Histopathology After Repeated Intrathecal Injections of Preservative-Free Ketamine in the Rabbit: A Light and Electron Microscopic Examination

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Epidural and spinal administration of ketamine has been used in humans. Single-dose studies have shown that preservative-free ketamine lacks neurotoxic effects, but there are no studies after repeated administrations. The aim of this study was to examine the effects of daily administration of preservative-free ketamine. Fourteen New Zealand albino rabbits were assigned to two groups receiving either intrathecal preservative-free ketamine 5 mg, 0.5 mL 1% solution (eight rabbits) or saline 0.5 mL (six rabbits) once a day for 14 consecutive days. The rabbits had a total subcutaneous implanted intrathecal catheter, which was introduced during general anesthesia. On Day 15 the rabbits were anesthetized and *in vivo* fixated by transcardial perfusion with Tyrode's solution followed by a mixture of 2% glutaraldehyde and 1% formaldehyde in a 0.1 mol/L phosphate buffer. A segment 5 cm on each side of the catheter tip was removed and kept in a cold solution of the fixative. Light microscopic, electron microscopic, and morphometric examinations showed no differences between the spinal cords from the rabbits injected with ketamine versus saline. Intrathecal ketamine produced motor impairment for a period of 15 min. We conclude that repeated intrathecal administration of preservative-free ketamine confirms the lack of neurotoxicity from single-dose studies.

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Although selective spinal analgesia with opioids has been used in the clinic for more than a decade, the major problems with respiratory depression, development of tolerance, and inefficiency against certain types of pain remain unsolved. Ketamine has been used both intrathecally (1) for surgical anesthesia and epidurally in humans (2–7). Although the analgesic action of epidural ketamine in humans is controversial, it has been shown to produce analgesia in both postoperative and cancer patients, without motor blocking effects, in the dose range used. Is ketamine an alternative to intrathecal and epidural opioids? If so, it should diminish either the risk of respiratory depression or the development of tolerance, or it should exert analgesic properties against opioid-resistant pain. The use of systemic or spinal ketamine is also of interest owing to its capacity to interact with the *N*-methyl-D-aspartate receptor. Thus, ketamine may be used for "preemptive analgesia" to decrease facilitation (wind-up) of spinal neurons (8). Preceding the clinical trials clarifying these questions, a record of nontoxicologic properties must be produced (9).

Five toxicologic examinations after intrathecal ketamine administration are reported in the literature (10–14). Although no signs of a toxic effect on the spinal cord have been shown after preservative-free ketamine, they are all single-dose studies in which light microscopy and fluorescence microscopy have been used as the methods to determine toxicologic effects.

This study was performed to examine the effect after repeated intrathecal administration of preservative-free ketamine and to examine any possible effects by the most sensitive method available: qualitative assessment by light and electron microscopy, combined with a quantitative morphometric investigation.

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Methods

The study was approved by the Danish Committee for Animal Research under the Department of Justice and performed in accordance with ethical guidelines for investigations in conscious animals. Fourteen female New Zealand albino rabbits, weighing 3.0–4.0 kg, were used in the study. The rabbits were kept in spacious cages, two rabbits per cage, with free access to food and water.

Intrathecal catheters (Portex®; outside diameter 0.9 mm) were inserted surgically in the area between L7 and S1, during general anesthesia induced by a combination of intramuscular ketamine 25 mg and xylascine 10 mg, supplemented with subcutaneous lidocaine infiltration. The tip of the catheter was introduced intrathecally through an incision of the ligamentum flavum and the meninges between the bases of the spinous process of L7 and caudal articular process of L6 or between L6 and L5. The catheter was introduced rostrally 5 cm. The free end of the catheter was mounted with a rubber membrane and passed subcutaneously to the area of the neck. The skin incision was then closed, and the rabbits were equipped with a total subcutaneous implanted, intrathecal injection system (15). Any signs of motor dysfunction after catheter implantation resulted in exclusion from the study. Catheter position was tested with 0.5 mL lidocaine 1%, which invariably results in paralysis of the lower abdomen and hindlegs if the catheter is positioned intrathecally, whereas it only produces motor weakness when positioned epidurally.

