



Original Investigation

How Safe is the Use of Intrathecal Fluorescein? An Experimental Study

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ABSTRACT

AIM: To investigate the effects of fluorescein-sodium on neural tissues.

MATERIAL and METHODS: Twenty-one Wistar rats were randomly divided into three experimental groups: control (group 1) and fluorescein-sodium groups with different doses (groups 2 and 3). In the control group, craniectomy following with durotomy was performed with the help of a loupe microscope, and a dry sponge was overlaid to the brain tissue. In the study groups, the open dura was covered with a sponge soaked with 0.02 mg (group 2) and with 0.2 mg (group 3) fluorescein sodium following craniectomy. Three weeks postoperatively, rats were sacrificed for the histopathologic evaluations.

RESULTS: Fluorescein-induced apoptosis occurs in a dose-dependent manner in rats' neurons. It was determined that neuron and neuroglial cell TUNEL staining was statistically different among the three groups ($p < 0.001$). Our results indicated that fluorescein induces apoptosis, resulting in increased nuclear factor kappa beta (NF- κ B) expression in a dose-dependent manner.

CONCLUSION: Fluorescein sodium is used frequently during surgery for CSF fistulas. However, information in the literature about its safety is insufficient. Our study holds promise for the development of new studies on the reliability of this agent.

KEYWORDS: Fluorescein-sodium, Rhinorrhea, Neurotoxic effect, CSF fistula

ABBREVIATIONS: CSF: Cerebrospinal fluid, NF- κ B: Nuclear factor kappa beta, IP: Intraperitoneal, H-score: Histocore, PBS: Phosphate-buffered saline, TdT: Terminal deoxy-nucleotidyl transferase, H&E: Hematoxylin and eosin, AI: Apoptotic index, DAB: 3',3'-diaminobenzidine

INTRODUCTION

Cerebrospinal fluid (CSF) leaks can be caused by many different etiologies, most commonly history of trauma, iatrogenic or nontraumatic bone invasions, empty sella syndrome, congenital bone defects, infections and meningoencephalocele, meningeal diverticula, and dural sac weakness. Diagnosis of CSF rhinorrhea starts with the well-interviewed patient history and continues with the confirmation

of the onset of leakage with the help of laboratory tests. β 2-Transferrin which is also known as Tau protein is an isoform of transferrin and only found in perilymphatic fluid and CSF. With the help of this parameter, some patients who do not have genuine CSF leakage do not undergo unnecessary invasive tests (13). After the detection of β 2-transferrin, patients must undergo endoscopic examination to determine the dural defect. The source of leakage may be from the different parts

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of the cranial fossa; anterior, middle or posterior. Traumatic or incidental fractures are found mostly at the cribriform plate which has adherent duramater (9,10).

Many treatment methods have been defined for management of CSF by neurosurgeons and otorhinolaryngologists. Bed rest, laxative treatment, and lumbar drainage are some defined treatment methods. Some studies report that these leaks carry a meningitis risk as high as 30% and should be repaired (3,4). Although transsphenoidal and transcranial approaches have been defined for repair of dural defects, an endoscopic transnasal transsphenoidal approach is mostly preferred in the anterior skull base. This approach is less invasive and has achieved successful results with low complication rates (12).

Fluorescein sodium is commonly used by ophthalmologists to detect intractable structures of the human eye. At some centers, the same dye is frequently used by neurosurgeons and otorhinolaryngologists to detect CSF fistulas with very successful results (17). We investigated the changes in brain tissue with use of fluorescein and determined its relationship with these complications. Although some complications have been reported, an experimental study showing the histologic effects of fluorescein has not been performed previously to our knowledge. We believe our work will shed light on this issue.

