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Intratumoral Administration of Methotrexate Bound to Activated Carbon Particles (MTX-CH): Antitumor Effectiveness Against Human Colon Carcinoma Xenografts and Acute Toxicity in Mice

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Running title: Effectiveness and Acute Toxicity of MTX-CH

Abbreviations: CH40, a suspension that mixed with activated carbon (Carbon 40®; Mitsubishi Chemicals Co. Ltd., Tokyo, Japan) and polyvinylpyrrolidone (PVP K-30®; Nakarai Chemicals Co. Ltd., Kyoto, Japan) in saline; FBS, fetal bovine serum; LD 10, 50 and 90, 10%, 50% and 90% lethal dose values; MTX, methotrexate; MTX-AQ, methotrexate aqueous solution; MTX-CH, methotrexate bound to activated carbon particles; MTX-HSA, methotrexate-human serum albumin.

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Abstract

We previously developed a new formulation of methotrexate (MTX) that is adsorbed onto a suspension of activated carbon particles (MTX-CH) and reported the usefulness of local administration in murine tumors. The present study examines the effects of human colon carcinoma (LoVo) xenografts and the acute toxicity of MTX-CH compared with MTX aqueous solution (MTX-AQ) in mice. 1) Therapeutic experiments: LoVo cells were implanted into the backs of BALB/c nude mice. When the cells had developed into tumors, we performed an intratumoral administration of a weekly dose of 30 mg/kg. The MTX concentration in the tumor was compared between the MTX-CH group and MTX-AQ group. 2) Experiments on acute toxicity: MTX-CH and MTX-AQ were injected subcutaneously in BDF1 mice, and intoxication symptoms, changes in body weight, and date of death were recorded. In the therapeutic experiments, intratumoral administration of MTX-CH was how much more effective in suppressing the tumor growth compared with MTX-AQ. In experiments of acute toxicity, the death time of the MTX-CH group was delayed to a greater extent and the 50% lethal dose (LD50) values of MTX-CH were lower than those of MTX-AQ. The LD50 values of MTX-CH are 75 times higher than the efficacious dose of 30mg/kg. The present results suggest that intratumoral administration of MTX-CH is useful for local therapy and the therapeutic dose of MTX-CH can be safely injected subcutaneously.

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Introduction

Intratumoral administration therapy is useful for the patients with digestive tract cancer, who cannot receive surgical treatment due to a locally advanced unresectable tumor or with surgical risks such as cardiac diseases, aging, and so on (Cascinu et al., 1998; Kuwayama et al., 1984). However, locally injected drugs in aqueous solution form are rapidly absorbed through blood capillaries into the circulatory blood; therefore, they are not effective at the injected sites (Ballard., 1968). In order to retain the drug in the tumor for a long period, we previously developed a new formulation of methotrexate (MTX) that is adsorbed onto a suspension of activated carbon particles (MTX-CH) (Hagiwara et al., 1992; Hagiwara et al., 1994). Activated carbon adsorbs a large amount of MTX onto the surface of particles, and releases most of the adsorbed MTX into the tissues. The adsorption of the isotherm of MTX into activated carbon shows that the adsorbed MTX remains in dynamic equilibrium with the concentration of free MTX around the activated carbon particles. When the free MTX is consumed by

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washout or binding to tissue and the concentration of MTX in a free state decreases around the carbon particles, the carbon releases the adsorbed MTX thus replacing the decreased concentration (Hagiwara et al., 1994). We previously reported on the effectiveness of local administration of MTX-CH in the early stages of esophageal cancers or gastric cancers. In the case of most of these patients, the primary lesion disappeared completely without any side effects (Hagiwara et al., 1996; Hagiwara et al., 1997; Hagiwara et al,. 2000). Of late, there has been an increase in the number of colon cancer patients. Furthermore, the population of senior citizens in Japan has also increased. Therefore, we found a need to treat colon cancer patients on whom surgical procedures cannot be performed due to locally advanced unresectable colon cancer or due to surgical risks such as cardiac diseases, aging, etc. We had previously investigated the effects of the MTX-CH intratumoral injection on murine colon tumors and reported that it suppresses tumor growth (Ito et al., 2003). In the present study, we examined the effects of human colon carcinoma

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xenografts (LoVo) and the acute toxicity of MTX-CH compared with MTX aqueous solution (MTX-AQ) in mice.

Materials and methods

Drug preparation. Activated carbon (Carbon 40®; Mitsubishi Chemicals Co. Ltd., Tokyo, Japan) that contains very small carbon particles (21 nm) was mixed with polyvinylpyrrolidone (PVP K-30®; Nakarai Chemicals Co. Ltd., Kyoto, Japan) (40,000 daltons) in saline and kneaded with three rollers to create a suspension (CH40). The composition of CH40 was as follows: 50 mg/ml of activated carbon and 20 mg/ml of polyvinylpyrrolidone. The activated carbon suspension was sealed in a glass tube and sterilized at 120°C for 10 mins. Fifty milligrams of MTX (Methotrexate®; obtained as a gift from Wyeth Lederle Japan Co. Ltd., Tokyo, Japan) was dissolved in 1 ml of CH40, and the resulting solution was shaken for 12 h to allow the dissolved MTX to be adsorbed onto the carbon particles (MTX-CH), thus demonstrating that in the preparation of MTX-CH, almost 99% of MTX is adsorbed on activated carbon (1994; Hagiwara

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et al.). As a comparison, the same concentration of MTX was dissolved in saline (MTX-AQ).

