의학석사 학위논문

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The Neurological Safety of Epidural Pamidronate in Rats

by

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1. 인터넷을 통한 온라인 서비스와 보존을 위하여 저작물의 내용을 변경하지 않는 범위 내에서의 복제를 허용함.
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3. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판허락을 하였을 경우 1개월 이내에 제주대학교 중앙도서관에 알림.
4. 배포, 전송된 학위논문은 이용자가 다시 복제 및 전송할 수 없으며 이용자가 연구목적이 아닌 상업적 용도로 사용하는 것을 금함에 동의함.

논문제목: The Neurological Safety of Epidural Pamidronate in Rats

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Abstract

Background: Pamidronate is a potent inhibitor of osteoclast-mediated bone resorption. Recently, the drug has been known to relieve bone pain. More recently, IV bisphosphonates treatment group reported significant improvement in neuropathic pain syndrome known as CRPS/RSD.

We believed it possible that direct epidural administration of pamidronate could have various advantages over oral administration with respect to dosage, side effects, and efficacy. Therefore, we evaluated neuronal safety of epidurally-administered pamidronate.

Methods: Twenty-seven rats weighing 250-350 g were equally divided into three groups. Each group received epidural administration with either 0.3 ml (3.75 mg) of pamidronate (group P), 0.3 ml of 40% alcohol (group A), or 0.3 ml of normal saline (group N). Pinch-toe test, motor function evaluation, and histopathologic examination of the spinal cord to detect conditions such as chromatolysis, meningeal inflammation, and neuritis, were performed on the 2nd, 7th, and 21st day following administration of

each drug.

Results: All rats in group A showed an abnormal response to the pinch-toe test and decreased motor function during the entire evaluation period. Abnormal histopathologic findings, including neuritis and meningeal inflammation were observed only in the group A rats. Rats in group P, with the exception of one, and group N showed no significant sensory/motor dysfunction over a 3-week observation period. No histopathologic changes were observed in groups P and N.

Conclusions: Direct epidural injection of pamidronate (about 12.5 mg/kg) showed no neurotoxic evidence in terms of sensory/motor function evaluation and histopathologic examination.

Keywords: epidural injection, neurotoxicity, pamidronate, bisphosphonate

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Introduction

Bisphosphonate is a pyrophosphate derivative that binds to the inorganic matrix of bone, and thereby strongly inhibits bone resorption. The drug was previously considered for treatment of osteoporosis for conditions in which hormone replacement therapy is restricted. In recent years, however, it has been reported that pamidronate is effective in alleviating the pain of vertebral compression fracture and bone metastasis in cancer patients with osteoporosis,[1,2] and IV bisphosphonates relieve neuropathic pain in patients with complex regional pain syndrome(CRPS/RDS).[31] Currently, it is used clinically by oral administration or intravenous injection. Once it has been orally administered, pamidronate has a lower profile of bioavailability, and its intestinal absorption is affected by intake of food and water. In addition, intravenous administration causes gastrointestinal disturbances, including nausea, vomiting, and diarrhea, as well as susceptibility to electrolyte imbalance, renal dysfunction, anemia, and hypertension. Moreover, the drug is expensive, and requires hospitalization for long-term intravenous injection.[1,2]

Spinoaxial administration of the drug is expected to reduce dosage, as compared with oral or intravenous infusion, thereby minimizing systemic

side effects and maximizing efficacy. This type of administration method has been of great interest in the field of pain medicine. Pain clinicians prefer the epidural administration of analgesics to intrathecal administration because it is less invasive and is likely to reduce the risk of neurotoxicity, especially when the administration is conducted over an extended period[28]. Although the drug is used safely for systemic administration, it has been well established that animal experiments must be performed to assess efficacy and safety if the drug is to be administered intrathecally or epidurally in a clinical setting.[3] However, no animal studies have been performed on the possible neurotoxicity of spinoaxially injected pamidronate, and thus we undertook the present study to evaluate the neurotoxicity of epidurally injected pamidronate in a rat model.

We then performed behavioral observations and histopathologic analysis to assess neurotoxicity.