■ MATERIAL and METHODS

Ege University Animal Research Ethics Committee approval was obtained for our experimental study and performed according to the "Guide for the Care and Use of Laboratory Animals" in the Animal Laboratory (ARGEFAR) of Ege University (approval number/date 2017-001/22.02.2017). We used the similar model from our prior study on the neurotoxic effects of povidone-iodine (2). In daily life during the operation, fluorescein was administered intrathecally from the lumbar region of the patient with catheters placed intrathecally in Trendelenburg position, allowing fluorescein to flow into the intracranial compartment. Since the subarachnoid space in mice was quite narrow, the fluorescein could not be confirmed to reach the intracranial compartment and we believed it more appropriate to apply it directly to the intracranial compartment, contacting brain tissue directly in the intracranial subarachnoid area.

Twenty-one adult Wistar albino rats weighing 350 g were housed in separate cages with a standard laboratory conditions. Three groups of 7 rats (4 male, 3 female) were randomly selected. Group 1 was the control group, while rats in groups 2 and 3 were given fluorescein sodium at different doses.

On day 21, rats were anesthetized with an intraperitoneal (IP) ketamine hydrochloride–xylazine combination (5–10 mg/kg) and 15g/kg subcutaneous cefazolin sodium was given prophylactically 1 hour preoperatively. After positioning and fixation, surgery was performed under sterile conditions using a loupe microscope. A linear scalp incision was performed on the right frontal midpupillary line. A burr hole and nearly 1 × 1 cm craniectomy were performed with a lancet and micro-surgical

set. A dural incision was made using microtweezers and a microscalpel. In Group 1, the dural defect was covered with a Spongostan (Ethicon, Inc., Somerville, NJ, USA), a hemostatic agent used in neurosurgical operations, and then the skin was sutured with 3/0 polypropylene. The dural defect was covered with a Spongostan soaked with 0.02 mg (approximately 4.5 mg/75 kg; group 2) and 0.2 mg (approximately 45 mg/75 kg; group 3) fluorescein. Skin was sutured in the same manner. Rats were placed back in their cages separately and housed for 3 more weeks.

Histologic Examination

IP 40 mg/kg ketamine and 4 mg/kg xylazine injection is performed for the anesthesia of the animals and fixed with 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS) perfusion. Hematoxylin and eosin (H&E) staining system was performed to stain the 5 mm sectioned cerebrum paraffin blocks. Photographs of sections were obtained with Olympus C-5050 digital camera (Olympus Optical Co., Tokyo, Japan).

Immunohistochemistry

We used the similar immunohistochemistry protocol of the studies performed by Acikgoz et al. and Oktem et al. for our study (1,14). Motorized rotary microtome (MR-2145; Leica Biosystems, Wetzlar, Germany) was used to cut the 5mm thick sections. Sections were washed with phosphate-buffered saline and treated with 2% trypsin (Sigma-Aldrich Corp., St. Louis, USA) for 15 minutes in 50 µM Tris-buffer following delineation with a Dako-pen (Dako, Glostrup, Denmark). Hydrogen peroxide (H₂O₂) (3%) was used to inhibit the peroxidase activity. Next step was the incubation of sections with the primary antibodies against nuclear factor kappa beta (NF-κβ; 1/100) for 24 hours and then with biotinylated secondary antibody following streptavidin in conjugated to horseradish peroxidase (Zymed Histostain-Plus peroxidase kit; 85-9043; Zymed Laboratories, San Francisco, CA, USA). For the detection of apoptosis sections were incubated with DAB for 5 minutes to observe immune labeling (DAB; Dead-End Colorimetric [TUNEL] system; Promega, Madison, WI, USA). Sections were counterstained with Mayer's hematoxylin (Zymed Laboratories) and were dehydrated with a graded ethanol cleared in xylene, and mounted with the help of Entellan (Merck, Darmstadt, Germany). Three histologists independently assessed the positive cells. Scores were calculated as; 100 cells per 10 view-fields of sections at ×20 magnification and the photographs were obtained using Olympus C-5050 camera.

