Anaesth Intensive Care 2006; **34:**

The Neurotoxic Effects of Intrathecal Midazolam and Neostigmine

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SUMMARY

In parallel with improvements in understanding of pain neurophysiology, many chemicals have recently been investigated for spinal anaesthesia and analgesia. However, studies discussing the effects of these drugs on neural tissue indicate that knowledge about some aspects of neurotoxicity is limited. Forty-nine New Zealand Albino rabbits, weighing 2.2±0.2 kg, were randomly assigned to seven groups of seven animals each. Single dose groups received intrathecally through the atlantooccipital membrane 0.9% saline 1.5 ml; midazolam 100 μg/kg (low dose midazolam group) or 500 μg/kg (high dose midazolam group); neostigmine 10 μg/kg (low dose neostigmine group) or 50 μg/kg (high dose neostigmine group). Two groups had seven days of repeated dosing with either midazolam 100 μg/kg/day (repeat midazolam group) or 10 μg/kg/day neostigmine (repeat neostigmine group). The animals were sacrificed on day 8, and two spinal cord sections from the fourth cervical level and fourth lumbar level were removed and prepared for histopathological study. Transmission electron microscopic evaluations were performed on transverse spinal cord sections by a neuropathologist blinded to the group allocation. Twenty myelinated axons and neurones in the cervical and lumbar sections were investigated for the histopathological study. This study indicates that midazolam and neostigmine have different neurotoxic effects that depend on the dose and the repetition of dosing when these drugs are administered intrathecally.

Key Words: Spinal anaesthesia, midazolam, neostigmine, neurotoxicity

A growing understanding of the neuropharmacology of the spinal cord processing of nociceptive input has led to intense interest in the administration of spinal drugs for anaesthesia and pain management. The direct application of receptor-specific therapeutic agents to the spinal cord can potentially interrupt specific pain pathways and limit systemic side effects[1]. However, intrathecal application of a drug may cause neurotoxicity, and studies demonstrating lack of neurotoxicity are necessary before these agents can be safely used in humans. Most spinal drugs currently in clinical use have been poorly studied for spinal cord and nerve root toxicity. Opioids cause two major problems: respiratory depression and tolerance. Some local anaesthetics have prominent neurotoxic effects and could not provide selective spinal analgesia[2]. Development of spinal drugs that optimize antinociceptive effects and minimize adverse effects therefore seem a desirable alternative to opioids or local anaesthetics. Midazolam, unlike other benzodiazepines, is water-soluble and modulates the affinity of gamma-aminobutyric acid (GABA) for its receptor while enhancing its control of chloride channel activity[2]. Neurotoxicity studies of centrally administered midazolam in animals have yielded conflicting results[3-8]. Neostigmine acts indirectly as a muscarinic agonist by inhibiting acetylcholinesterase and thus produces analgesia. Neurohistopathological studies of centrally administered neostigmine (with or without paraben preservatives) revealed no spinal cord toxicity[9,10]. The purpose of the present study was to further evaluate whether midazolam and neostigmine have neurotoxic effects when administered intrathecally.

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Accepted for publication on November 11, 2005.

MATERIALS AND METHODS

Experimental Protocol

Upon the approval of all the protocols by the animal-rights Ethics and Research Committee of Ankara University, 49 New Zealand albino rabbits, weighing 2.2±0.2 kg, were randomly assigned to seven experimental groups of seven. Single dose groups received intrathecally, through the atlantooccipital membrane, 0.9% saline 1.5 ml; midazolam 100 μg/kg (low dose midazolam group) or 500 μg/kg (high dose midazolam group); neostigmine 10 μg/kg (low dose neostigmine group) or 50 μg/kg (high dose neostigmine group). Two groups had seven days of repeated dosing with either midazolam 100 μg/kg/day (repeat midazolam group) or neostigmine 10 μg/kg/day (repeat neostigmine group). Saline, which does not significantly alter the pH and osmolarity of the cerebrospinal fluid, was used as a vehicle for the dosing groups and drugs. The volume administered intrathecally was held constant among all groups (1.5 ml). The animals were sacrificed on day 8, and two spinal cord sections from the fourth cervical and lumbar levels were removed and prepared for histopathological study.