Materials and methods

1. Study subjects

Following approval of the Institutional Review Board of Animal Experiments, male Sprague-Dawley(SD) rats weighing approximately 250-350 g were randomly divided into 3 groups, which were administered 0.3 mL (3.75 mg) of epidural pamidronate (group P, n = 9) and the same volume of epidural alcohol (group A, n = 9) or normal saline (group N, n = 9). Each group comprised nine rats, and a total of twenty-seven rats were examined in the current study. All rats had free access to food and water and they were bred following a 1-week adaptation period with a 12-hr photo cycle. All rats showing abnormalities during behavioral observation were excluded from the current analysis.

2. Placement of a catheter in the epidural space

Anesthesia was induced by placing a rat in a closed box containing 4% sevoflurane in oxygen (3 L/min) with spontaneous ventilation. Approximately three minutes later, 2-3% sevoflurane was administered with a loose-fitting mask, and thereby anesthesia was maintained. An epidural catheter was prepared by making a knot at 2.5 cm from the tip of

a 17 cm micro-plastic catheter (PE-10; Natsume Co., Japan). After applying a sterile dressing, an epidural puncture was then performed. That is, skin was incised at a length of 2-3 cm and the area adjacent to the supraspinous ligament was dissected. Between the 13th thoracic spine and the first lumbar spine, the ligamentum flava was exposed with no damage. Using microsurgical scissors, a small-sized hole was made in the ligamentum flava. A catheter was then inserted and progressed toward the tail at a distance of approximately 2.5 cm, toward the tip of a catheter placed between the 4/5 lumbar spine. Animals were excluded from the current experiment if blood or CSF was aspirated through the catheter. The remaining part of the catheter passed through the dermal layer and was then extracted in the junction between the cervical and thoracic spine. All scars were rinsed with saline. A catheter was passed through the center of a knot using a 4-0 silk suture, and tightly fixed to the adjacent tissues. Using 1-2 drops of surgical glue (alpha cyanoacrylate; Aron-Alpha, Toagosei, Japan), the puncture site was sealed. To confirm correct catheter positioning, 2% lidocaine at 0.15 ml was gradually injected through the catheter after complete recovery from anesthesia. We defined a correct epidural catheter replacement that anterior legs showed normal movement, but posterior legs did not move temporarily. If rats died of respiratory distress when given lidocaine, it was considered subarachnoid

or intravascular injection; such cases were excluded from this study. After confirming correct epidural catheter placement, we examined gait, spinal deformity, and behavioral abnormalities for 3 days. If the rats showed abnormal findings during the 3-day observation period, they excluded from this study. Each rat was then isolated and raised in stabilized conditions.[4,5)]

3. Drug infusion to the epidural space

Three days following insertion of the catheter into the epidural space, ambulatory posture, vertebral deformity, and abnormal behavior were examined. Further laboratory procedures progressed in rats with no abnormal findings. Twenty-seven male SD rats, weighing 250-300 g, were successfully prepared for this study, and these rats were divided equally 3 groups. Under general anesthesia, animals in group P were injected with pamidronate (Panorin®, pamidronate disodium 15 mg/1 ml Ampule, Hanlim, Seoul, Korea) 3.75 mg(0.3 mL, 12.5 mg of pamidronate dissolved in 1 mL of saline). In group A, 40% alcohol was injected, and in group N, 0.9% normal saline was gradually injected at a dose of 0.3 ml for approximately one minute, except for the volume of a catheter. All drugs were newly prepared prior to injection and managed using an aseptic method. Upon recovery from anesthesia, rats were isolated to be safely bred at a 12-hr

light/dark cycle. Considering the extent of spreading of contrast medium by fluoroscopy, the total spreading spinal segments of 0.3 mL of contrast medium was 10 or 11. It was enough to affecting the entire spinal cord segment cropped for histopathological examination.

4. Assessment of sensory and motor nerve disturbance through behavioral observation

Acute toxicity was assessed 2 days after injection and chronic toxicity was evaluated 7 and 21 days after injection. Rats were evaluated for sensory and motor nerve disturbance and abnormal behavior by one investigator blinded to the experimental procedure at the same time on each day. Pinch-toe testing and motor function evaluation were started 2 days after drug injection to exclude the possibility of the systemic effect of pamidronate.