At the beginning of the study, the animals were randomized into two groups: eight rabbits in the ketamine group and six rabbits in the control group. The control group received isotonic saline 0.5 mL once a day for 14 consecutive days, and the ketamine group received racemic ketamine without any adjuvants (Ketalar®; Parke-Davis, Morris Plains, NJ) (pH 5.1) 10 mg/mL, 0.5 mL (equivalent to 1.5 mg/kg) once a day for 14 days. After each injection the dead space of the system was cleared with 0.3 mL of isotonic saline. Any neurologic effects were registered. This ketamine dose produced analgesia in another study in our laboratory (55.5% ± 15.6% increase in intestinal distention threshold; $P < 0.03$) and is the same dose as used in a previous study (12).

To evaluate ketamine's effect on motor function, a motor dysfunction index (MDI) was introduced: 0, normal motor function; 1, dyscoordination during walk; 2, inability to walk, but sufficient motor function to maintain a sitting position; 3, inability to sit, but movement of the hindlegs; 4, total paralysis. This test was applied on Day 1 only.

One day after the last injection, the animals were anesthetized with pentobarbital and transcardially perfused with Tyrode's solution (temperature 38°C, pH 7.4) containing heparin 10,000 U/L, until the solution on the output side was without any blood. Immediately after this procedure, the animals were perfused with approximately 1 L of a mixture of 2% glutaraldehyde and 1% formaldehyde in 0.1 mol/L phosphate buffer (temperature 38°C, pH 7.4). Five centimeters of the spinal cord caudal and rostral to the tip of the catheter was then removed and kept in a cold solution of the fixative. The lumbar segment of L5, at the tip of the catheter, defined as the tissue between the root entry zones, was removed and its total length measured under a dissection microscope, followed by a calculation of the tissue volume. This segment was then serially sectioned by means of a vibratome (Oxford Instruments, United Kingdom) into 100-µm-thick transverse sections. After a short rinse in a 0.1 mol/L sodium cacodylate buffer (pH 7.4), the sections were postfixed for 30 min in 2% osmium tetroxide dissolved in the buffer, dehydrated in graded ethanol, and embedded in epoxy resin (agar 100) between Teflon-sprayed slides and 100-µm-thick acetate foil (16). In each animal, after being examined in the light microscope, 10 randomly selected tissue blocks containing either the dorsal or the ventral horn were excised and reembedded for both fine and ultrafine sectioning. The fine sections were stained with toluidine blue and the ultrafine ones with uranyl and lead citrate. The fine sections were prepared for quantitative analysis of the dorsal horn morphology. The variations in cell number in laminae I–III, mean cell volume (μm^3), and cell ratio (%) in a reference volume (V_{ref}) in the dorsal horn were used as sensitive variables of spinal neurotoxicity (17–19). The V_{ref} contained laminae I–III (18) of a rostrocaudal column throughout the whole L5 segment. The outline of the area of the V_{ref} was delineated as shown in Figure 2A. The V_{ref} was calculated in each animal according to the Cavalieri principle (20) in the following way: in the light microscope the outline of a transverse area of the laminae I–III was drawn with the aid of a drawing tube attached to the microscope. The mean area of about 10 samples from each animal, measured by means of a graphic pen-data device, was considered the base of a cylinder, the height of which was the length of the L5 segment, estimated from the number of sections made from the segment. The estimation of the V_{ref} was done to prevent sources of error due to tissue swelling or shrinking after fixation and tissue preparation. The number of cells (Q) was calculated within the V_{ref} according to the dissector principle by counting the number of nucleoli present in a dissector (21). This method has been described earlier (18). The volume of a dissector (V_{dis}) is easily computed: the area of the dissector, drawn with the drawing tube, times the height of the fine section (2 µm). Within the whole L5 segment of each animal, a systematic random sample of 30 pairs of dissectors was analyzed in the

light microscope. The total number of cells (N) was calculated according to the following formula (19):

$$N = \Sigma Q \times \frac{V_{ref}}{V_{dis}}.$$

Q is the actual number of cells counted, V_{ref} is the reference volume, i.e., the total volume of laminae I–III of the dorsal horn of the L5 segment, V_{dis} is the volume of the dissectors, and N is the total number of cells within the reference volume. To calculate the mean cell volume, the ratio of the V_{ref} that constitutes the volume of the cell (cell ratio) was estimated by means of the point-counting method (22). In each animal, a random sample of 12 sections was analyzed with a point-sampling screen. Estimation of cell ratio was computed as P_c/P_r, where P_c is the number of points falling on the cells and P_r is the total number of points falling within laminae I–III of the V_{ref}. Thus the mean cell volume was calculated according to the formula:

