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Dose-dependent mortality involving convulsions due to subarachnoid Urografin® injection in rats

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ABSTRACT

An ionically hypertonic contrast medium Urografin® was inadvertently administered into the subarachnoid space of an individual and this resulted in convulsions and acute respiratory failure. We examined the effects of subarachnoid Urografin® injections on the rat central nervous system. The onset and frequency of the convulsions, as well as fatality, were dependent on the amount of Urografin® administered. No convulsions were observed in rats receiving injections of hypertonic NaCl solution or saline. The results confirmed that subarachnoid injections of Urografin® cause convulsions and death, as previously reported in human cases, and our study ascertained the causal relationship between the above malpractice and fatal outcomes.

1. Introduction

Myelography is a type of radiographic examination that is routinely performed using a non-ionic contrast medium to detect pathologies of the spinal cord [1]. However, inadvertent administration of Urografin®, which is an ionically hypertonic contrast medium used for retrograde urography and percutaneous cholangiography, into the subarachnoid space results in convulsions and acute respiratory failure [2]. These effects can lead to death if they are not treated immediately. The cases of eight survivors and three mortalities have been reported in the literature [2–10]. The names of the products used in these cases were different, but the main active ingredient in all of the products was meglumine amidotrizoate, or Urografin® (Table S1). The victims were subjected to forensic autopsy as part of the protocol for medical malpractice cases.

The mechanisms underlying the adverse effects of subarachnoid meglumine amidotrizoate injections on the central nervous system (CNS) are unclear. Previous autopsy cases indicated the presence of slight brain edema, but have not led to specific findings in the CNS [9,10]. We hypothesized that neurotoxicity of meglumine amidotrizoate and/or dehydration of central nervous tissue by the hypertonic solution may have possible adverse mechanisms, although no previous study has addressed these possibilities. Obtaining information regarding the effects of meglumine amidotrizoate on the CNS is essential for forensic autopsies of victims and is needed to prove a causal connection between medical malpractice and death. Because some patients survived after a subarachnoid meglumine amidotrizoate injection, the factors that determine a fatal outcome should also be identified to help in the evaluation of emergency medical treatments used after inadvertent administration.

It is therefore important to elucidate the changes induced in the CNS by meglumine amidotrizoate using an animal model. We used Urografin® to induce CNS lesions in rats and analyzed the resulting neurological and histological changes to assess the impact of meglumine amidotrizoate on the CNS.

2. Materials and methods

2.1. Animals and surgery

The Ethical Review Committee of Animal Experiments at Tokyo Women's Medical University approved the animal procedures. Male Wistar rats weighing 400–500 g were allocated randomly to one of seven groups. The Urografin® groups were injected with 10 μL (n = 3), 7.5 μL (n = 6, including 3 dead animals), or 5 μL (n = 3) of 60% Urografin® (Bayer Yakuhin, Ltd.; Osaka, Japan). The hypertonic sodium chloride (NaCl) solution group was treated with 7.5 μL NaCl solution with an osmolar ratio of 6 relative to saline (n = 3). The saline group was treated with 7.5 μL Isotonic Sodium Chloride Solution "Hikari" (Hikari Pharmaceutical Co., Ltd.; Tokyo, Japan) (n = 3). The sham group only underwent catheter insertion (n = 3) and the control group received no intervention (n = 3). The main active ingredient of Urografin® is meglumine amidotrizoate, which is an ionically hypertonic contrast medium with an osmolar ratio of 6 relative to saline. Thus, we used hypertonic NaCl solution to determine whether the adverse effects of Urografin® were caused by neurotoxicity of the

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ingredient or by the hypertonicity of the medium.

Rats were anesthetized using an intraperitoneal (i.p.) injection of pentobarbital (40 mg/kg) and 3% isoflurane (Wako Pure Chemical Industries, Ltd.; Osaka, Japan) delivered using a facemask and an inhalation anesthesia apparatus (Narcobit-E; Natsume Seisakusho, Tokyo, Japan). Subarachnoid administration was performed as described in the Technical Notes section of "Recommendations for Intrathecal Administration, rev 3.00e" (Primetech Corporation, Tokyo, Japan), with some modifications [11,12]. After hair on the occipital and cervicodorsal areas was shaved, we cut open the occipital scalp and the cervicodorsal skin and incised the muscles to expose the cisternal membrane. A 24-gauge Surflo® I.V. Catheter (external and internal diameters of 0.7 and 0.47 mm, respectively; Terumo Corporation; Tokyo, Japan) was inserted through the cisternal membrane into the cisterna magna and the subarachnoid space. The reagents were injected into the subarachnoid space using a microsyringe (SGE Analytical Science Pty., Ltd.; Ringwood, Victoria, Australia). The muscle, occipital scalp, and cervicodorsal skin were sutured following the injection. We confirmed that the drugs were in the subarachnoid space using 1% Evans blue dye. The rat's rectal temperature was maintained at 37 °C using a warming pad and a feedback probe connected to a Small Animal Warmer and Thermometer (Bio Research Center Co., Ltd.; Aichi, Japan) during the operation.

Convulsions were monitored using a video recorder (Sony HDR-AS15; Sony Corporation, Tokyo, Japan), and the number of convulsions was recorded over a 5-min period.

