Ann Med Res

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Gadolinium chelates inhibit intracellular calcium signaling in rat trigeminal ganglion neurons similar to rat dorsal root ganglion neurons, but less effective

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ARTICLE INFO

Keywords:
Gadolinium
Contrast media
Trigeminal ganglion
Dorsal root ganglion
Calcium signaling

Received: Jun 17, 2022
Accepted: Oct 11, 2022
Available Online: 23.11.2022

DOI:
10.5455/annalsmedres.2022.06.183

Abstract

Aim: Gadolinium (Gd^{3+}) based magnetic resonance (MR) contrast agents (GBMCA) are manufactured with a chelation process, where organic ligand molecules form a stable complex around Gd^{3+}. The aim of the present study was to investigate the possible impact of free gadolinium and GBMCA in (trigeminal ganglion (TG) and dorsal root ganglion (DRG)) neuron cell cultures on intracellular calcium (Ca^{2+}) since these agents could dissociate and lead to the release of toxic Gd^{3+} ions in metabolic conditions.

Materials and Methods: Ganglion cells were examined for drug responses and high KCl^{+} nonspecific depolarization in Fura 2 AM-loaded neurons by monitoring changes in $[Ca^{2+}]i$ using a digital microscopic image analysis system. GBMCA and molecular gadolinium doses of 0.1 mmol/kg were prepared at the same pH and temperature and applied to the cells.

Results: Application of Gd^{3+}, gadobenate dimeglumine, gadobutrol, and gadodiamide to rat ganglia neurons led to significant decreases in basal intracellular calcium levels while gadoterate meglumine administration did not have a significant impact. As expected, Gd^{3+} was more effective when compared to GBMCA. While the impact was almost full with gadodiamide, Gd^{3+} and other GBMCAs had irreversible effects at different levels. The impact of gadoterate meglumine was neglectable when compared to Gd^{3+} and other GBMCAs, although it exhibited slightly gadodiamide-like effects. Compared with rat DRG neurons, the effect of administration of Gd^{3+}, gadobenate dimeglumine, gadobutrol and gadodiamide was lower on TG neurons, whereas gadoterate meglumine had no significant effect on TG and DRG neurons.

Conclusion: In conclusion, gadolinium chelates released gadolinium and irreversibly reduced the intracellular calcium levels in identical TG and DRG cells at different levels.

Introduction

Humans have been exposed to Gd^{3+}-based MR contrast agents (GBMCA) during last three decades due to medical requirements. Since free gadolinium (Gd^{3+}) is extremely toxic, GBMCA is produced by chelation process where organic ligand molecules are complexed with gadolinium. Ligands should provide a highly soluble, stable and therefore nontoxic complex for human consumption. During the ingestion of Gd^{3+}-chelate complexes in the body, certain de-chelation may be observed between Gd^{3+}, ligand and the complex. This depends on the thermodynamic stability constants of Gd^{3+} chelates. Several Gd^{3+} chelates in common clinical use exhibit significant variations in stability constants [1-3]. The nephrogenic systemic fibrosis (NSF) cases that were associated with the stability of these chelates were reported [1].

Intracellular free Ca^{2+} ($[Ca^{2+}]i$) is one of the most important and decisive biological signals and control important events such as excitability, exocytosis, motility, apoptosis, gene transcription and cell differentiation in living cells [4,5]. The intracellular Ca^{2+} induces signals due to a temporary increase in $[Ca^{2+}]I$ associated with the Ca^{2+} concentration gradient and its movement through the protein channels [5,6].

It was demonstrated that gadolinium affects Ca^{2+} binding membrane proteins, inhibits calcium channels, and an inhibitory effect of Gd^{3+} was also described due to its in-

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teraction with Ca^{2+} binding area in the endoplasmic reticulum Ca^{2+} ATPase [6-11].
Neurons located in the trigeminal ganglion (TG) convey somatic sensory information from peripheral tissues to the CNS similar to the dorsal root ganglion (DRG). Certain studies reported that a substantial population of TG neurons and DRG neurons express the same receptors in rats, rabbits, and monkeys. There has been little effort to further investigate the electrophysiological properties of TG neurons, which may be due to the assumption that the electrophysiological properties of TG neurons would be identical to those described for DRG neurons.TG and DRG neurons have been widely used in $[Ca^{2+}]$i analysis due their easy applicability and availability [12-14].
In a recent (our) study, it was demonstrated that Gd^{3+} chelates dissociated in in vivo conditions and inhibited intracellular calcium signaling in DRG and GT1-7 neurons [15,16]. In the present study, we addressed the following question: is there any difference between the effects of GBMCA on TG and DRG neurons?

