a role in cell cycle and proliferation. Accordingly, some previous studies have indicated that MDZ may block the cell cycle at the G2/M phase by inhibiting the expression of cyclin A, cyclin B, and cyclin-dependent kinase 1, and may inhibit cell proliferation by blocking the cell cycle at the G0/G1 phase (10,24).

The extensive body of literature has indicated that MDZ leads to toxicity in neurons, particularly in the developing human brain. A previous study showed that the use of MDZ, both in isolation and in combination with ketamine, resulted in neuronal injury in neonatal rats (28). Similarly, some other studies have demonstrated that the use of MDZ in combination with other anesthetic agents, such as isoflurane, resulted in neuronal toxicity along with permanent learning difficulties, and another study suggested that long-term exposure to MDZ during early development may result in persistent changes in the structure and function of the brain (9,27). Additionally, MDZ has been shown to impair GABA neurotransmission in the developing brain, thereby leading to caspase-3 activation and ultimately, to apoptosis through the intrinsic pathway (3). To our knowledge, the current study is the first in the literature to investigate the effects of MDZ in both neonatal and adult rats. However, the findings herein were dissimilar to those of the studies that have suggested that MDZ leads to apoptosis in neonatal rats. Interestingly, it was found herein that the Bax levels in the neonatal rats were slightly increased, while the procaspase-3 and caspase-3 levels were slightly decreased when compared to the control group. Moreover, it was also found that the Bax, procaspase-3, and caspase-3 levels were significantly higher in the neonatal rats that were administered both MDZ and thiopental when compared to the control group and to the groups that were administered MDZ or thiopental alone ($p<0.05$). In the adult rats, however, the Bax and procaspase-3 levels were lower and the caspase-3 levels were significantly higher in the rats that were administered MDZ when compared to the rats that were administered thiopental and the control rats ($p<0.05$). Similar to apoptosis, the cyclin B levels exhibited a number of differences between the neonatal and adult rats. In line with the studies that have suggested that MDZ inhibits cell proliferation, our findings revealed that the cyclin B levels in the neonatal rats that were administered MDZ were 10% lower when compared to all of the other neonatal groups. In the adult rats, however, the cyclin B levels in the rats that were administered MDZ were 17% lower than those of the rats that were administered thiopental, although these levels were 8% higher than those in the control group. Taken together, these findings implicated that although MDZ has no effect on apoptosis in neonatal rats, it may induce apoptosis in adult rats through the extrinsic pathway rather than the intrinsic pathway. Additionally, the findings also supported the studies that have suggested that MDZ has a role in apoptosis independent of its effects on

GABAA. However, the findings herein were dissimilar to those of the studies reporting on the effects of MDZ in neonatal rats, which could be attributed to the differences among the studies regarding the drug dosage, and experimental duration and conditions. In addition, the finding of the current study that implicated that the extrinsic pathway is partially activated in developing neurons could also be attributed to these differences. Accordingly, the effects of MDZ on cell proliferation in newborn rats may be more prominent than apoptosis, and MDZ may lead to neurotoxicity by inhibiting cell proliferation. Nevertheless, further comprehensive studies investigating cell proliferation, necrosis, apoptosis, and GABA levels are needed to substantiate these findings.