1) Pinch-toe test

A hind paw was pinched using a forcep (01-1155, Solco, Seoul, Korea). Then, using a pinch-toe test, motor and sensory nerve damage was assessed.[6-8] When the hindpaw was pinched by a forcep to such an extent that resistance of bone could be perceived (deep pinch), observed normal avoidance responses were as follows:

(1) Avoidance of lower extremities

(2) Crying

(3) Attempt by the animal to bite the forcep

In the lower limbs on both sides, if all three of the above categories were observed at a minimum 5-minute interval up to three times during a maximum 6-seconds (cut off time), the case was determined to be normal. Otherwise, corresponding cases were determined to have an abnormal response that indicated the possibility of motor and sensory nerve damage.[6)]

2) An assessment based on ambulatory pattern and lower limb deformity

To examine motor nerve disorders, with the application of motor function based on ambulatory pattern and the deformity of lower extremities,[9)] all rats were divided into the following grades. Grade 1 = normal gait with no evidence of motor paresis; Grade 2 = normal gait with slight hindpaw deformity, such as plantar flexion of toes; Grade 3 = slight gait disturbance with motor weakness and/or an inverted hindpaw; and Grade 4 = prominent limping gait with a dropped hindpaw. The rats corresponding to ≥ Grade 2 were all considered to have motor nerve injury.

5. Histopathologic examination

Tissue samples were collected from the spinal cord on day 2 for assessment of acute toxicity, and on day 7 and 21 for chronic toxicity. Rats were killed under general anesthesia (as described above) and 4% paraformaldehyde in 0.1 M phosphate buffer was injected into the heart. Euthanasia was thus induced and the cadaver was fixated. In the center of the area where the tip of a catheter was placed, approximately three segments of the spinal cord, both superiorly and inferiorly, including the nerve root, were removed. Tissue samples were fixated in a 10% formalin solution for three days and then decalcified using 10% formic acid for two weeks. Tissue specimens were rinsed with a buffer solution (pH 7.4) and then fixated using 2% osmium tetroxide, which was dissolved in a buffer solution for 30 minutes. Dehydration was performed using a graded ethanol, followed by embedding of the tissue sample using epoxy resin (agar 100) between teflon-sprayed slides and thick acetate foil with a thickness of 100 μm. Tissue samples containing the ventral and dorsal horns of the spinal cord were dissected and then re-embedded for fine sectioning. Tissue samples were then stained using hematoxylin-eosin and Luxol fast blue dyes for light microscopy.

Histopathological findings for assessment of neurotoxicity include chromatolysis in the motor neurons of the ventral horn of the spinal cord,

neuritis, meningeal inflammation, adhesion in periosteum, dura mater and medulla, enlargement of dura mater, peripheral neuropathy, myelin loss, and local infarction. To rule out bias, histopathologic change was analyzed by one pathologist blinded to the study.

6. Statistical analysis

Assessment of motor and sensory nerve disorders and histopathologic examination of the spinal cord were performed and their results were tested using the ANOVA between the three groups. Any cases with a significant difference were analyzed using the Fisher exact test between the two groups, which was followed by Bonferroni correction (SigmaStat ver. 2.0, Jandel corporation). A P value < 0.05 was considered statistically significant.

Results

In the P group, with the exception of one rat, and in the N group, no rats showed motor or sensory change, or any behavioral change throughout the study period. All rats in group N and all rats except one in group P had a normal gait at the time point for all examinations (Table 1). During the overall period, however, most rats in Group A showed decreased activity and appetite. In group A, all rats responded to some of three reactions, or no reaction to the pinch-toe test, and had a gait disturbance of Grade 3 or more. ($P < 0.05$, Table 1).

No significantly abnormal histopathologic findings were observed in the P and N groups. However, in group A, various histopathological forms of neurological deficit, such as local neuritis, myelin loss, and meningeal inflammation occurred at each time point of drug administration ($P < 0.05$, Table 2 and Figs.1 and 2).