Materials and Methods

In the present study, changes in intracellular free calcium levels were investigated with ratio-metric intracellular calcium imaging on cultured neurons. All experiments were conducted in Firat University Physiology laboratories.

The local Ethics Committee (Firat University Animal Experiments Ethics Committee, date: 24.03.2011, decision no: 70) approved the study protocol. Short-term primary trigeminal ganglia neuron and dorsal root ganglia neuron cultures were obtained from twenty 1- 2 days old Wistar rats.

Preparation of primary rat TG and DRG neuron cultures

The animals were decapitated and connective tissue surrounding the central nervous system (CNS) is removed to reveal the brain. A transection was made through the pontine flexure to dissect both hemispheres and the midbrain. The hindbrain, including the upper cervical spinal cord, the connective tissue and meninges, was then dissected. Care was taken not to damage the trigeminal ganglia on both sides and their processes. The maxillary process/pad of whiskers was clipped from the remainder of the head without damaging the infraorbital (IO) nerve. The IO nerve crosses through the infraorbital foramen to the mustache pad just below the eye. To avoid damaging the IO nerve, the eyepiece was left intact. Ganglia were extracted and the connective tissue around the TG was removed with sharp needles. The TG were extracted and temporarily stored in the neurobasal culture medium containing B27 (Invitrogen), 5 mM glutamine and penicillin-streptomycin (5000 IU/ml, 5000 mg/ml). In addition, the spinal cord and the DRG were extracted at the cervical level., thoracic, lumbar and sacral (˜45-50/pup) and temporarily stored in culture medium. Then enzymatic collagenase (0.125% in culture medium for 13 min) and trypsin (0.25% in PBS for 6 min) treatments were performed on all samples. Cells were mechanically dissociated by grinding and plated onto 12 mm round polydilysinalamine-coated glass coverslips (BD BioCoat, Bedford, MA, USA). Cells were stored in culture medium including nerve growth factor supplementation (NGF 2.5 S, SigmaAldrich, Germany) at 37°C in a humidified 95% air/5% CO_2 incubator (Heracell, Kendro Lab. GmbH, Germany).TG and DRG neurons were incubated for at least 4 h prior to imaging experiments. Cell culture coverslips were used in calcium imaging experiments 3 hours after seeding and up to 24 hours in culture.

Ratiometric intracellular calcium imaging

TG and DRG neurons were loaded with the Ca^{2+}-sensitive dye Fura2-acetoxymethyl ester (Fura 2AM, 1 µM, Invitrogen) for approximately 60 min at 37 °C in a humidified incubator with 5% CO_2. Cells were washed 3 times with free Fura2 standard solution for at least 10 min for de-esterification and removal of unincorporated Fura 2AM. The contents of the imaging bath solution included 130.0 NaCl, 2.0 $CaCl_2$, 1.0 $NaHCO_3$, 5,0 Glucose, 10,0 HEPES, 3,0 KCl und 0,6 $MgCl_2$, (in mM).The pH was adjusted to 7.4 with NaOH. The osmolarity was adjusted to 310-320 mOsm with sucrose. Imaging experiments were performed in a dark room at room temperature. Glass coverslips with Fura2-loaded cells were placed in an imaging/perfusion chamber with a perfusion valve. System Warner Instruments, Hamden, CT, USA and No. 41; which included an inverted microscope (Nikon TE2000S, Japan).The volume of the bath chamber was 600 µl.