1) Therapeutic experiments in mice

Animals and cancer cell line. The female nude mice (BALB/c, 5 weeks old) were obtained from Shimizu Laboratory Animal Center (Kyoto, Japan). The mice were maintained under standard conditions (specific pathogen free, room temperature of 24°C, relative humidity of 60%, day/night cycle of 12 h) and were allowed free access to standard mouse food and tap water during the experiment. Animal care, housing, and surgery followed the Rules and Regulations of the Committee for Animal Research, Kyoto Prefectural University of Medicine, Japan. The human colon adenocarcinoma cell line (LoVo) was used as an experimental tumor. This cell line was obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. The cell line was maintained in Ham's F12 medium (GIBCO BRL, Tokyo, Japan) containing 20% heat-inactivated fetal bovine serum

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(FBS; GIBCO BRL, Tokyo, Japan) at 37°C in a humidified incubator with 5% CO_2.

Experimental model. LoVo cells (2×10^6 cells/200 μl/site) were subcutaneously implanted in 5-week-old female BALB/c nude mice. The drug administration was started five days after the implantation, when the cells had developed into tumors of about 5 mm in diameter (defined as Day 0).

i) Intratumoral administration therapy. A total of 40 mice were divided into five groups of eight mice each. The mice in the first group received no treatment and served as controls. The mice in the second group received an injection of MTX at 30 mg/kg of body weight into the tumors in the form of MTX-CH (0.6 ml/kg of body weight as MTX-CH, MTX-CH group). The mice in the third group received an injection of same dose of MTX in the form of MTX-AQ at the same injection site (MTX-AQ group). The mice in the fourth group received an injection of same volume of CH40 (0.6 ml/kg of body weight) at the same injection site (CH40 group). The mice in the fifth group were injected with the

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same volume of saline at the same injection site (saline group). The drugs were administered on Day 0 and on every 7th day until Day 21. The major and minor axes of the tumors were measured before every administration and on Day 28. In order to assess the effect of the drugs, the rate of change was calculated individually. The method of calculation of the rate of change of tumors is shown in Fig. 1A.

Statistical analysis. The Mann–Whitney test was used to compare data between five groups by the computer software SAS/STAT Version 8 （SAS Institute Inc.）. Statistical significance was defined as p-value less than 0.05.

ii) The MTX concentration in the tumor. A total of 48 mice were divided into eight groups of six mice each. Four groups of mice received injections of MTX at 30 mg/kg of body weight into the tumors in the form of MTX-CH. The remaining groups (four groups) received injections of the same dose of MTX in the form of MTX-AQ at the same injection site. The four groups of mice were killed with an overdose of pentobarbital sodium (Nembutal®; Dainippon

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Pharmaceutical Co. Ltd., Osaka, Japan) at 6, 12, 24, and 48 h after the injection, respectively, and then the tumors were excised. The excised tumors were minced, mixed with Tris Buffer, and homogenized. After the centrifugation, the concentration of MTX was measured by the fluorescence polarization immunoassay procedure (Slordal et al., 1986; Pesce et al., 1986). In this procedure, the carbon particles and tissue fractions were removed by centrifugation, and the supernatant was subjected to an assay of MTX concentration. Therefore, the MTX concentration shown in this study is believed to indicate the action of MTX on the tissue in the region, and is believed to be free of MTX that is bound to carbon particles.

Statistical analysis. One-way ANOVA was used to compare data between two groups using the computer software SAS/STAT Version 8（SAS Institute Inc.）. Statistical significance was defined as p-value less than 0.05.

2) Acute toxicity in mice

Animals. Male BDF1 mice (5 weeks old) were obtained from Shimizu

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Laboratory Animal Center (Kyoto, Japan). BDF1 mice are strong and useful in investigating the toxicity of anticancer drugs (Harrison SD et al., 1978). These mice were maintained under the same conditions and animal care as in 1) Therapeutic experiment.

Study design. A total of 104 male BDF1 mice were divided into 13 groups of eight mice each. Five groups were given MTX-CH, and the other five groups were given MTX-AQ. The remaining three control groups were given only physiological saline, only CH40, or nothing. Both MTX-CH and MTX-AQ doses ranging from 1388 mg/kg to 2880 mg/kg were given in five dose levels, each increasing 1.2-fold. The group that was given only CH40 was administered a dose of 2880 mg of activated carbon/kg of the body weight corresponding to the amount of activated carbon in MTX-CH (methotrexate of 2880 mg/kg dose). The group that was given only the physiological saline was administered a dose of 57.6 ml/kg of the body weight corresponding to the amount of physiological saline in MTX-CH (methotrexate of 2880 mg/kg dose). The drugs were

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subcutaneously injected in the mice using a 26-G needle. The mice were observed 14 days after the drug administration, and the intoxication symptoms, changes in body weight, and date of death were recorded. The surviving animals were killed by an overdose of pentobarbital sodium (Nembutal®; Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) on day 14. All animals, including those that died of intoxication and those that survived the observation period of 14 days, were autopsied for observation of macroscopic and microscopic changes in their body organs. The heart, lung, liver, kidneys, spleen, small intestine, and large intestine of each mouse were removed and weighed. Then the absolute organ weight was used to calculate the relative organ weight with respect to the total body weight to better evaluate the change in organ weight due to toxicity. The weighed organs were then fixed with 10% buffered formalin, embedded in paraffin, sliced into 4 μm microscopic specimens, and stained with hematoxylin and eosin. These microscopic specimens were microscopically examined for pathologic changes associated

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with drug toxicity.