2.2. Sample preparation

Samples for histological analyses were obtained from rats receiving injections of 7.5 μL Urografin® (n = 3, survivors), hypertonic NaCl solution (n = 3), or saline (n = 3), and rats in the sham (n = 3) and control (n = 3) groups. The rats were sacrificed under anesthesia using 150 mg/kg pentobarbital (i.p.) and 3% isoflurane (Wako Pure Chemical Industries) delivered using the facemask of an inhalation anesthesia apparatus (Narcobit-E) 48 h after surgery. The brain and spinal cord were removed, post-fixed in 4% paraformaldehyde phosphate buffer solution (Wako Pure Chemical Industries) at 4 °C for 2 days, and immersed in a 15% sucrose solution for one day. The brains were then placed in a 30% sucrose solution for another 2 days at 4 °C. The brains and spinal cords were cut coronally into 4 mm-thick sections using a rat brain slicer (Neuroscience Inc., Tokyo, Japan). The coronal brain and spinal cord sections were processed using an Optimal Cutting Temperature (OCT) compound (Tissue-Tek; Sakura Finetek Japan Co., Ltd., Tokyo, Japan), embedded, sectioned (10-μm thickness), and mounted on siliconized slides (Matsunami Glass Ind., Ltd., Tokyo, Japan) [13].

2.3. Histological analysis

Histological analysis was performed on one section from each region for each stain. We analyzed sections from the hippocampus (−3.30 mm from bregma), pons (−10.04 mm from bregma), medulla, and the second cervical, sixth thoracic, and second lumbar spinal cord. Coronal sections were stained with hematoxylin and eosin (H & E) and Nissl substance.

Four antibodies were used for immunohistochemistry: a polyclonal antibody against ionized calcium binding adaptor molecule 1 (Iba 1, 019-19741; Wako Pure Chemical Industries; 1:4000) as a marker of microglia; a monoclonal antibody against glial fibrillary acidic protein (GFAP, MAB3402; Millipore, Tokyo, Japan; 1:20,000) as a marker of astrocytes; a polyclonal antibody against transient receptor potential cation channel, subfamily V, member 4 (TRPV4, ACC-124; Alomone Labs, Jerusalem, Israel; 1:600); and a polyclonal antibody against aquaporin 4 (AQP4, AQP-004; Alomone Labs; 1:15,000). TRPV4 is an osmotic sensor and functions in the transduction of osmotic stimuli [14]. AQP4 is a water channel in the brain and is downregulated following injections of hypertonic solutions [15]. The sections were incubated in the primary antibodies at 4 °C overnight and then incubated at room temperature for 30 min in the presence of a universal immunoperoxidase polymer for rat tissue sections for anti-rabbit or anti-mouse primary antibodies (Histofine Simple Stain Rat MAX PO [Multi]; Nichirei Biosciences Inc.; Tokyo, Japan). This was followed by enzymatic development using 3,3′-diaminobenzidine (DAB) and counterstaining with hematoxylin. To optimize the experimental conditions and reduce variability, all sections were treated in the same manner, which enabled us to perform semiquantitative measurements of the different stains [16].

Sections stained for each antibody were photographed using a camera attached to a light microscope (FSX100; Olympus Corporation, Tokyo, Japan). Areas measuring 0.44 mm × 0.32 mm (length × width) and 0.11 mm × 0.08 mm (length × width) from each color image were scanned.

To count Iba 1-immunopositive microglial cell bodies, photomicrographs were obtained using the stitching function of the camera and the light microscope (FSX100) according to the manufacturer's protocol. The stitching function was carried out on 5 × 5 pictures and a 1.98 mm × 1.55 mm (length × width) area at the center of each region was scanned as a color image. We used ImageJ to count Iba 1-immunopositive microglial cell bodies in the color images [17,18]. First, we separated the nuclei stained by hematoxylin and the microglia stained by DAB in the color images using the Color Deconvolution plugin. We then used the 'adjust', 'threshold', and 'analyze particles' commands in ImageJ for each color. The 'analyze particles' settings were as follows: sizes of 20-infinity for the hematoxylin-stained nuclei and 400-infinity for the activated microglia indicated by the DAB staining. Circularities for both colors were set to 0.00–1.00. Iba 1-immunopositive microglia were classified as one of two distinct morphological phenotypes. Cells with a ramified morphology exhibiting long and thin processes were considered as deactivated. Cells with amoeboid morphology and enlarged cell bodies were considered activated [19]. Using the above method, we counted activated microglia with cell bodies larger than a predetermined size. We also determined the total numbers of cell nuclei present. We then divided the numbers of activated microglia by the numbers of nuclei [20].

Apoptotic cells in sections obtained from each region were detected using an *in situ* Apoptotic Detection kit (Takara Bio Inc.; Shiga, Japan) according to the manufacturer's protocol. This kit utilizes the terminal deoxynucleotidyl transferase-mediated dUTP biotin nick-end labeling (TUNEL) method. Apoptotic cells were photographed using a camera attached to a light microscope (FSX100). Areas of 0.11 mm × 0.08 mm (length × width) from color images containing the apoptotic cells were scanned and the numbers of apoptotic cells were recorded.

2.4. Statistical analysis

All values are reported as the mean ± standard deviations. A two-way analysis of variance (ANOVA) was used to assess differences among the cerebrum, pons, medulla, and the second cervical, sixth thoracic, and second lumbar spinal cord sections. We also assessed differences among the control, sham, saline, hypertonic NaCl solution, and Urografin® groups. If we found significant differences, we followed our analysis with a Bonferroni post hoc test. Differences were considered significant when p values were less than .05.

3. Results

3.1. Subarachnoid injection of reagents

We confirmed that the reagents were in the subarachnoid space. The brain and spinal cord sections were stained with Evans blue following the administration of Urografin® containing 1% Evans blue dye (Fig. 1).