Fura2-loaded TG and DRG cells were alternately illuminated at 340 nm and 380 nm with a 175 W ozone-free xenon lamp (Sutter Instruments Co., Novato, CA, USA) optically coupled to the microscope with a liquid conductor. A computer controlled filter wheel (Lambda10; Sutter Instruments, Novato, CA) for switching between the 340 and 380 nm filters in the light path. The light passed through a Sflour 40x objective (Oil: N.30, W.22 mm, Nikon) attached to a Nikon TE2000S inverted microscope and a 510 nm bandpass emission filter (Fura2 filter set, Semrock Brightline, Invitrogen). Finally, the light reached a cooled charge-coupled device (CCD) camera (C474295; Hamamatsu Photonics, Japan). The excitation exposure varied between 150 and 400 ms for each wavelength in each experiment. Image pairs were acquired and digitized every 5 seconds. The advanced imaging software "Simple PCI" (sPCI, Compix, USA) controlled camera, shutter and filter wheel filter switching speed and image acquisition. A computer controlled shutter was used to minimize light bleaching and allow cells to be exposed to excitation light only when needed for imaging. Analysis of intracellular calcium concentration and associated calculations were performed offline using image processor and data analysis software (sPCI, Hamamatsu Photonics). Time-dependent the fluorescence intensity of individual cells in the selected region of interest was determined using the imaging software.

An estimate of $[Ca^{2+}]$i was calculated based on the ratio of fluorescence intensity at 340/380 nm (corrected by subtracting background fluorescence) and expressed as the ratio (F340/F380). Results are calculated as normalized fluorescence ratio (NFR). The maximum Fura-2 emission ratio (340/380 nm) response to drug delivery was compared as a percentage of the initial potassium chloride

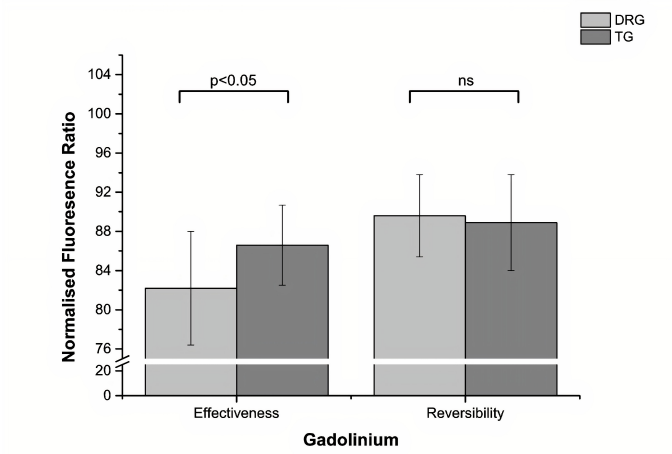

Figure 1. The effects of the gadolinium on intracellular calcium in rat TG and DRG neurons.

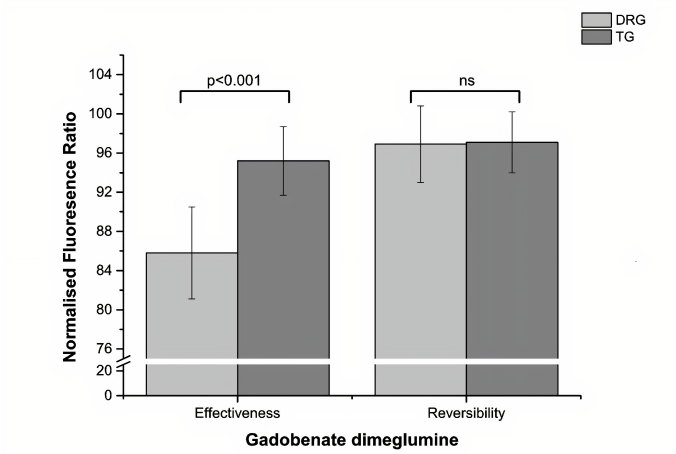

Figure 2. The effects of the gadobenate dimeglumine on intracellular calcium in rat TG and DRG neurons.