$$\text{mean cell volume} = \text{cell ratio} \times V_{ref} \times N^{-1}.$$

Randomly selected sections of the ultrafine sections were selected for electron microscopy. In each animal, approximately 50 sections were scanned for signs of cell toxicity: karyolysis, edema, and the general appearance of the mitochondria, microtubules, neurofilaments, synaptic vesicles, and spinal tracts. The preservation of the tissue as well as the myelin damage was also studied. Occasional splits of the myelin sheets were accepted, as this can be seen in untreated neuronal tissue.

Wilcoxon's ranked sum test was used for statistical evaluation of the effect on motor function and quantitative morphometric data. $P < 0.05$ was chosen as the level of significance. The coefficient of error—measuring the distribution of individual cell counts for each dissector—was estimated by dividing individual cell counts into two groups of samples (for details, see ref. 19). A coefficient of error less than 0.05 was accepted.

Results

Behavioral Results

The rabbits receiving saline displayed no signs of motor impairment or other neurologic deficits. In the ketamine group, the motor function was affected in all rabbits; MDI at 5 min was 2.8 ± 0.4 (mean ± SEM), $P < 0.05$, and at 15 min it was 1.0 ± 0.5, $P > 0.05$. At 30 min no effects on motor function were observed (Figure 1). All the rabbits in the ketamine group experienced this effect on motor function after each daily injection. No other signs of neurologic sequelae were observed. No rabbits were excluded because of neurologic deficits after catheter implantation. Catheter location was confirmed by the motor impairment after ketamine injection and by microscopy. All catheters were placed intrathecally throughout the study.

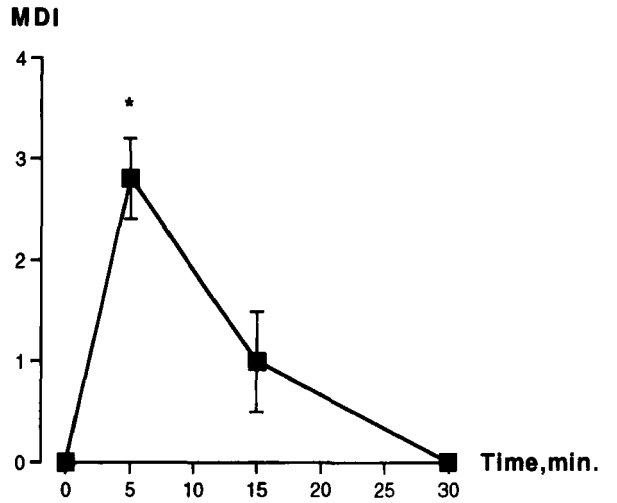

Figure 1. Effect of 5 mg (1.5 mg/kg) of intrathecal preservative-free ketamine on motor function. MDI = motor dysfunction index. Values are mean ± SD; $n = 6$. *$P < 0.05$.

Histopathology

Light Microscopy Findings. The tissue preservation was high in all animals. The spinal tracts demonstrated no visible signs of injury or spongy appearance of the myelin sheaths (Figure 2A). The neurons of the dorsal horn (Figure 2B) and the ventral motor neurons (Figure 3) had the same appearance in the ketamine-injected rabbits as in the control rabbits. No significant differences were noticed between the rabbits injected with ketamine and saline, with respect to tissue reaction. A slight compression of the spinal cord along the catheter was seen in three animals, two in the saline group and one in the ketamine group. In the area around the catheter, an increased number of dark inflammatory cells was observed. Very few dark inflammatory cells were seen in the gray matter of the investigated segments, but in two animals, one from each group, these cells were present in the leptomeninges.