Anaesthesia and monitoring

The animals were anaesthetized with ketamine 50 mg/kg and xylazine 8 mg/kg intramuscularly before intravenous access was gained in the right ear vein. Because their respiration was not depressed, they were allowed to ventilate spontaneously while ketamine 10 mg/kg/h was used for anaesthesia maintenance. An arterial cannula was inserted into the right ear artery; arterial blood pressure was measured and the electrocardiograph (ECG) was monitored (Hewlett Packard 783835, Germany). A rectal probe was placed to monitor core temperature, and a heating blanket maintained normothermia. Because the animals were not intubated, no oxygen monitoring was performed.

Surgical procedure and tissue fixation

The skin was shaved and disinfected prior to the insertion of the needle. Under surgical conditions, the animals were placed in lateral position on the operating table and percutaneous puncture of the intracisternal subarachnoid space through the atlantooccipital membrane was performed using a 22-gauge (G) needle. The subarachnoid position of the needle was confirmed by aspiration of 0.5 ml cerebrospinal fluid before injection of 1.5 ml of study solution. Utmost care was taken to avoid traumatization with the needle. The procedure was performed by one researcher, using an identical technique for each case, and no undesired trauma was noted at electron microscopy evaluation. The animals were sacrificed on day 8 with the administration of a thiopental overdose through an ear vein. After placing the animals in the prone position, a laminectomy was performed within 30 minutes of death. The dura was exposed, and 1 ml of a fixative containing 4% paraformaldehyde and 1.25% glutaraldehyde phosphate buffer solution was injected into the subarachnoid space from the lumbar to the cervical zone. After fixation, two slices of spinal cord from the fourth cervical and lumbar regions were obtained and stored in 2.5% glutaraldehyde for 24 hours. Samples were then washed in Sorenson's Phosphate Buffer (SPB) and stored in 1% osmium tetraoxide for 2 hours. Washing in SPB was repeated, and samples were dehydrated in alcohol solutions. After dehydration, samples were washed in propylene oxide twice, and were embedded into an araldite, DDSA, DMP-30, and dibutyl phthalate mixture. Finally, the samples were stored at 37°C for 48 hours, and 60 nm slices were taken with a LKB Novo ultramicrotome (Sweden). The slices were dyed with uranyl acetate and lead citrate.

Histopathological evaluation and scoring

In each sample taken from the lumbar and cervical region, 20 randomly selected myelinated axons and neurones were investigated by transmission electron microscopy. To evaluate the findings, the following scoring system was used:

For myelinated axons: 0—myelin layers normal; 1—separation of myelin configuration; 2—interruption of myelin configuration; 3—honeycomb appearance. For neurones: 0—normal; 1—small vacuoles (less than 20μ); 2—medium vacuoles (20-100μ); 3—large vacuoles (more than100μ).

Statistical evaluation

The Kruskal-Wallis test was used to evaluate the histopathological findings, while ANOVA was used to evaluate the blood pressure and heart rate values. $P<0.05$ was considered statistically significant.

RESULTS

There were no significant differences among the groups with respect to heart rate and blood pressure values ($P>0.05$) (Tables 1 and 2). Histopathological comparison of saline group with other groups revealed significant differences in cervical myelinated axons, cervical neurones, lumbar myelinated axons, and lumbar neurones ($P<0.001$). There were also significant differences between the low dose mida-

zolam and high dose midazolam group; the low dose neostigmine and high dose neostigmine group; the low dose midazolam and repeated dose of midazolam groups; the low dose neostigmine and repeated dose of neostigmine groups; the high dose midazolam and the repeated dose of midazolam groups; and the high dose neostigmine and repeated dose of neostigmine groups (all $P<0.001$). Cervical and lumbar histopathological scores for axons and neurones are presented in Tables 3, 4, 5 and 6.

ELECTRON MICROSCOPIC FINDINGS

In the saline group, separation of myelin configuration in a few axons and small vacuoles in some neurones, was detected. Unmyelinated axons were normal (Figure 1).

In the low dose midazolam group, most of the myelinated axons showed separation of myelin configuration and small vacuoles in neurones. Unmyelinated axons were normal (Figure 2, left column).

TABLE 1
Heart rate

Groups	5 minutes before cannulation	At spinal injection	5 minutes after spinal injection
SAL	258±6.5	262±6.8	263±6.4
LDM	258±4.7	260±6.3	260±6.6
LDN	260±5.6	261±8.2	262±5.6
HDM	259±4.5	259±5.4	260±4.5
HDN	258±6.3	260±5.6	262±5.5
RDM	259±5.7	261±6.4	261±8.2
RDN	260±5.7	262±8.3	264±6.3

SAL: Saline group, LDM: Low Dose Midazolam group, LDN: Low Dose Neostigmine group, HDM: High Dose Midazolam group, HDN: High Dose Neostigmine group, RDM: Repeated Dose of Midazolam group, RDN: Repeated Dose of Neostigmine group. Values are mean±standard deviation. Rate per minute monitored for a total duration of 15 minutes.