Table 1. Evaluation of Pinch-Toe Test and Motor Deficit Following Epidural Drug Injection

Days after injection	Group N	Group P	Group A
Pinch-toe test			
2nd day(n=9)	-	1(11)	9(100)*
7th day(n=6)	-	-	6(100)*
21st day(n=3)	-	-	3(100)*
Motor deficit[a]			
2nd day(n=9)	-	1(11)	9(100)*
7th day(n=6)	-	-	6(100)*
21st day(n=3)	-	-	3(100)*

Values are expressed as number (%) of abnormal rats.

[a]Group P : 0.3ml (3.75 mg) of epidural pamidronate and Groups A and N: the same volume of epidural alcohol and normal saline, respectively.

Grade 1 = normal gait with no evidence of motor paresis; grade 2 = normal gait with slight hind paw deformity, such as plantar flexion of toes; grade 3 = slight gait disturbance with motor weakness and/or an inverted hindpaw; grade 4 = prominent limping gait with a dropped hindpaw. Rats with a motor disturbance of grade 2 or above were considered to have a motor deficit.

* $P < 0.05$ versus corresponding data of Groups N and P

Table 2. Neuropathologic Findings of Spinal Cord and Nerve Under Light Microscopic Examination Following Epidural Drug Injection.

	Group N			Group P			Group A		
	2^{nd}(n=3)	7^{th}(n=3)	21^{st}(n=3)	2^{nd}(n=3)	7^{th}(n=3)	21^{st}(n=3)	2^{nd}(n=3)	7^{th}(n=3)	21^{st}(n=3)
Chromatolysis	-	-	-	-	-	-	-	-	-
Local neuritis	-	1	-	-	-	-	1	3*	2
Dural hypertrophy	-	-	-	-	-	-	-	1	2
Synechia	-	-	-	-	-	-	-	-	-
Periopheral neuropathy	-	-	-	-	-	-	1	3*	2
Myelin loss	-	-	-	-	-	1	0	3*	3*
Meningeal inflammation	-	-	-	-	-	-	-	1	2
Local infarction	-	-	-	-	-	-	-	-	-

Values are expressed as number of positive animals.

Group P: 0.3 ml (3.75 mg) of epidural pamidronate and Groups A and N: the same volume of epidural alcohol and normal saline, respectively. 2^{nd}, 7^{th}, and 21^{st}:days after epidural injection of test drugs.

* $P < 0.05$ versus corresponding data of Groups N and P.