(KCl^+) stimulation for each cell. the data directly for each cell. Responses with different KCl^+ agents were obtained from different cells.In TG and DRG neurons loaded with Ca^{2+}-sensitive Fura 2 AM dye, intracellular free calcium levels increased in response to high KCl^+ stimulation (30 mM). Calcium signals were also calculated as peak values of TG and DRG neurons compared to initial KCl^+ stimulation. Gd3+ and GBMCA reduced intracellular free calcium levels in response to KCl^+.Fura-2 AM was procured from Invitrogen (Switzerland). Fura-2-AM was dissolved in dimethyl-sulfoxide (DMSO). The final DMSO concentration in bathing solution did not exceed 0.2 % (v/v), which did not elicit any change in $[Ca^{2+}]i$ by itself in the control experiments. Gadolinium (Gadolinium ICP/DCP standard solution 10,006 µg/mL Gd3+ in 2 % HNO_3, 100 ml PN: 356220) was procured from Fluka (Taufkirchen, Germany). Global marketing agents such as gadodiamide (Omniscan™ 0.5 mmol/ml, Amersham Health, Cork, Ireland), gadoterate meglumine (Dotarem® 0.5 mmol/mL, Guerbet, Aulnay sous Bois, France), gadobutrol (Gadovist® 1.0 mmol/mL, Bayer Schering Pharma AG, Berlin, Germany) and gadobenate dimeglumine (MultiHance® 0.5 mmol/mL, Bracco S.p.A., Milan, Italy) were obtained. All agents were dissolved in the imaging bath solution and aliquots were frozen. Each stock solution was diluted to the required concentration (5 mM) few minutes before the bath application. It was assumed that the dose was equal to the in vivo concentration of chelates. Due to the use of in vivo agent exposure doses, single application was conducted. All agents were administered in the imaging bath solution for a total of 1 min.

Statistical analysis

Data are expressed as mean ± standard deviation. Differences between the means were analyzed with the one-way ANOVA using SPSS for Windows v.22.0 software (SPSS Inc., Michigan, IL, USA). The difference was accepted as significant when $p<0.05$.

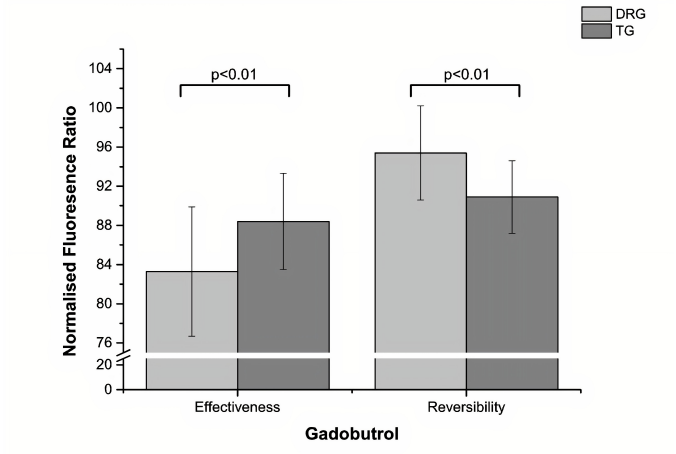

Figure 3. The effects of the gadobutrol on intracellular calcium in rat TG and DRG neurons.

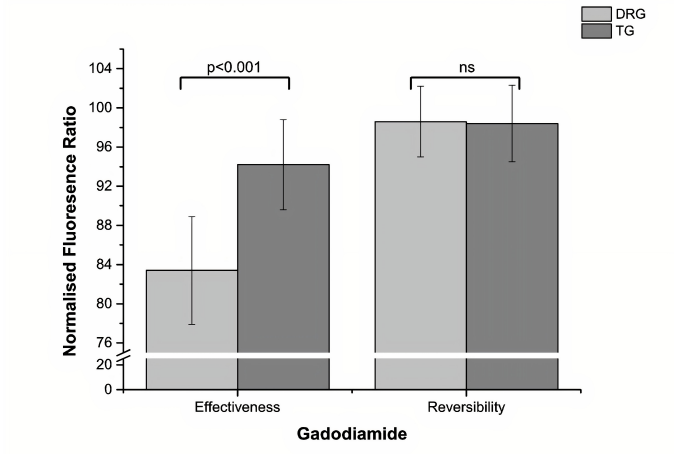

Figure 4. The effects of the gadodiamide on intracellular calcium in rat TG and DRG neurons.

Results

Gd^{3+} (% 86.6±4.1; $p<0.001$), gadobenate dimeglumine (% 95.2.8±3.5; $p<0.001$), gadobutrol (% 88.4±4.9; $p<0.001$)

Table 1. The effects of the gadolinium and gadolinium chelates on intracellular calcium in rat TG neurons when compared to initial (basal) KCl+ stimulation (100%).