Statistical analysis. The lethal dose values and 95% confidence interval (CI) were calculated by the probit method using computer software SAS/STAT Version 8（SAS Institute Inc.）.

Results

1) Therapeutic experiments

i) **Intratumoral administration.** The results of the intratumoral administration are shown in Fig. 1B. The tumors of the MTX-CH group and the MTX-AQ group at the end of the experiment are shown in Fig. 1C and Fig. 1D, respectively. During the 28-day-period of observation, the mean rate of change in tumors in the MTX-CH group was the lowest among all groups. The mean rate of change in tumors in the MTX-CH group was 192.6% on Day 28; whereas, the mean rates of change in tumors of the other groups were almost over 400%. The rate of change in tumors of the MTX-CH group was significantly lower than the other groups after Day 7 ($p<0.05$). In this experiment, no side effects were observed

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in the mice that were given MTX-CH or MTX-AQ.

ii**) The MTX concentration in the tumor.** The MTX concentration in the tumor is shown in Fig. 2. The mean MTX concentration in the tumors of the MTX-CH group was 100.82 nmol/g at 6 h after administration and slowly decreased to 5.74 nmol/g at 48 h after administration. In contrast, in the MTX-AQ group, the mean MTX concentration in tumors was 55.46 nmol/g at 6 h after administration, and it rapidly decreased to 0.47 nmol/g at 48 h after administration. The mean MTX concentrations in the tumors of the MTX-CH group at 6, 12, and 48 h after administration were significantly higher than those in the MTX-AQ group ($p<0.05$).

2) **Acute toxicity.**

i) Intoxication symptoms, changes in body weight, and date of death. Intoxication symptoms such as weakness, lethargy, and dishevelment were observed in all the mice that were given a dose of more than 1388 mg/kg of MTX-CH or MTX-AQ. In the mice that were given MTX-CH, these symptoms

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began on Day 2 or 3 and disappeared by Day 10. In the mice that were given MTX-AQ, these symptoms began on Day 2 or 3 and disappeared by Day 8. The changes in body weight are shown in Fig. 3. In the mice that were given MTX-CH, the body weight loss continued for the first 7-10 days and the body weight began to increase on Day 8-11 (Fig. 3A). In the mice that were given MTX-AQ, the body weight loss continued for the first 5-7 days and the body weight began to increase on Day 6-8 (Fig. 3B). In the mice that were given only physiological saline, only CH40, or nothing, there was an increase in body weights (Fig. 3C). In the mice that were given MTX-CH, most deaths were observed at doses higher than 2000 mg/kg. All deaths were observed within 10 days after drug administration. In mice that were given MTX-AQ, most deaths were observed at doses higher than 2400 mg/kg. All deaths were observed within 7 days after drug administration (table 1). No deaths were observed in mice given only physiological saline, only CH40, or nothing.

ii) Lethal dose values. The LD10, LD50, and LD90 values of MTX-CH were

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1998.7, 2288.5, and 2620.2 mg/kg, respectively. The LD10, LD50, and LD90 values of MTX-AQ were 2260.6, 2637.8, and 3078.0 mg/kg, respectively. The LD50 value of MTX-CH was 0.87 times lower than that of MTX-AQ. The 95% CIs are shown in table 2.

iii) Autopsy findings. Both the absolute organ weight and relative organ weight (ratio of organ weight to body weight) are shown in table 3. The absolute weights of the liver and kidneys from mice that died of toxicity were significantly lower compared with those of the control mice. The kidneys and liver of the dead mice were anemic when examined macroscopically, and the mucosa of the small intestine showed atrophy. Microscopically, acute tubular necrosis of the kidney (Fig. 4A) and toxic centrilobular necrosis of the liver (Fig. 4B) were observed. The mucosa of the small intestine was necrotic and degenerative (Fig. 4C). Acute tubular necroses, in particular, were severe. These microscopic findings were not observed in the mice that survived up to day 14. No difference was observed in the pathologic findings between the dead mice given the two

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different dosage formulations.

Discussion

New drug formulations of MTX such as methotrexate-human serum albumin (MTX-HSA) (Halbert et al,. 1989), polyamidoamine dendrimers encapsulating methotrexate (Kojima et al., 2000), liposomal methotrexate (Williams et al., 2000; Pignatello et al., 2003) and lipoprotein-mimicking biovectorized methotrexate (Utreja et al., 1999) were previously reported. The therapeutic effects of MTX-HSA and liposomal methotrexate were reported. Liposomal methotrexate was effective in treating rheumatoid arthritis in rats (Williams et al., 2000) and the human erythroleukemia cell line (Pignatello et al., 2003). In the phase I trial of MTX-HSA, no therapeutic response was observed in patients with colorectal cancer (Hartung et al., 1999). In the phase II trial of MTX-HSA, no objective responses were observed in patients with metastatic renal cell carcinoma (Vis et al., 2002). The antitumor activities of the MTX-HSA intravenous injection in various human cancer xenografts in nude mice were