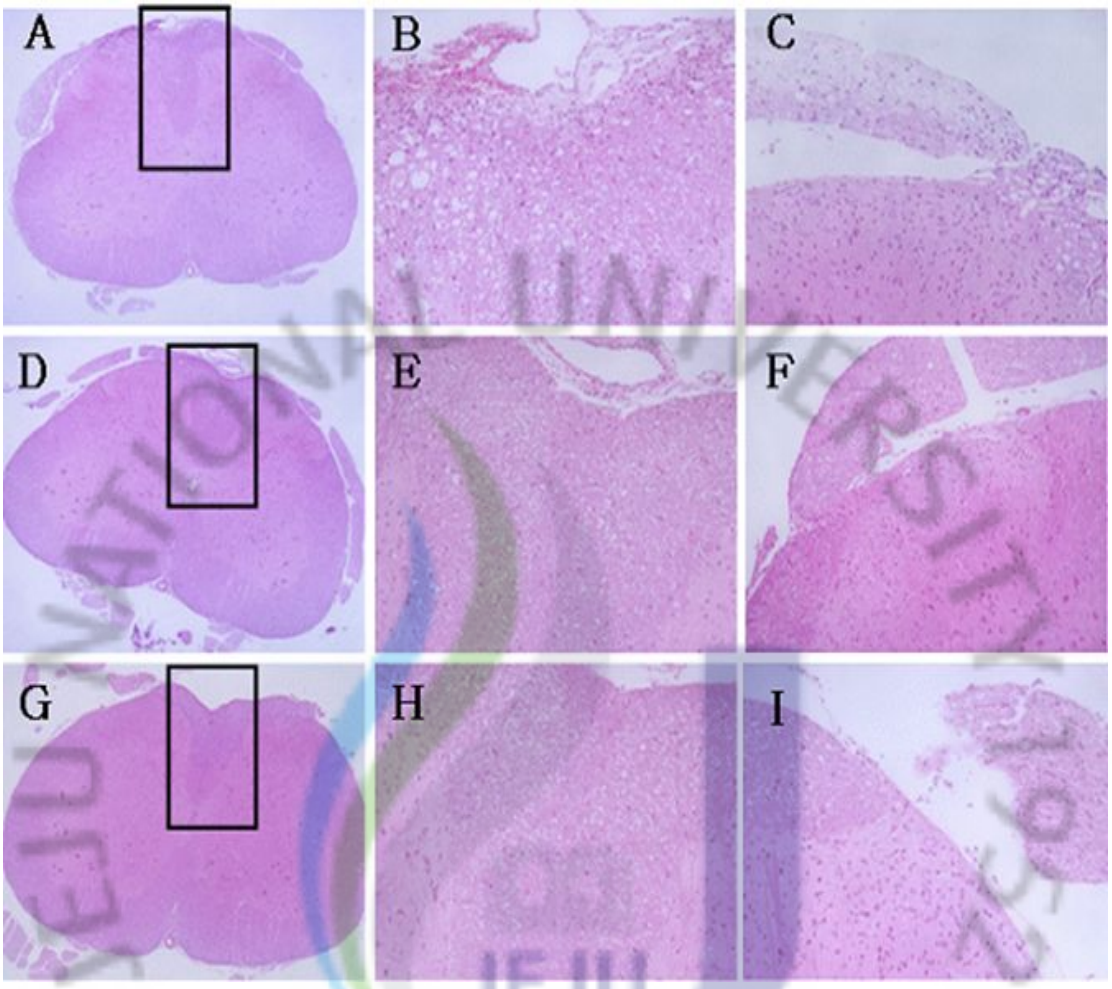

Fig. 1. Microscopic findings in spinal cord and nerve on the 2nd day (A, D, and G), 7th day (B, E, and H), and 21st days (C, F, and I) following epidural injection of alcohol (A-C), pamidronate (D-F), and normal saline (G-I). Hematoxylin and eosin stain. Figures in middle and right columns (x 200) show the high power view of the adjacent left side column (x 40) (A, D, and G). In the epidural alcohol group, marked vaculolar change of posterior funicules (B) and lymphocytic infiltration in the spinal cord (C) are visible. In the epidurally-injected pamidronate and normal saline groups, no vacuolation or inflammation are visible.