NF-κβ immunohistochemical semiquantitation was performed using the modified Histoscore which was calculated by the positive cells percentage and a semi-quantitative assessment of the intensity of staining (non-staining: 0, weak: 1, median: 2, strong: 3). The scores may range from 0 to 300. The expression rates were determined as low and high, depending to the Histoscore results.

TUNEL Assay

Deparaffinized sections were incubated with proteinase K (20 µMmL⁻¹) for 10 minutes. H₂O₂ (3%) was used for the inhibition

for the peroxidase activity. Next step was the incubation with equilibration buffer, Terminal deoxynucleotidyl transferase (TdT) enzyme (1 hour) and finally with anistreptavidin-peroxidase for 45-50 minutes. Staining with DAB and counterstaining with Mayers hematoxylin was performed. Quantification of apoptotic cells was performed by at least two independent observers and apoptotic cells were counted with $\times 40$ magnification of a light microscope. Apoptotic index (AI) was defined as apoptotic cell count per section (AI = total apoptotic cell count/100).

Statistical Analysis

Descriptive statistics are reported as median with interquartile range (IQR) values. The variables were investigated using analytical methods (Schapiro-Wilk's test) to determine whether they were normally distributed. Kruskal-Wallis tests were used for statistical comparison of data between groups. To check the significance of pairwise differences The Mann-Whitney U test was performed using correction of Bonferroni to adjust for multiple comparisons. Spearman's correlation and Kendall's Tau-b correlation were run to determine the relationship between Groups 1, 2, and 3, and TUNEL/NF- κ B-positive staining cell number values. In the interpretation of the correlation coefficient, 0.0–0.24 weak, 0.25–0.49 medium, 0.50–0.74 strong, 0.75–1.00 very strong. For statistical significance, $p < 0.05$ was considered as significant in the 95% confidence interval. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL, USA) program, version 21.0.

RESULTS

The median (IQR) numbers of neuroglial cells detected by NF- κ B staining was 45 (6), 28 (2), and 30 (6) in groups 1–3, respectively. The number of neurons stained with NF- κ B was 8 (2), 5 (2), and 3 (1), respectively (Figure 1A-C). The number of neuroglial cells stained with TUNEL were 19 (4), 9 (3), and 2 (1), respectively, while the number of neurons stained with TUNEL were 5 (2), 2 (0), and 1 (1), respectively (Figure 2A-C). Neuron and neuroglial cell staining was statistically different among the three groups in the type of TUNEL staining ($p < 0.001$; Figure 3).

Post hoc analyses of these results showed that the number of neuroglial cells stained with NF- κ B was higher in Group 1 than in the other groups ($p = 0.001$, $p = 0.019$ for groups 2 and 3, respectively). The number of neurons stained with NF- κ B and the number of neurons and neuroglial cells stained with TUNEL were significantly lower in the control groups (both statistically significant, $p < 0.001$; Table I).

Correlation analysis was performed to demonstrate increased toxicity with increasing doses of fluorescein. A very strong and significant correlation was found between the dose increase in the groups and the neuron and neuroglia TUNEL staining in the positive direction ($r = 0.838$, 0.855 ; $p < 0.001$, respectively). Apoptosis rate increased as fluorescein dose increased.

Increased doses of fluorescein showed a moderate strong correlation between NF- κ B-positive stained neuroglial cells