Quantitative Findings. The calculations of the reference volume revealed no statistically significant differences between the control animals (1.30 ± 0.08 mm^3) (mean ± SD) and the rabbits given injections of ketamine (1.30 ± 0.15 mm^3). The lowest and highest numbers were found in the ketamine-injected group. The length of the L5 segment was 8.2 ± 0.12 mm in the saline-injected rabbits and 8.3 ± 0.3 mm in the ketamine group. The total number of cells in laminae I–III of the dorsal horn demonstrated no significant differences between the saline- and ketamine-injected animals. The mean values of the cell numbers were

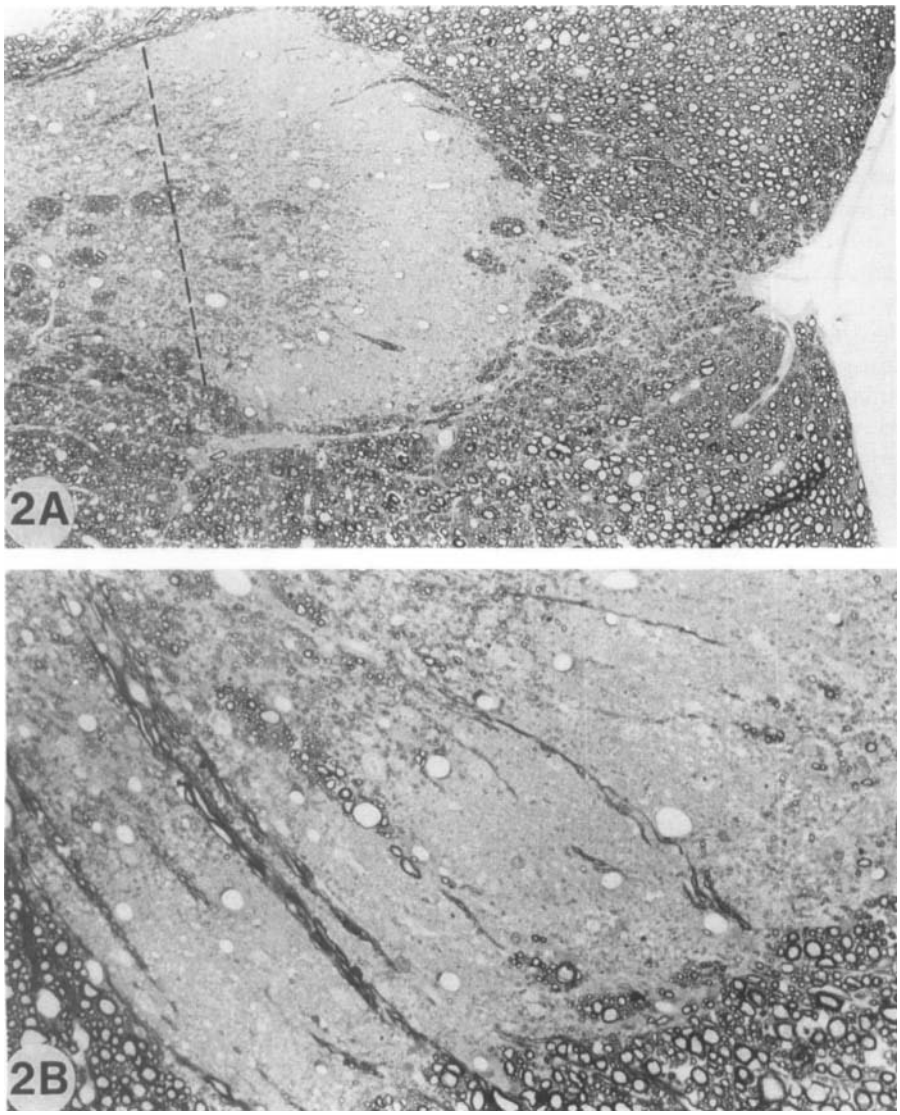

Figure 2. Light micrographs of toluidine-blue-stained plastic embedded sections from the lumbar spinal cord (L5 segment) after intrathecal injections of preservative-free ketamine for 14 days. 2A demonstrates normal appearance of the myelin sheaths, and 2B shows normal morphology of the dorsal horn. 2A: ×300; 2B: ×650.

$46 \pm 5 \times 10^3$ and $47 \pm 6 \times 10^3$ for the saline and ketamine groups, respectively. The individual values (mean ± SD) are shown in Table 1. The coefficient of error demonstrated $P < 0.05$ for both saline- and ketamine-injected animals. The ratio of neuronal cell bodies in the reference volume was 4.0% ± 0.4% for the ketamine group, which was slightly lower than the mean value of 4.1% ± 0.4% in the saline-injected animals. This difference was not statistically significant.