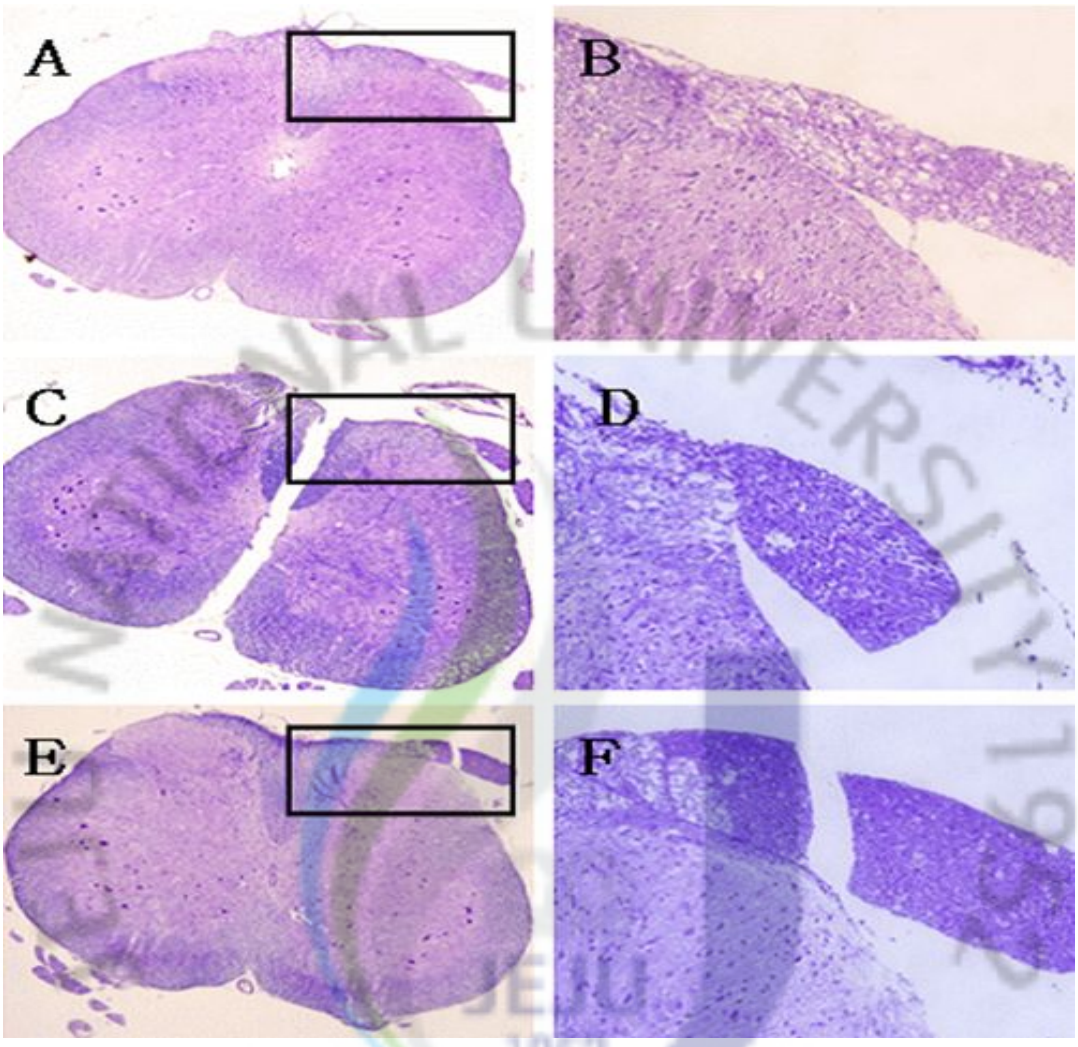

Fig. 2. Microscopic findings in spinal cord and nerve following epidural injection of alcohol (A and B), pamidronate (C and D), and normal saline (E and F). Luxol fast blue stain. Right-side figures (x 200) (B, D, and F) show the high power view of the adjacent left side column (x 40) (A, C, and E). In the epidural alcohol group, pale and diminished myelin are visible, and presented as a purple color (B). In the epidurally-injected pamidronate or normal saline groups, no definite abnormal morphologic findings of myelin are visible.

Discussion

Bisphosphonates show specific, powerful binding to the inorganic matrix of bone, and thereby suppress the maturation and activity of osteoclasts, ultimately causing apoptosis of osteoclasts.[10,11] One of the bisphosphonates, pamidronate, has an amino group, and thereby strongly inhibits bone resorption.[1,2] It is a drug that treats hypercalcemia, osteolytic bone metastasis, and Paget's disease due to malignant tumors by inhibiting bone resorption by osteoclasts.

In recent years, pamidronate has been reported to have excellent efficacy in treatment of chronic lower back pain due to osteoporotic fracture, alleviating pain due to bone metastasis of breast cancer, lowering severe bone pain in Paget's disease, improving the pain of cancer metastasis, and controlling the pain in complex regional pain syndrome (CRPS).[2 12-16]

Bonabello et al. reported that the antinociceptive effects occurred with no respect to peripheral opioid receptors in both the central and peripheral nervous systems, with no association in bone lesions.[17] It has also been reported that bisphosphonates modify such substances as Prostaglandin E_2, sensitizing anti-IL-1, anti-IL-6, and anti-TNF-alpha

effects, as well as the traumatic pain receptor, and thereby have an analgesic effect in addition to inhibiting osteolclastic activity.[18] In addition, it has also been reported that the afferent nerve terminal suppresses the release of various neuropeptides following the onset of trauma in patients with complex regional pain syndrome (CRPS).[16] In addition, such effects as inhibition of protein prenylation, inhibition of neoangiogenesis, and immune modulation have an influence on alleviation of pain .[19]