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reported (Wosikowski et al., 2003). According to the report, the MTX-HSA intravenous injection suppresses the tumor growth of human colon carcinoma (Colo 205, WiDr), although the tumor does not disappear. These new drug formulations of MTX were designed to improve drug transport system and were administered intravenously. The antitumor effects of MTX depend on the duration of its active exposure period rather than on its concentration (Keefe et al., 1982). We developed MTX-CH as a sustained-release drug based on the action mechanism of MTX as a locally administered therapy. MTX-CH slowly released the adsorbed MTX. In the present study, when the MTX-CH was locally injected into the tumor, the concentration of MTX in the tumor was maintained at significantly higher levels for longer periods in the MTX-CH group than in the MTX-AQ group. The high variability of MTX concentration is observed at the earliest time point for the MTX-CH group. The tumor has rich blood vessels and lymph ducts. Therefore, if the injected MTX-CH was absorbed through blood capillaries or lymph ducts at an early time point, the

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remains of MTX-CH in the tumor decreased. We considered that the initial variability of the MTX concentration in the MTX-CH group was observed due to this reason. The weekly intratumoral administration of MTX-CH suppressed the tumor growth of human colon carcinoma xenografts in nude mice for long time, although the tumor did not disappear. We consider the appropriate cases opting for treatment by intratumoral MTX-CH injection therapy as being those with locally advanced unresectable cancer or those that face surgical risks such as cardiac diseases, aging, etc. We had previously reported on the effects of intratumoral injection of MTX-CH in the early stages of esophageal cancer and gastric cancer with surgical risks. In the case of most of these patients, the primary lesion disappeared completely without any side effects. We anticipate that the MTX-CH intratumoral injection therapy is more effective than systemic chemotherapy in these cases. Furthermore, in the case of patients with locally advanced cancer on whom neither surgical procedures can be performed nor systemic chemotherapy can be administered, the intratumoral injection of MTX-

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CH suppresses the tumor growth and prevents stenosis or bleeding, thus improving the quality of life of the patients. Endoscopic intratumoral administration of anti-cancer drugs is a low-invasive procedure and is useful for unresectable digestive tract cancer (Cascinu et al., 1998; Kuwayama et al., 1984). It can be expected that intratumoral administration of MTX-CH will be useful as one of the clinical tools in patients with colon cancer who cannot receive surgical treatment due to various reasons.

In this acute toxicity experiment, no differences in the starting time of body weight loss and intoxication symptoms were observed between the MTX-CH group and the MTX-AQ group. However, the recovery of body weight and the intoxication symptoms of the MTX-CH group were more delayed than in the MTX-AQ group, and the death time of the MTX-CH group was at a later time than that of the MTX-AQ group. We hypothesize that the above-mentioned prolongation of toxicities in the MTX-CH group is due to the slow release of MTX from MTX-CH. In the present study, no remarkable differences were

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observed in the intoxication symptoms and pathologic findings on autopsy between the MTX-CH group and the MTX-AQ group. Based on these findings, we propose that no new types of toxicities are introduced by the change in dosage form into either MTX-CH or MTX-AQ and no remarkable differences are observed between acute toxicities of MTX-CH and MTX-AQ. The MTX in the blood plasma causes systemic toxicities. We had previously reported that the MTX concentration in the blood plasma of rats, given a subcutaneous injection of MTX-CH, decreased slowly in comparison with the rats that were given MTX-AQ (Hagiwara et al., 1994). Since MTX-CH slowly releases free MTX over a long period, the MTX concentration in blood plasma remains at a high level in mice that were given MTX-CH. On the other hand, in the mice that were given the same dose of MTX in the form of MTX-AQ, the MTX concentration in the blood peaked at a high level at an early point in time, and then fell rapidly to a non-toxic level. The toxic effects of MTX depend on the length of its active exposure period rather than on its concentration (Richard et al., 1998). The

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lethal toxicities of MTX were higher in mice that were given MTX-CH as compared to mice that were given the same dose of MTX in the form of MTX-AQ. Therefore, the LD10, 50, and 90 values of MTX-CH were lower than those of MTX-AQ.

As regards the lethal dose, however, the LD50 value of MTX-AQ was only 1.15 times higher than that of MTX-CH. This implies that the lethal toxicity of MTX-CH was only 1.15 times higher than that of MTX-AQ. As regards the efficacious dose against tumors, however, MTX was efficacious at 30 mg/kg in the form of MTX-CH in this therapeutic experiment. The LD50 value of MTX-CH was 75 times higher than the efficacious dose of 30 mg/kg. In our experiments, MTX-CH at 30 mg/kg induced no adverse effects. Further studies for the subacute and chronic toxic effects of MTX-CH need to be performed.

In conclusion, MTX-CH is superior to MTX-AQ in the control of tumor growth by intratumoral administration and can be safely injected subcutaneously at the efficacious dose of 30 mg/kg.

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Figure Legends

Fig. 1.

(A) The method for calculation of the rate of change of tumors.

∗, ∗∗: Statistical significance of MTX-CH vs. MTX-AQ (∗: $p<0.05$; ∗∗: $p<0.01$).

(B) The results of intratumoral administration therapy (mean ± SE).

(C) The tumors of MTX-CH group at the end of the experiment.

(D) The tumors of MTX-AQ group at the end of the experiment.

Fig. 2.

The MTX concentration in the tumor (mean ± SE).

∗: Statistical significance of MTX-CH vs. MTX-AQ ($p<0.05$).

Fig. 3.

(A) Body weight changes in the mice that were given MTX-CH at doses of 1388 mg/kg, 2000 mg/kg, and 2880 mg/kg (mean ± SE).