TABLE 2
Mean arterial pressure

Groups	5 minutes before cannulation	At spinal injection	5 minutes after spinal injection
SAL	84.5±3.4	84.1±3.2	83.3±3.5
LDM	83.6±3.6	83.3±3.6	84.8±3.7
LDN	87.4±3.1	88.6±4.1	87.9±3.7
HDM	86.9±3.5	87.9±3.5	88.4±4.1
HDN	85.1±3.6	86.1±3.7	86.8±7.9
RDM	87.8±3.3	88.3±4.1	89.1±3.2
RDN	88.8±3.5	89.2±3.2	88.5±3.9

SAL: Saline group, LDM: Low Dose Midazolam group, LDN: Low Dose Neostigmine group, HDM: High Dose Midazolam group, HDN: High Dose Neostigmine group, RDM: Repeated Dose of Midazolam group, RDN: Repeated Dose of Neostigmine group. Values are mean±standard deviation. Values in mmHg monitored for a total duration of 15 minutes.

TABLE 3
Cervical myelinated axons

	SAL	LDM	LDN	HDM	HDN	RDM	RDN
0	117	11	32	0	0	0	0
1	23	129	108	124	49	17	28
2	0	0	0	16	91	98	102
3	0	0	0	0	0	25	10

0: Myelin layers are normal, 1: Separation in myelin configuration, 2: Interruption in myelin configuration, 3: Honeycomb appearance
*SAL: Saline group, LDM: Low Dose Midazolam group, LDN: Low Dose Neostigmine group, HDM: High Dose Midazolam group, HDN: High Dose Neostigmine group, RDM: Repeated Dose of Midazolam group, RDN: Repeated Dose of Neostigmine group.

TABLE 4
Cervical neurones

	SAL	LDM	LDN	HDM	HDN	RDM	RDN
0	121	14	27	0	0	0	0
1	19	126	113	129	54	8	138
2	0	0	0	11	86	79	2
3	0	0	0	0	0	53	0

0: Normal, 1: Small vacuoles, 2: Medium vacuoles, 3: Large vacuoles.
SAL: Saline group, LDM: Low Dose Midazolam group, LDN: Low Dose Neostigmine group, HDM: High Dose Midazolam group, HDN: High Dose Neostigmine group, RDM: Repeated Dose of Midazolam group, RDN: Repeated Dose of Neostigmine group.

TABLE 5
Lumbar myelinated axons

	SAL	LDM	LDN	HDM	HDN	RDM	RDN
0	115	13	28	0	0	0	0
1	25	127	112	126	58	17	29
2	0	0	0	14	82	99	95
3	0	0	0	0	0	24	16

0: Myelin layers are normal, 1: Separation in myelin configuration, 2: Interruption in myelin configuration, 3: Honeycomb appearance
SAL: Saline group, LDM: Low Dose Midazolam group, LDN: Low Dose Neostigmine group, HDM: High Dose Midazolam group, HDN: High Dose Neostigmine group, RDM: Repeated Dose of Midazolam group, RDN: Repeated Dose of Neostigmine group.

TABLE 6
Lumbar neurons

	SAL	LDM	LDN	HDM	HDN	RDM	RDN
0	121	17	27	0	0	0	0
1	19	123	113	127	58	7	139
2	0	0	0	13	82	73	1
3	0	0	0	0	0	60	0

0: Normal, 1: Small vacuoles, 2: Medium vacuoles, 3: Large vacuoles.
SAL: Saline group, LDM: Low Dose Midazolam group, LDN: Low Dose Neostigmine group, HDM: High Dose Midazolam group, HDN: High Dose Neostigmine group, RDM: Repeated Dose of Midazolam group, RDN: Repeated Dose of Neostigmine group.