No statistical difference was found in the mean cell volume between the two groups (Table 1).

Electron Microscopy Findings. No morphologic differences could be detected between the saline- and the ketamine-injected animals, in the electron microscope. The neuronal cell bodies demonstrated normal shapes and intact cell membranes and had their nuclei in typical central positions with distinct nucleoli (Figure 4). The mitochondria, microtubules, and neurofilaments of the axons displayed a normal appearance as well. Synaptic contacts were seen mostly between boutons containing small, round vesicles and small- to medium-sized dendrites (Figure 5). The typical axonal spheroids described by Leonhardt (23) were not identified in the dorsal or ventral horns. Microglial cells were seen only occasionally in the gray matter, with no visible difference between the two groups. Phagocytosed cells within the neutrophil cells could not be detected. The perivascular cells and perivascular astrocytes showed normal morphology (Figure 6).

Discussion

The present study shows that repeated administration of intrathecal preservative-free ketamine, 5 mg, in rabbits has no neurotoxic effect when examined by both light and electron microscopy, including the sensitive

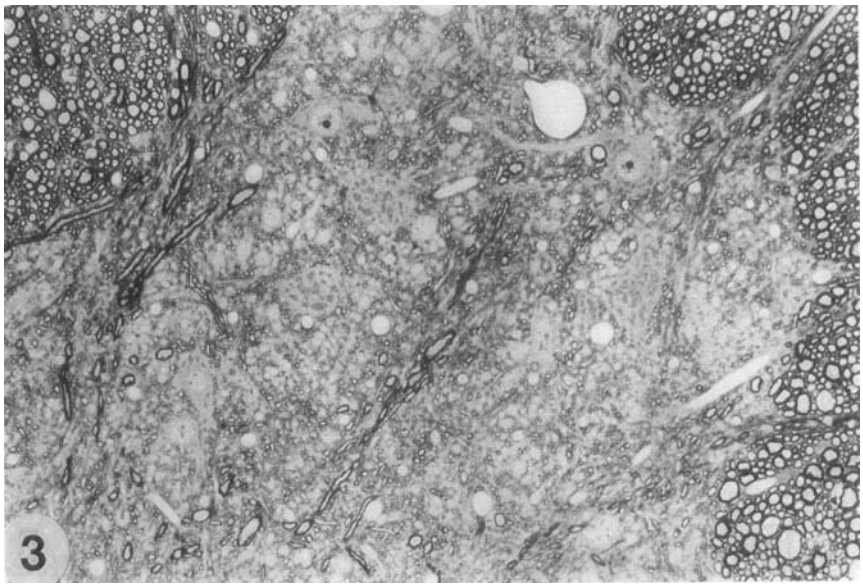

Figure 3. High-power micrograph of the ventral horn showing two normal motor neurons with their distinct nucleoli. Preservative-free ketamine-injected rabbits. Stained with toluidine blue; ×600.

Table 1. Comparison of Cell Numbers, Mean Cell Volume, and Cell Ratio in Rabbits Receiving Ketamine (K) Versus Saline (S)

Rabbit	Cell no.	Mean cell volume	Cell ratio
K1	48,000	700	3.5
K2	52,000	900	3.5
K3	50,000	900	4.2
K4	56,000	900	4.0
K5	46,000	1200	4.0
K6	38,000	1100	4.6
K7	46,000	1400	3.8
K8	38,000	1200	4.1
Mean ± SD	47,000 ± 6000	1000 ± 200	4.0 ± 0.4
S1	49,000	800	4.0
S2	40,000	900	4.8
S3	42,000	1000	4.0
S4	54,000	900	3.5
S5	47,000	900	4.3
S6	44,000	1200	4.1
Mean ± SD	46,000 ± 5000	950 ± 100	4.1 ± 0.4