(B) Body weight changes in the mice that were given MTX-AQ at doses of 1388 mg/kg, 2000 mg/kg, and 2880 mg/kg (mean ± SE).

(C) Body weight changes in the mice that were given only CH40, only physiological saline, or nothing (mean ± SE).

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Fig. 4.

(A) Microscopic view of the kidney. Necrosis of the renal tubules, particularly of the proximal convoluted tubules may be widespread throughout the kidney. Most of the epithelium lining the collecting tubules has necrosed and sloughed into the lumen. The surviving cells have made considerable attempts at repair and the tubules are already lined by flat elongated cells. Hematoxylin-eosin X 400.

(B) Microscopic view of the liver. Several hepatocytes around the central vein are in different stages of fatty degeneration or necrosis. Hematoxylin-eosin X 400.

(C) Microscopic view of the small intestine. The mucosal layer is flattened and the villi are much shorter and wider than normal. Hematoxylin-eosin X 200.

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TABLE 1
Mortality of mice

(A) Mortality of MTX-CH group

Dose of MTX-CH (mg/kg)	No.of dead mice/no.mice tested (mortality rate in %)	Date of death (days after administration)
1388	0/8 (0)	-
1666	0/8 (0)	-
2000	1/8 (12.5)	7
2400	5/8 (62.5)	7,7,7,8,8
2880	8/8 (100)	7,7,7,8,8,9,9,9

(B) Mortality of MTX-AQ group

Dose of MTX-AQ (mg/kg)	No.of dead mice/no.mice tested (mortality rate in %)	Date of death (days after administration)
1388	0/8 (0)	-
1666	0/8 (0)	-
2000	1/8 (12.5)	5
2400	2/8 (25)	5,7
2880	6/8 (75)	4,4,5,5,5,6

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TABLE 2
Lethal dose values

(A) Lethal dose values of MTX-CH

Lethal probability (%)	Dose (mg/kg)	95% CI (mg/kg)
10	1998.7	1557.9 – 2162.1
50	2288.5	2093.8 – 2515.0
90	2620.2	2417.8 – 3404.8

(B) Lethal dose values of MTX-AQ

Lethal probability (%)	Dose (mg/kg)	95% CI (mg/kg)
10	2260.6	1564.5 – 2464.4
50	2637.8	2395.8 – 3023.9
90	3078.0	2795.2 – 4869.8

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TABLE 3
Organ weight changes in dead mice and control

Mean weight of organ(g)[mean organ weight / body weight (mg/g)]

Dose (no. of mice)		Heart	Lung	Liver	Kidney	Spleen
Control (n=8)		0.243[0.91]	0.159[0.59]	1.628[6.03]	0.581[2.15]	0.056[0.21]
MTX-CH	2000 mg/kg(n=1)	0.189[1.05]	0.142[0.79]	0.884[4.91]	0.318[1.76]	0.038[0.21]
	2400 mg/kg(n=5)	0.191[0.98]	0.139[0.71]	0.998[4.99]	0.334[1.88]	0.037[0.18]
	2880 mg/kg(n=8)	0.181[1.01]	0.133[0.74]	0.826[4.59]	0.324[1.81]	0.028[0.16]
MTX-AQ	2400 mg/kg(n=2)	0.188[1.04]	0.134[0.74]	0.899[4.99]	0.326[1.82]	0.038[0.18]
	2880 mg/kg(n=6)	0.186[1.05]	0.135[0.76]	0.854[4.85]	0.331[1.88]	0.035[0.19]