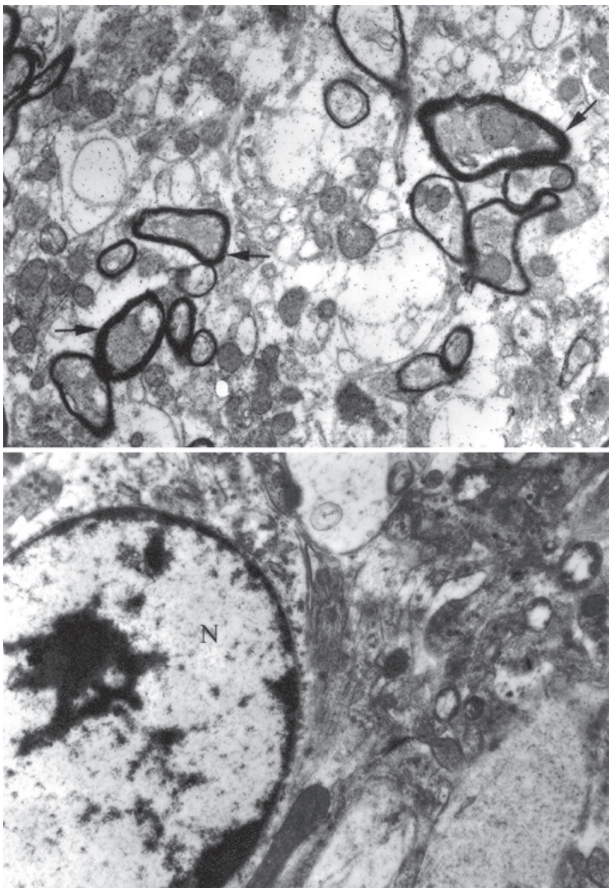

FIGURE 1: Up: Normal myelin layer (arrows). Down: Normal neurone (N: neurone).

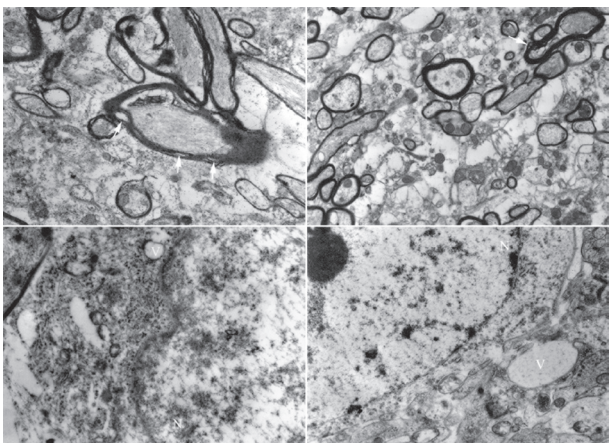

FIGURE 2: Left up: Separation of myelin configuration (arrows). Left down: Small vacuoles in neurones (N: neurone, v: vacuole). Right up: Separation of myelin configuration (arrows). Right down: Small vacuoles in neurones (N: neuron, v: vacuole).

In the low dose neostigmine group, most of the myelinated axons had separation of myelin configuration and small vacuoles were seen in neurones. Unmyelinated axons were normal (Figure 2, right column).

In high dose midazolam group, most of the myelinated axons had separation of myelin configuration, small vacuoles in neurones were detected and some of the myelinated axons had interruption to the myelin configuration, while some neurones had medium vacuoles. Unmyelinated axons were normal (Figure 3, left column).

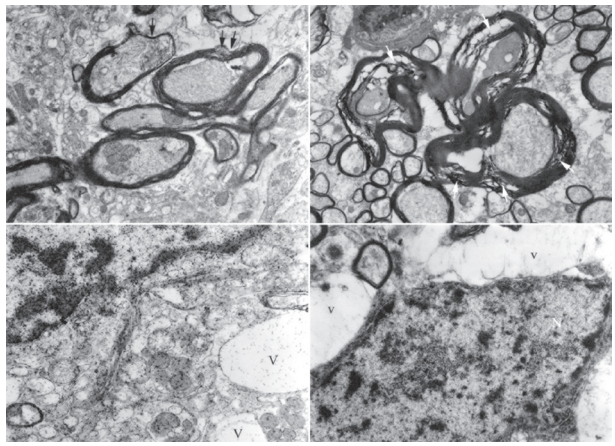

FIGURE 3: Left up: Interruption in myelin configuration (arrows). Left down: Medium vacuoles in neurones (v: vacuole). Right up: Interruption in myelin configuration (arrows). Right down: Medium vacuoles in neurones (N: neurone, v: vacuole).

In the high dose neostigmine group, most of the myelinated axons had interruption of myelin configuration and medium vacuoles were observed in neurones. Unmyelinated axons were normal (Figure 3, right column).