Agent	Normalized fluorescence rate			P			N
	Basal KCl	Agent + KCl	Control KCl	Basal/Agent	Agent/Control	Basal/Control	
Gadolinium	100.0 ± 0.0	86.6 ± 4.1	88.9 ± 4.9	<0.001	NS (0.159)	<0.001	11
Gadobenate dimeglumine	100.0 ± 0.0	95.2 ± 3.5	97.1 ± 3.1	<0.001	NS (0.127)	<0.001	15
Gadobutrol	100.0 ± 0.0	88.4 ± 4.9	90.9 ± 3.7	<0.001	NS (0.069)	<0.001	21
Gadodiamide	100.0 ± 0.0	94.2 ± 4.6	98.4 ± 3.9	<0.001	<0.01 (0.004)	NS (0.235)	14
Gadoterate meglumine	100.0 ± 0.0	99.4 ± 1.1	99.8 ± 1.2	NS (0.057)	NS (0.198)	NS (0.516)	19

NS: non-significant.

Table 2. The Effectiveness and Reversibility of the effects of gadolinium and gadolinium chelates on intracellular calcium in rat TG and DRG neurons when compared to initial (basal) KCl+ stimulation (100%).

Agent	DRG		TG		P		N	
	Effectiveness	Reversibility	Effectiveness	Reversibility	Effectiveness	Reversibility	DRG	TG
Gadolinium	82.2 ± 5.8	89.6 ± 4.2	86.6 ± 4.1	88.9 ± 4.9	<0.05 (0.042)	NS (0.699)	15	11
Gadobenate dimeglumine	85.8 ± 4.7	96.9 ± 3.9	95.2 ± 3.5	97.1 ± 3.1	<0.001	NS (0.857)	47	15
Gadobutrol	83.3 ± 6.6	95.4 ± 4.8	88.4 ± 4.9	90.9 ± 3.7	<0.01 (0.007)	<0.01 (0.002)	21	21
Gadodiamide	83.4 ± 5.5	98.6 ± 3.6	94.2 ± 4.6	98.4 ± 3.9	<0.001	NS (0.855)	57	14
Gadoterate meglumine	98.7 ± 3.4	99.3 ± 2.3	99.4 ± 1.1	99.8 ± 1.2	NS (0.394)	NS (0.394)	25	19

NS: non-significant.

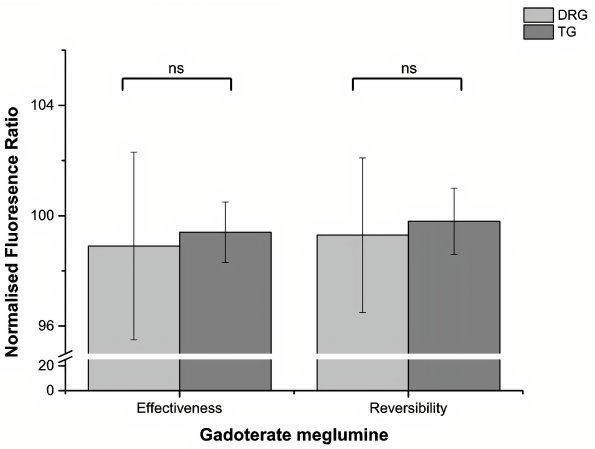

Figure 5. The effects of the gadoterate meglumine on intracellular calcium in rat TG and DRG neurons.

and gadodiamide (% 94.2±4.6; p<0.001) administration to rat TG neurons loaded with Ca^{2+}-sensitive dye Fura 2 AM led to significant decreases in basal intracellular calcium levels, while gadoterate meglumine did not have a significant impact (% 99.4±1.1; p=0.057). As expected, Gd^{3+} was more effective when compared to GBMCA in inducing a decrease in intracellular calcium levels. The effect of reversibility was roughly complete with gadodiamide (% 98.4±3.9; p=0.235). Gd^{3+} (% 88.9±4.9; p<0.001), gadobenate dimeglumine (% 97.1±3.1; p<0.001) and gadobutrol (% 90.9±3.7; p<0.001) exhibited irreversible effects at various levels. The effect of gadoterate meglumine (% 99.8±2.1; p=0.516) was neglectable when compared to Gd3+ and other GBMCA.

When compared to rat DRG neurons similarly loaded with the Ca^{2+}-sensitive dye Fura 2 AM, the impact of Gd3+ (% 82.2±5.8) (p<0.05) (Figure 1), gadobenate dimeglumine (% 85.8±4.7 (p<0.001) (Figure 2), gadobutrol (% 83.3±6.6) (p<0.01) (Figure 3) and gadodiamide (% 83.4±5.5) (p<0.001) (Figure 4) administration was lower on the TG neurons, while gadoterate meglumine (% 98.7±3.4) did not have a significant impact on TG and DRG neurons (p=0.394) (Figure 5).