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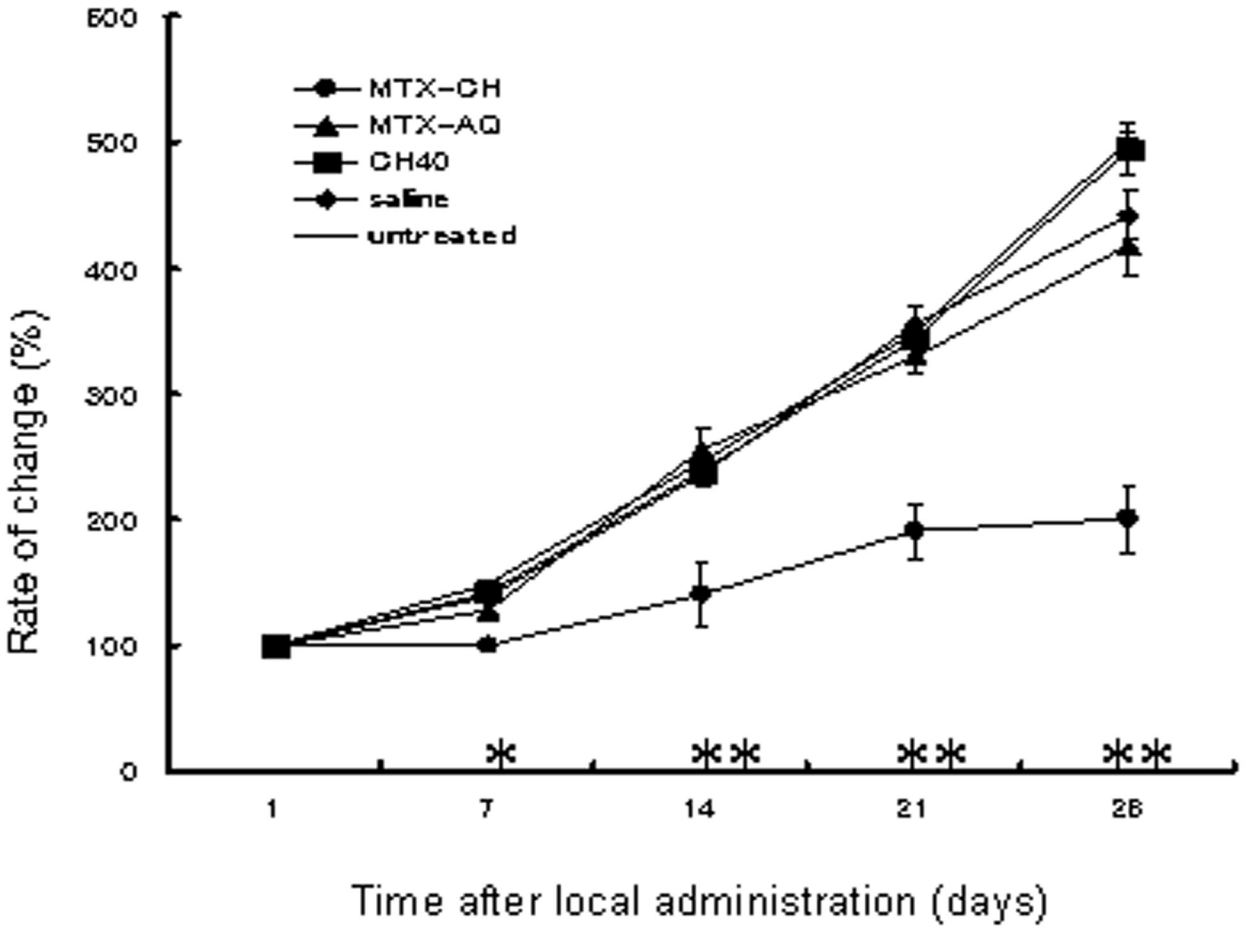
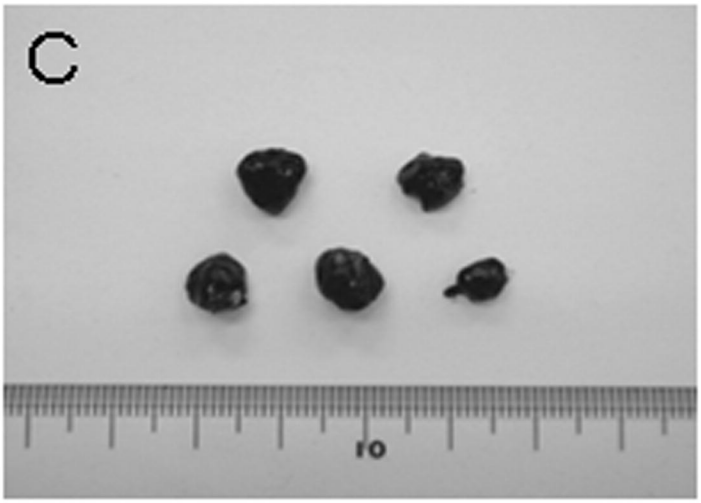
Fig. 1.

A

$$\text{Rate of change}(\%) = \frac{\text{Major} \times \text{minor axis at that time}}{\text{Major} \times \text{minor axis on Day 0}} \times 100$$

B





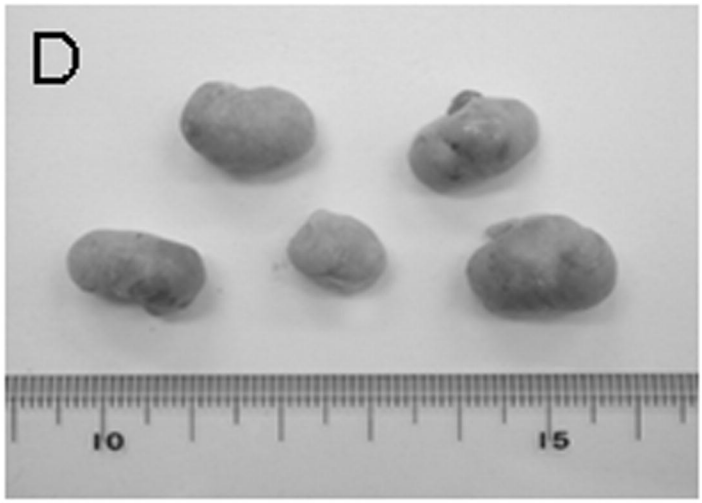


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Fig. 2.