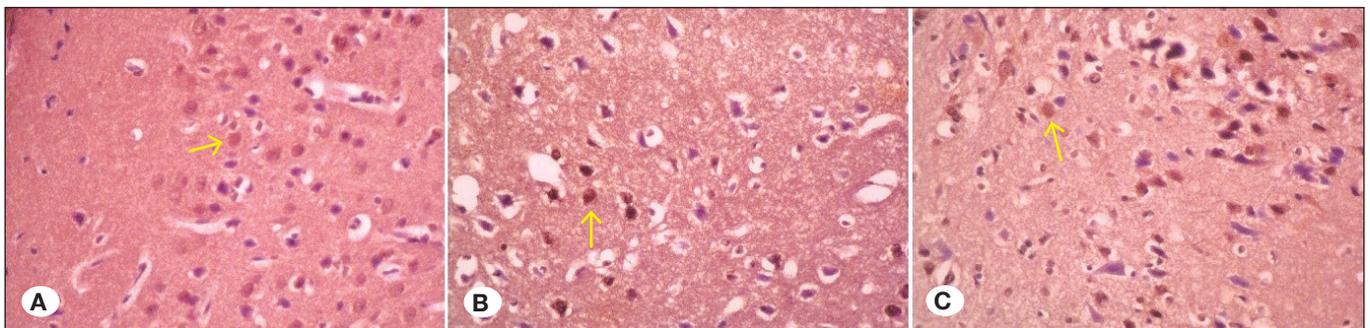


Figure 1: Immunohistochemical image of the change in NF- κ B protein. Positive-stained cell nuclei appear to be stained dark brown. Yellow arrow marks one positive stained cell. Original magnification $\times 40$ (A: Group 1, B: Group 2, C: Group 3).

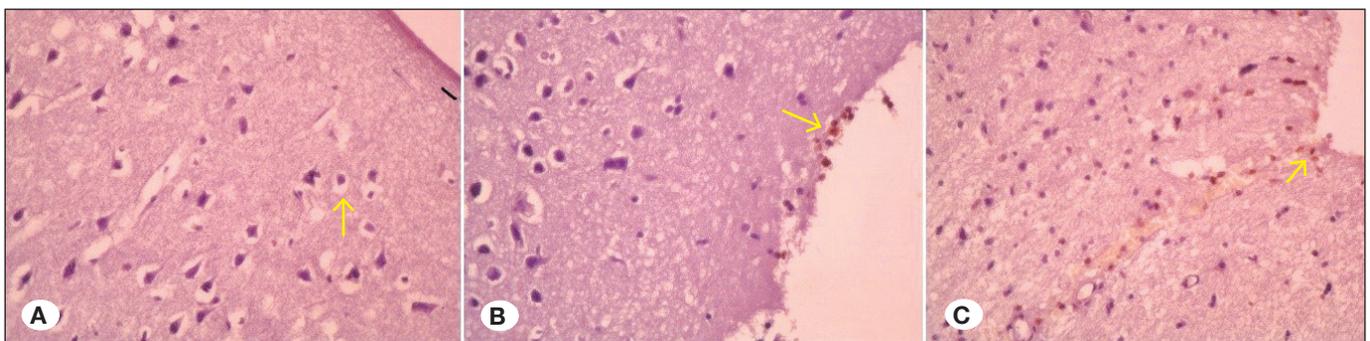


Figure 2: TUNEL assay results. Apoptotic cells appear to have brown-colored cytoplasm (yellow arrow). Original magnification $\times 40$ (A: Group 1, B: Group 2, C: Group 3).

Table I: Statistical Results of the Groups: Median (IQR)

	Group 1	Group 2	Group 3	p	Post hoc p		
					Group 1-2	Group 1-3	Group 2-3
Neuroglia cells stained with NF-κB	45 (6)	28 (2)	30 (6)	0.001	0.001	0.019	1
Neurons stained with NF-κB	8 (2)	5 (2)	3 (1)	<0.001	0.1	<0.001	0.1
Neuroglia cells stained with TUNEL	19 (4)	9 (3)	2 (1)	<0.001	0.102	<0.001	0.102
Neurons stained with TUNEL	5 (2)	2 (0)	1 (1)	<0.001	0.284	0.05	<0.001