Thiopental has also been shown to promote apoptosis, in a similar way to MDZ. A previous in vitro study investigating human lymphocytes and Jurkat cells suggested that thiopental has a direct role in promoting apoptosis (11). Another study evaluated the same cells and reported that thiopental has the potential to inhibit nuclear factor kappa B, thereby leading to apoptosis and affecting cell proliferation (15). An in vivo rat study showed that thiopental increased the expression of apoptotic proteins in the brain, such as caspase-3 and -9 (23). In contrast, there have been some studies proposing that thiopental inhibits apoptosis, similar to MDZ. A previous study demonstrated that thiopental inhibits apoptosis by inducing heat shock protein 70 in T lymphocytes (20). The findings of the current study supported the findings of the studies that have proposed that thiopental promotes apoptosis, in that the Bax, procaspase-3, and caspase-3 levels in the neonatal rats were significantly higher in the rats that were administered thiopental when compared to the rats that were administered MDZ and the control rats ($p<0.05$). In the adult rats, however, these levels were significantly lower in the rats that were administered thiopental when compared to the rats that were administered MDZ, although these levels were higher than those of the control rats. More importantly and expectedly, in both the neonatal and adult rats, the levels of apoptotic markers were significantly higher in the rats that received both MDZ and thiopental when compared to the other rats ($p<0.05$). These findings implicated that thiopental, unlike MDZ, activates the intrinsic pathway of apoptosis and the increased apoptosis caused by the combined use of MDZ and thiopental could be attributed to the activation of both intrinsic and extrinsic pathways, and also to the synergy induced by the combined use of both drugs through GABA or some other mechanisms. In the current study, the cyclin B levels exhibited no significant difference between the NT and control groups, whereas these levels increased significantly in the NMT group when compared to the other groups ($p<0.05$). In the adult rats, on the other hand, the cyclin B levels were significantly lower in the AT group when compared to the AMT group, although they were higher when compared to the MDZ and control groups.

ROS are generated as a result of mitochondrial injury in neuronal cells. When ROS production exceeds the cellular antioxidant capacity, it results in increased oxidative stress in neurons, thereby leading to apoptosis in the cells (12). MDZ has been shown to inhibit ROS production in numerous studies. For instance, MDZ inhibited the apoptosis induced by ketamine by reducing ROS generation (13). Study conducted of infant rats was shown midazolam induced total antioxidant capacity and inhibited apoptosis (2). Another study suggested that MDZ protects tumor cells against toxicity by inhibiting ROS production and exerts its effect in B35 neuroblastoma cells through Akt-phosphorylation (7). Contrary to these studies, MDZ and inhalation anesthetics have been shown to increase ROS production in neonatal rats and have toxic effects on developing brain cells (5). On the other hand, thiopental, similar to MDZ, has also been shown to increase oxidative stress in the rat brain and other organs (1,4). The findings of the study herein supported the studies that have proposed that MDZ induces ROS production. In the current study, both the TOS and OSI levels were significantly higher in the neonatal and adult MDZ groups when compared to the control groups ($p<0.05$ for both). Similarly, both the TOS and OSI levels were significantly higher in the NT group when compared to the control group ($p<0.05$ for both). In the adult rats, however, the TOS and OSI levels were lower in the AT group when compared to the MDZ group, although they were higher than those of the control group. More importantly and unexpectedly, the TOS and OSI levels in both the NMT and AMT groups were significantly lower when compared to the MDZ and thiopental groups ($p<0.05$). Taken together, these findings implicated that both MDZ and thiopental may cause apoptosis and neurodegeneration by increasing oxidative stress, and that that thiopental and MDZ may trigger ROS production in neonatal and adult rats, respectively, in a similar fashion as in apoptosis. On the other hand, the reduced oxidative stress induced by the combined use of MDZ and thiopental could be attributed to the increased antioxidant capacity.

CONCLUSION

The results indicated that both MDZ and thiopental promoted apoptosis and ROS production, and thereby resulted in neurotoxicity in the rats, with MDZ showing a greater effect in the adult rats and thiopental showing a greater effect in the neonatal rats. Accordingly, it is tempting to consider that in anesthetic practice, the use of MDZ could be safer for neonates and the use of thiopental could be safer for adults.

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AUTHORSHIP CONTRIBUTION

Study conception and design: CS, EO

Data collection: GG, IMG, SY

Analysis and interpretation of results: CS, EO, GG, IMG

Draft manuscript preparation: CS, EO, GG, IMG, SY, YET, LAD

Critical revision of the article: CS,YET LAM

Other (study supervision, fundings, materials, etc...): CS, EO, GG, IMG, SY, YET, LAD

All authors (CS, EO, GG, IMG, SY, YET, LAD) reviewed the results and approved the final version of the manuscript.

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