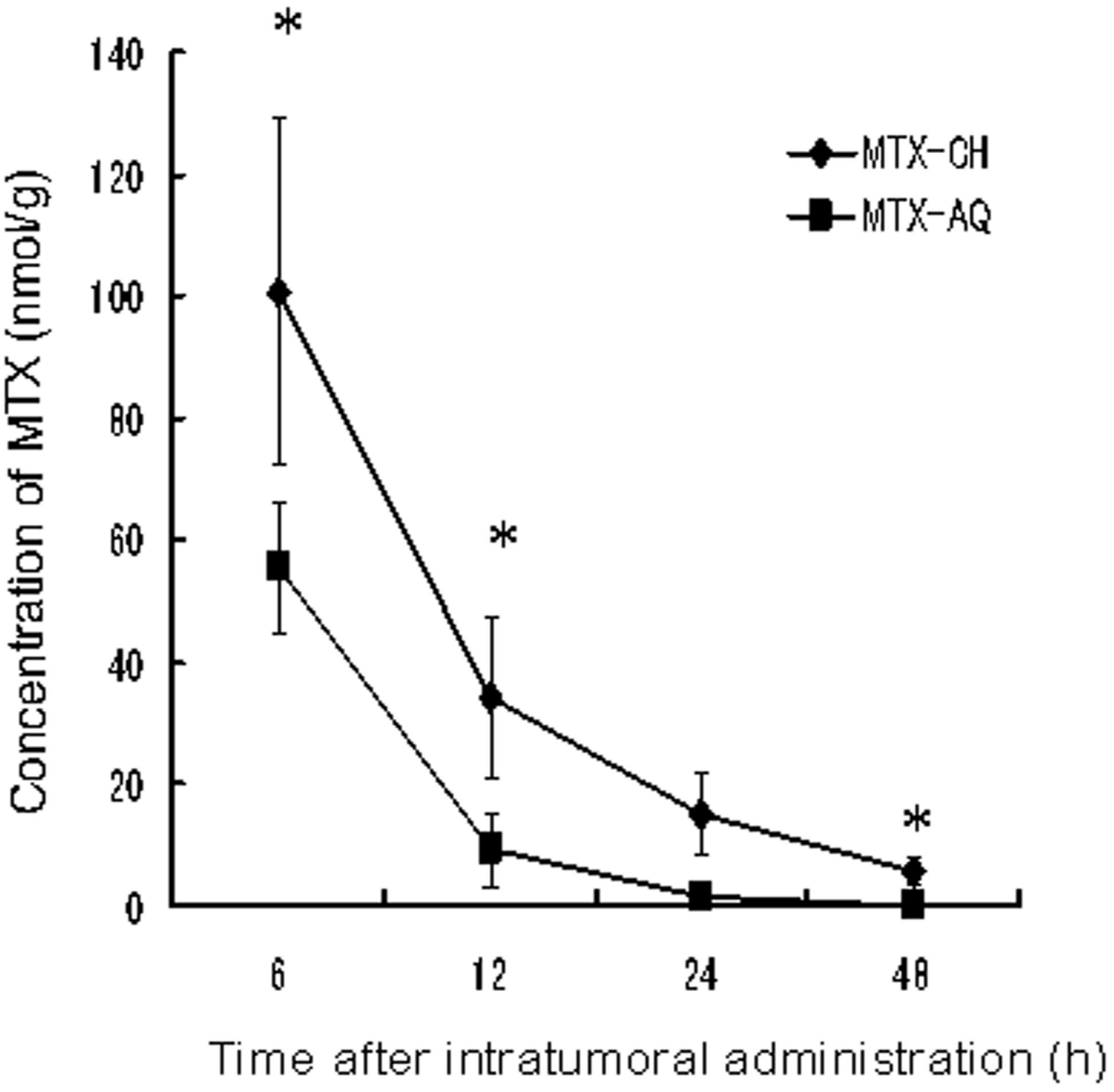

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Fig. 3.