The reversibility effects of agents on TG neurons were not significant for Gd^{3+} (% 89.6±4.2) (p=0.699), gadobenate dimeglumine (% 96.9±3.9) (p=0.857), gadodiamide (% 98.6±3.6) (p=0.855) and gadoterate meglumine (% 99.3±2.3) (p=0.394) except gadobutrol (% 95.4±4.8) (p<0.01) when compared to DRG. The duration of the effect of gadobutrol was similar to that of Gd3+. All findings are presented in Tables 1 and 2.

Discussion

Based on the stability constants and trans-metalation kinetics, these agents (GBMCA) can dissociate, leading to the release of toxic Gd^{3+} [1].

In the present study, we demonstrated this phenomenon with the free gadolinium effect on TG and DRG neurons via the fluorescence ratio-metric intracellular calcium imaging method. The divalent cation calcium (Ca^{2+}) is used by cells as a second messenger to control various physiological and pathophysiological cellular processes including reproduction, muscle contraction, secretion, metabolism, neuronal excitability, embryogenesis, immunization, wound healing, tumor metastasis, cell proliferation, migration, apoptosis and necrosis. This important event is realized through the Ca^{2+} entry route and it could be blocked by Gd^{3+} [17-20].

In healthy subjects, passive glomerular filtration does not

alter the excretion of soluble Gd^{3+} chelates within 1-5 hours, but their clearance is reduced and their half-life may exceed 30 hours in patients with renal failure. [1].
Cacheris et al. clearly demonstrated that the amount of Gd released, and not the total dose of a single Gd-chelated agent, determines the toxic outcome [21]. High thermodynamic stability constants, measured in water, are neither necessary nor sufficient conditions for in vivo stability [22]. The GBMCA dissociation rates are low at pH 7.4 and the complexes dissociate much faster in acidic conditions [23]. However, different figures have been reported in the literature [3,23].
We investigated the differences between the effects of GBMCA on the intracellular calcium in cultured TG and DRG neurons in in vitro conditions in the present study, due to the recent reports about the inhibitory effects of these agents on DRG neurons.
In the current study, we observed that intracellular calcium levels decreased due to the inhibition of calcium flow by gadolinium chelates in TG neurons. The effects of the agents on TG neurons were similar but not significant when compared with DRG neurons. The effects of gadodiamide, gadobenate dimeglumine, and gadobutrol were reversible in both TG and DRG neurons, however, the effects of gadobutrol on TG neurons were not significant when compared to the DRG neurons based on effectiveness and reversibility. It was determined that effects of gadoterate meglumine on intracellular calcium were insignificant in both TG and DRG neurons.

Gadolinium chelates releases gadolinium and irreversibly reduces the intracellular calcium content similar to TG and DRG cells at different levels. These chelates may release gadolinium in physiological conditions and subsequently induce changes in intracellular calcium levels and could lead to possible toxic effects. The differences between their effects on different cells may lead to different outcomes in human physiology. This is consistent with the apparently higher incidence of NSF cases associated with one chelate compared to the other molecules.

For pathophysiologic mechanisms of this effect and possible therapeutic approaches, further comprehensive studies are needed. Future studies that would compare various GBMCA, various physiological conditions and the same GBMCA, and various cell types are required to identify all structures–activities-correlations and any pathophysiologic effects.

Acknowledgements

The present study was sponsored by Firat University Scientific Research Projects Department (FUBAP Project No: 1972).

Declaration of interest

The authors contributing to this research declare that there is no conflict of interest that could harm the objectivity of the research.

Data availability

Data supporting the results of this study are available from the corresponding author upon reasonable request.

Author contribution statement

M.B. and H.K. contributed to the design, writing, and literature review of the study, while B.B. and M.O. contributed to the material with the collection and analysis of data.

Funding

This work was sponsored by Firat University Scientific Research Projects Department (FUBAP Project No: 1972).

Ethics approval

Ethical approval for this study was obtained from Fırat University Animal Experiments Ethics Committee (dated 24.03.2011, decision no: 70).

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