Currently, pamidronate is administered via oral route or intravenous infusion. It can be administered up to a maximal dose of 300 mg/d for oral administration and 90 mg/d for intravenous infusion.[20] However, there are a few limitations to administration via the oral route, and disadvantages of oral administration of bisphosphonate are as follows: (1) a lower rate of intestinal absorption (1-10%),[21] (2) affection by food ingestion, (3) obligation to administer during the fasting state and (4) gastrointestinal disturbances, such as nausea or abdominal pain. Due to these disadvantages, patient compliance has been reported to be lower,[21] and injection methods are favored. Intravenous injection has many advantages in terms of cost-effectiveness and patient compliance. A persistent intravenous infusion conversely induces osteochondrosis, and also produces such adverse effects as phlebitis, febrile sensation, chilling sensation, myalgia, malaise, thrombophlebitis, and hypophosphatemia .[20-21]

In the early stage of administration, adverse effects, such as increased bone pain, transient leukocytopenia [22] and necrosis of the jaw bone [20,23,24] used to evolve. In addition, drugs are expensive and hospitalization is needed for an intravenous infusion, which can also be problematic.

From these causes, we considered epidural treatment of bisphosphonate as an alternative. An epidural approach is currently used most prevalently to control pain, and it is more effective than other administration routes in cases in which the spinal cord is the target of drug administration [25] . Even a minimal dose can produce the same effect, and it is considered that systemic adverse effects and risk of neurotoxicity can be reduced. It is also advantageous in that inhibition of the necessary spinal level can be selective. Pamidronate would also be expected to have an excellent effectiveness when directly administered to the area adjacent to the spinal cord. However, administration of any drug into the spinal cord or an epidural space should be evaluated for potential occurrence of neurotoxicity in the spinal cord.[3,26] Although the addictives that can cause neurotoxicity with higher probability, such as anti-oxidants, anti-disinfectants, or excipients could be ruled out, direct administration of drugs to the area adjacent to the spinal cord can cause direct contact between a high-dose of drugs to the nerve when compared with other administration routes. Therefore, neurological safety must first be

demonstrated.

We administered 3.75 mg of pamidronate into the epidural space. In humans, this 3.75 mg (approximately 12.5 mg/kg) is equivalent to an epidural dose of 750 mg. Moreover, the required dose for the epidural route is about 1/30 of that required for oral administration.[29] Thus, the 3.75 mg of epidural pamidronate administered may be equivalent to an oral administration of about 22,500 mg for a human adult. Because maximal recommended oral dose for pamidromate is 1,200 mg in human adults, and because the dura mater of small animals has greater diffusibility than that of human[30], we decided that a 3.75 mg dose of epidural pamidronate was sufficient for neurotoxicity evaluation purposes in the rat.

Determination of drug volume has been known to correspond with an epidural infusion of 10-15 ml in humans and 0.1 ml in SD rats.[27] In SD rats weighing 250-350 g, a contrast medium of 0.3 ml in volume is distributed across 10-11 segments of the spinal cord. It can therefore be predicted that most drugs with a viscosity lower than that of the contrast medium would be distributed in more extensive areas. It can therefore be inferred that the dosage is sufficient for examination of sensory and motor change in the lower limbs, including the 3-6th lumbar nerve root.

Although statistically significant neurotoxicity findings or ambulatory

disorder were not observed on light microscopy, an ambulatory disorder was observed in a rat in the P group on day 2 (Grade 4). Because no special abnormal findings were observed on light microscopy, it can therefore be inferred that physical injury caused by catheter use, migration of the catheter into the subarachnoid space, and intravascular migration of the catheter would be a factor responsible for the presence of abnormal findings, rather than drug-induced basic changes in nerves.

In conclusion, epidural administration of pamidronate 12.5 mg/kg did not produce significant neurotoxic adverse effects on behavioral observation and histopathologic examination in SD rats, which implies that pamidronate could be directly administered epidurally in a clinical setting. However, similar studies in other animal species are warranted to examine the reliable safety of an epidural application of pamidronate before use in a clinical setting.

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