morphometric method. Previous results concerning the spinal toxicity of intrathecal ketamine are conflicting (10–14). Preservative-free ketamine, in concentrations less than 1%, is without specific toxicologic effects (10,14), whereas preservative-containing ketamine either is without toxic effect (10,11) or produces minor changes when compared with saline and lidocaine (11). The use of ketamine 5% results in both neurologic and toxicologic changes (13). Malinovsky et al. (14) found that the preservative chlorbutanol produces neurotoxic changes and is responsible for the minor changes induced by the commercial solution of ketamine containing this preservative, while preservative-free ketamine and *d*-ketamine show no signs of toxic effect when examined in the light microscope. Thus they conclude that a preservative-free solution of 1% ketamine does not produce any signs of toxicity on the spinal cord when compared with saline. All the studies mentioned above examined single intrathecal injections and qualitative morphologic analysis with light microscopy. The results from the present study, where a more sensitive method was used, are in agreement with the previous results published in the literature on preservative-free ketamine.

Although chronic intrathecal or epidural catheterization produces significant histologic changes around the catheter (15,24), these changes can easily be distinguished from any toxic effects on the spinal cord when the study includes a control group receiving saline. Thus significant histopathologic changes between the group receiving the test drug and the group receiving saline injections are necessary in order to define a drug as having neurotoxic properties. The use of a single-injection technique also induces pathologic changes due to the injection technique (10,12). However, it does not reveal any information about long-term use of a drug which is important to have before introducing a drug in the treatment of pain in humans, which often lasts for from several days (postoperative pain) to several months (cancer and chronic pain syndromes). We advocate examination after chronic administration of the test drug through an indwelling catheter to provide the necessary information to determine whether a drug is safe for intrathecal and/or epidural use in humans.

The histologic preparation in the present study was chosen partly because it causes minimal cell and tissue shrinkage (16). Minimizing this disadvantage provides a more accurate estimation of cell numbers and makes it possible to estimate cell volume relative to total tissue

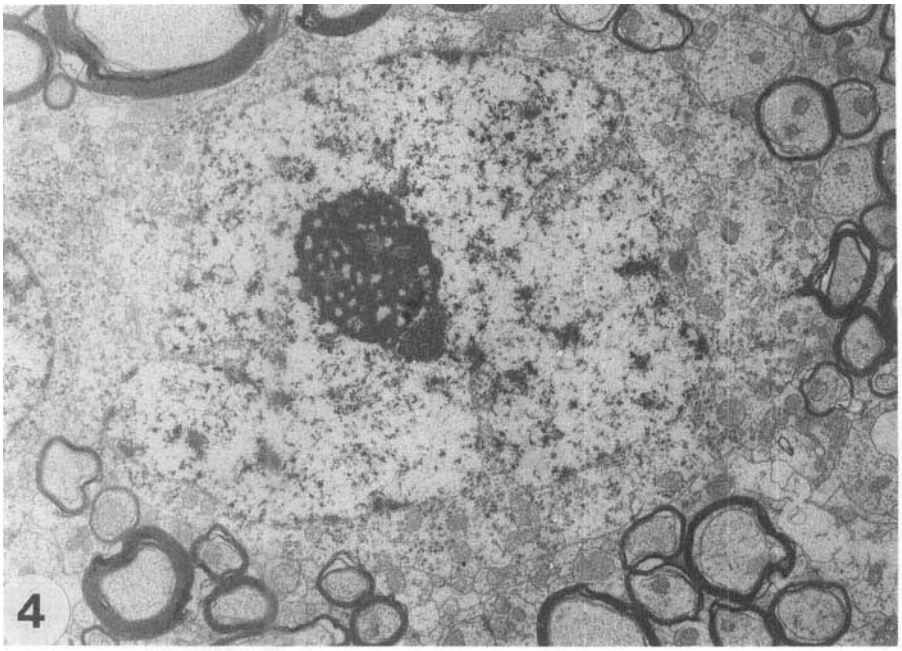

Figure 4. Electron micrograph of a normal neuron in lamina II of the dorsal horn after 14 of preservative-free ketamine injections. ×20,000.