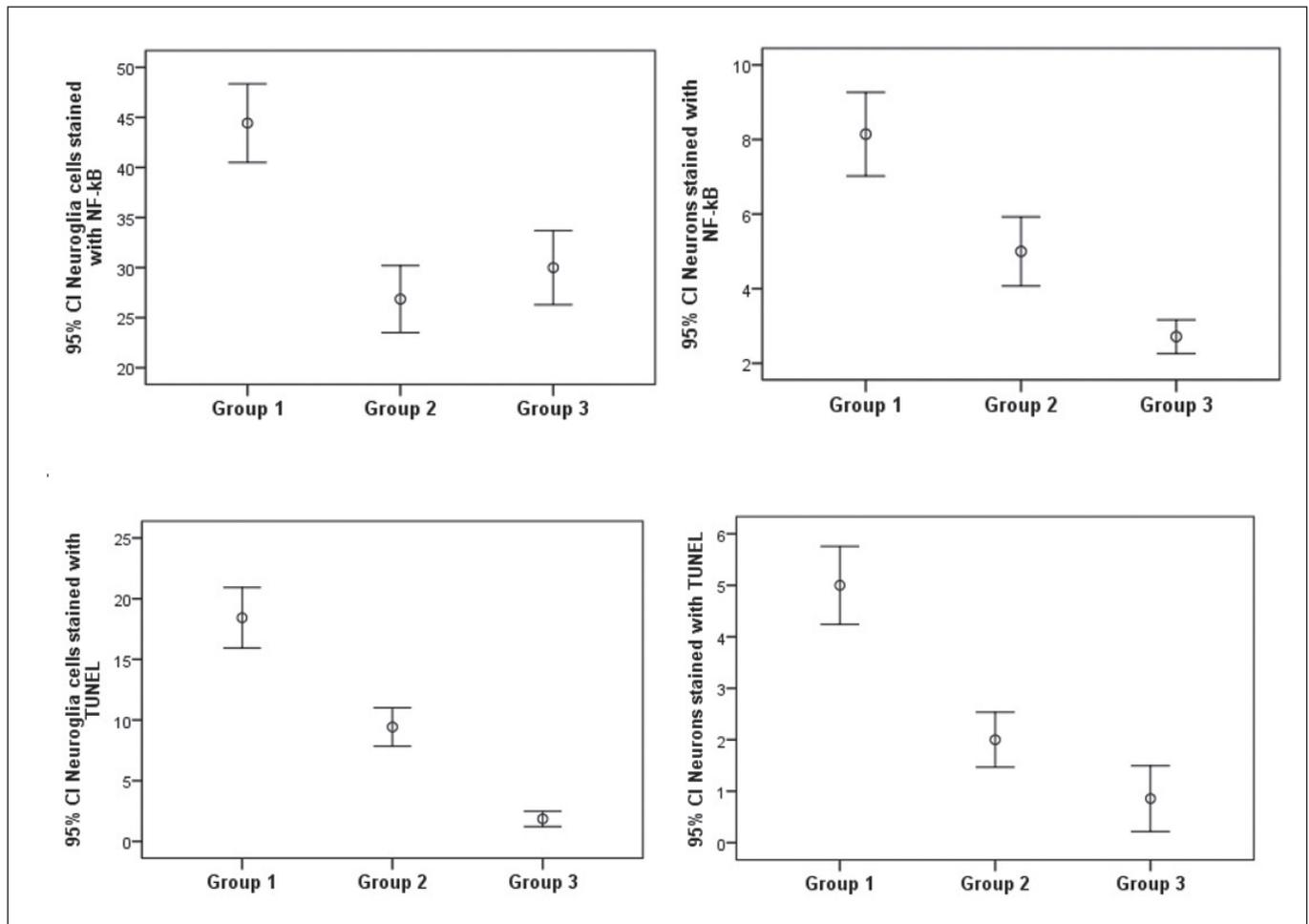


Figure 3: Statistical analysis results of neuron and neuroglial cells staining with NF-κβ and TUNEL.

and very strong correlation between neuronal cell numbers ($r=0.451$, $p=0.013$ and $r = 0.882$, $p<0.001$, respectively).