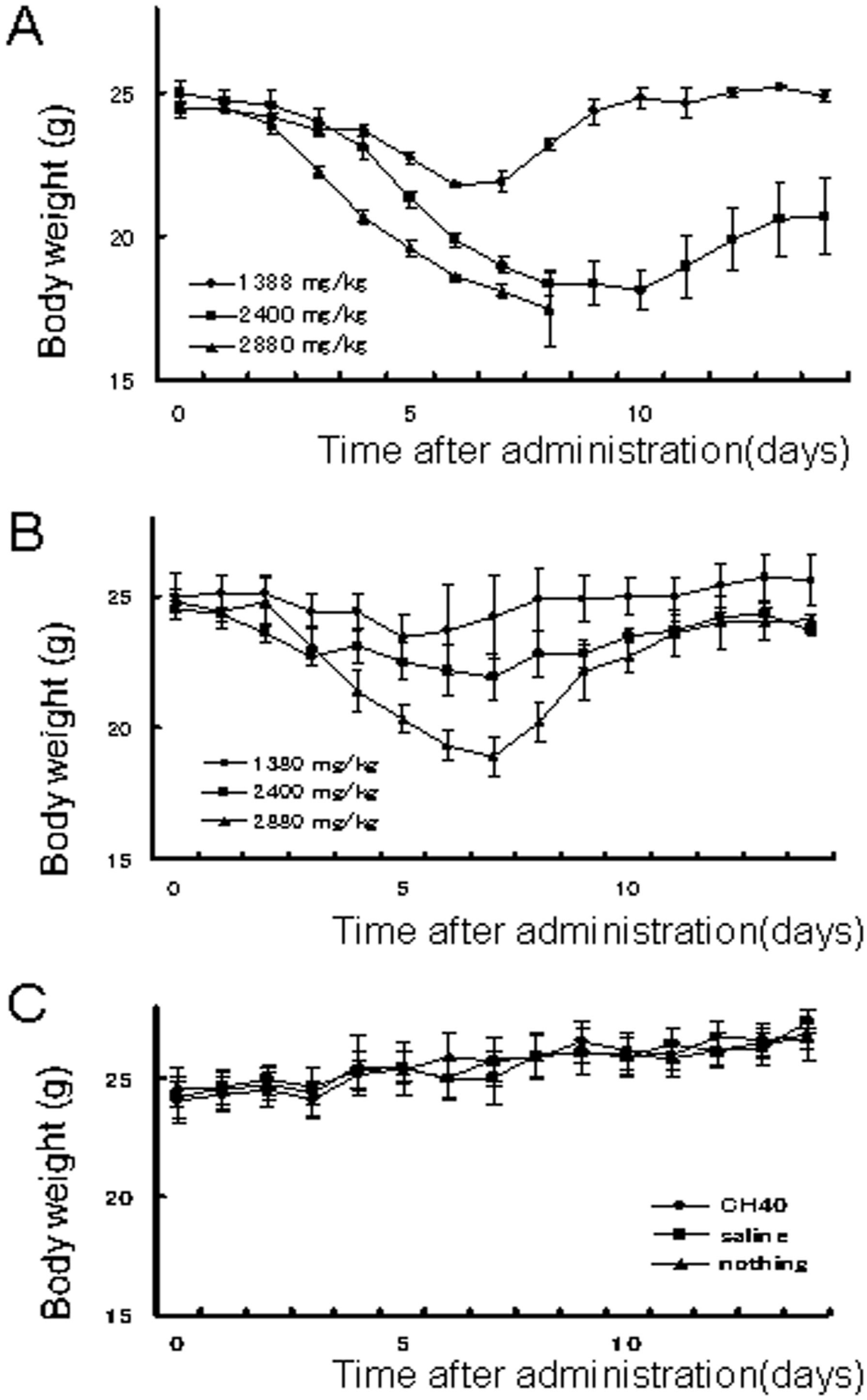

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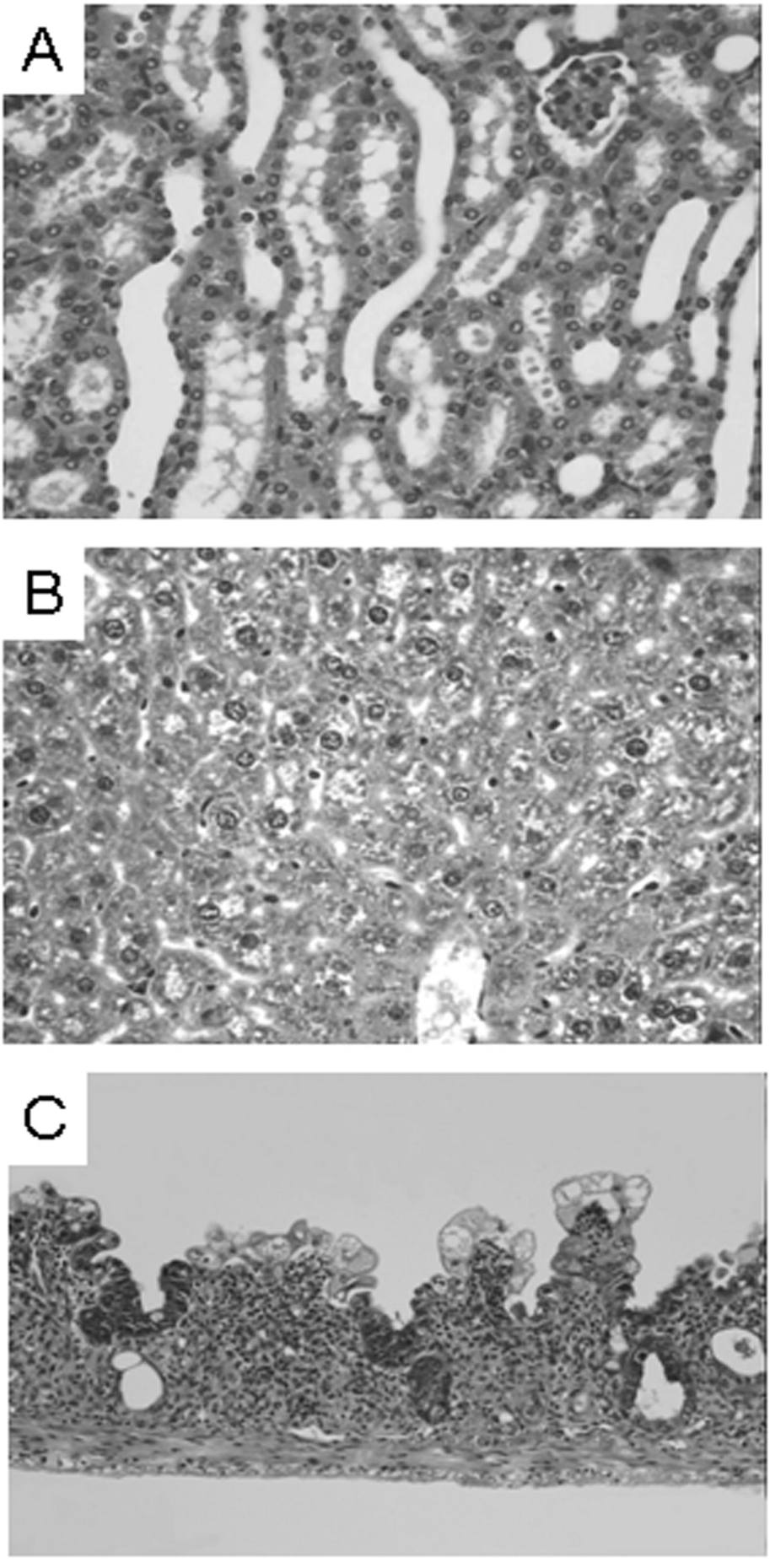
Fig. 4.



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