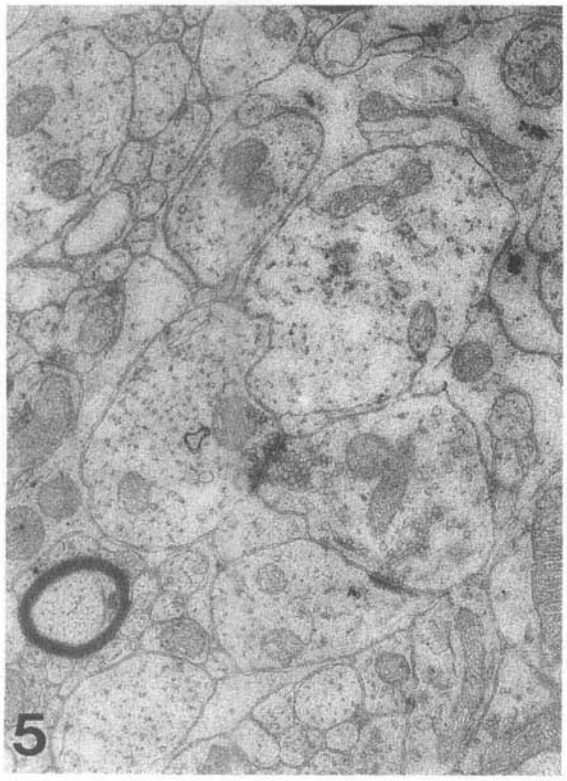

Figure 5. Electron micrograph showing synaptic contacts between a button containing round vesicles and two dendrites in lamina II of the dorsal horn after 14 daily injections of preservative-free ketamine. Normal morphology. ×27,000.

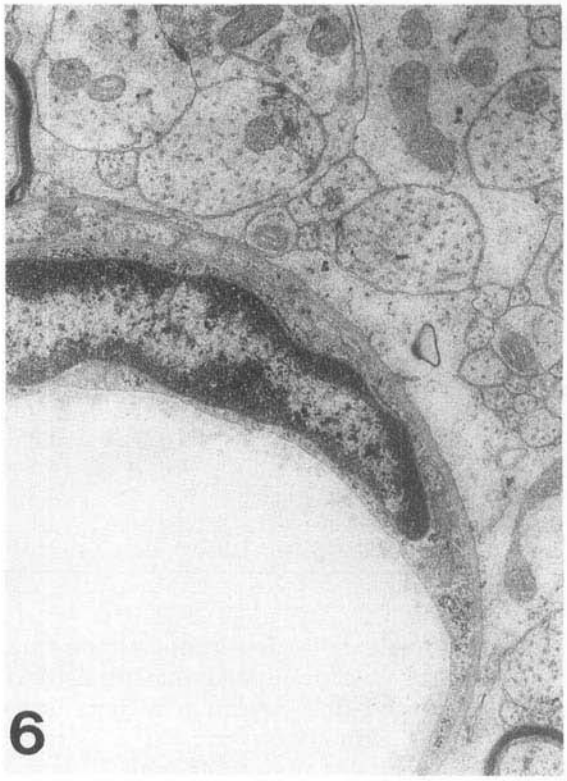

Figure 6. Electron micrograph of a small vessel demonstrating a normal endothelial cell in the ventral horn of a preservative-free ketamine-injected rabbit. ×25,000.

volume (19). The mean cell volume seems to be the best indicator of which cell type will most likely be affected by neurotoxicity (25). The results of our study show no significant differences between saline controls and ketamine in light and electron microscopy and in the morphometry examinations. These observations support the view that preservative-free ketamine is safe for intrathecal use in humans, even for repeated injections over a period of time.

There are no studies on toxic reactions after the use of ketamine in the vicinity of the spinal cord in humans. Only a local anesthetic action has been described after intrathecal administration (1) and after intravenous injection (26). The same local anesthetic effect was observed in this study after 5 mg of intrathecal ketamine. The rabbits fully recovered every day without any objective signs of neurologic sequelae or motor impairment.

Before introducing a drug for spinal use in humans, morphologic neurotoxicologic studies for safety screening should be performed in several different species, to minimize the risk of overlooking a drug's possible toxic effect. Including the results from this study, intrathecal or epidural preservative-free ketamine has been shown to be devoid of neurotoxic effects after both single and repeated administration in rabbits (present study and refs. 12 and 14) and single doses in rats (13), monkeys (10), and baboons (11).

In conclusion, these observations support previous studies showing that spinal preservative-free ketamine lacks neurotoxicologic properties in several species after both single and repeated injections.

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