In the repeated dose of midazolam group, the worst results were observed. All myelinated axons had significant destruction and honeycomb appearance was observed. Neurones had large vacuoles and there was a decrease in organelles within the cell. Unmyelinated axons were normal (Figure 4, left column).

In the repeated dose neostigmine group, most of the myelinated axons displayed interruption in myelin configuration; however, there were only small vacuoles in most neurones. Unmyelinated axons were normal (Figure 4, right column).

DISCUSSION

This study shows that both midazolam and neostigmine, when administered intrathecally, have neurotoxic effects in rabbits. These effects are augmented if the doses are repeated or increased.

Previous findings with respect to the spinal toxicity of intrathecal midazolam have been conflicting[4,8,11]. Initial rat studies with intrathecal catheter implantation and administration of 0.15 mg/kg for 15 days or

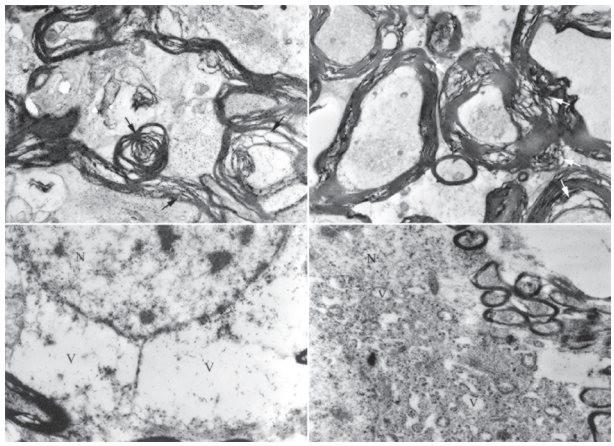

FIGURE 4: Left up: Destruction and honeycomb appearance (arrows). Left down: Large vacuoles in neurones (N: neurone, v: vacuole). Right up: Interruption of myelin configuration (arrows). Right down: Small vacuoles in neurones (N: neurone, v: vacuole).

isolated exposures to 0.1-0.3 mg/kg of midazolam prepared in saline solution showed no neurotoxic reaction in light or electron microscopy[11]. However, Malinovsky et al[4] reported that 0.1 mg/kg of intrathecal midazolam had a neurotoxic effect in three of nine rabbits. A light microscopic study in the rat demonstrated no spinal cord toxicity after 15 days of daily doses of intrathecally administered midazolam[4]. To investigate these contrasting results, a state-of-the-art study on the rat was performed using light microscopy, electron microscopy, cell morphology, and transcardial tissue fixation after daily intrathecal administration of approximately 0.3 mg/kg midazolam for 20 days[8]. The rat spinal cords showed strong evidence of neuronal death and cellular abnormalities in most midazolam exposed groups, even with light microscopy. Another study showed that spinally administered midazolam, even in large doses, does not cause acute neurotoxicity or inflammation of the spinal cord. Spinal cord toxicity may, however, manifest not just histopathologically but also neurophysiologically. Cicek et al[12] have investigated the effects of different doses of epidural midazolam on spinal somatosensory evoked potentials (SSEP) and have found that epidurally administered midazolam up to 150 μg/kg caused no change in SSEP records, while 250 and 500 μg/kg doses led to decreases.

The respiratory depression of the animals could not be quantitatively and precisely measured because they were not intubated. More precise values may be obtained in further studies.

Neurohistopathological analyses of rats and dogs after long-term intrathecal neostigmine administration (with and without preservatives) revealed no spinal toxicity. Phase I safety assessments in human volunteers have been performed for both preservative-free and paraben-containing hyperbaric preparations of spinal neostigmine without clinical evidence of neurotoxicity[13,14]. In our study we observed neurotoxic effects of neostigmine, especially with 50 μg/kg single dose and 10 μg/kg repeated doses.

The studies by Tucker et al showed support for the clinical safety and efficiency of intrathecal midazolam, particularly when used in combination with fentanyl[15,16]. Although some of our results were not consistent with the results of other studies, it should be noted that neurotoxic effects are dose related and that high doses of drugs, and even neurotransmitters, may be neurotoxic. We think our results should stimulate further discussion.

CONCLUSION

Before introducing a drug for spinal use in humans, morphologic, physiologic, and clinical studies should be performed on several different species to minimize the risk of missing a possible toxic effect. This study supports previous studies showing that midazolam has neurotoxic effects and it contradicts the studies of neostigmine reporting no neurotoxic effects after intrathecal administration.

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