H&E Staining

In all groups, pyramidal cells, granular cells, and neuron bodies of fusiform cells, as well as central neuroglial cells, axonal extensions, and blood capillaries were distinguished in the gray matter region of the brain tissue. In the inner white matter region, axons extending from neurons in gray matter, glial cells associated with axons, and blood capillaries are observed. In Group 2, the relative increase in the number of

neuroglial cells, especially in the gray matter region (groups 1 and 3), was noted (Figure 4A-C).

DISCUSSION

In 1960, intrathecal fluorescein was described by Kirchner and Proud to localize the origin of otorrhea and rhinorrhea (6). This technique is used if other imaging methods were insufficient for diagnosis or to observe the origin of leakage during surgery. In 1926, Dandy described the first case of CSF fistula repair (16). After this date, repair with craniotomy become widespread.

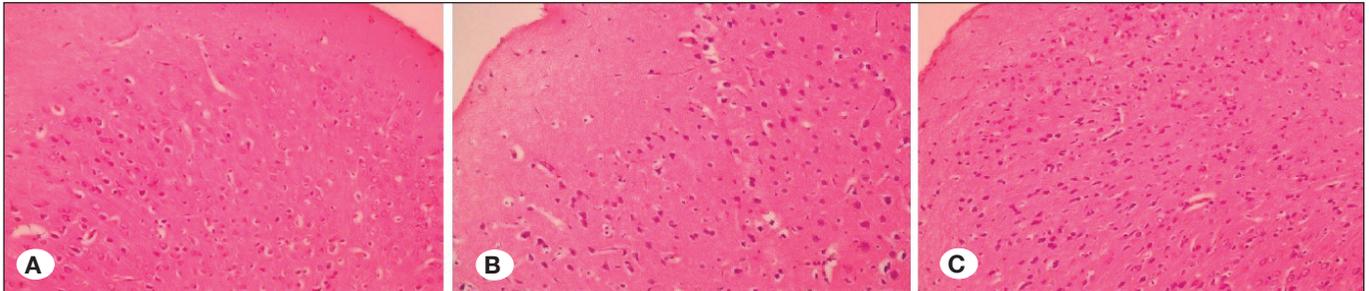


Figure 4: Histologic changes after fluorescein administration. Relative increase in the number of neuroglial cells observed. H&E, original magnification $\times 20$ (A: Group 1, B: Group 2, C: Group 3).

With time, endoscopic approaches were identified and became the gold standard, with a success rate of 60% to 100%. The success of the process largely depends on the detection of the leakage source and the use of fluorescein has become widespread. However, there is no clear consensus in the literature about the reliability, dosage, and infusion details of this dye. Although various imaging modalities are ambitious in determining the leak location, some surgeons prefer to see the leakage during surgery.

Several side effects of using intrathecal fluorescein sodium have been reported to date. Color changes in tears and urine, headache and neck pain, nausea, vomiting, fever and dizziness, cranial nerve deficits, lower extremity weakness, and numbness are the most common side effects. In the literature, there are rare but more serious adverse effects and complications, such as epileptic seizures, opisthotonos, hemiparesis, dysphasia, myelopathy, pulmonary edema, and death (3,6,9,11). The etiology and time of seizure activity remains unclear, suggesting that fluorescein may trigger direct central neuro-activation or meningeal irritation, or may have caused aseptic meningitis. Also, contamination of CSF with erythrocytes during lumbar drainage can cause seizure-like activities (6). In 1997, Wolf et al. described a large cohort of 925 patients (18) and reported fluorescein-related seizure in three patients (18). Although complications are rare, they can be serious. Some complications mentioned above may be due to lumbar puncture, but there are not enough data in this regard. In 1978, Moseley et al. surveyed the American Association of Neurological Surgeon members for the use of intrathecal fluorescein sodium and showed that all complications were transient (3). To avoid complications, John et al. described perioperative intranasal application of fluorescein sodium to the nasal cavity after exposure, reporting that yellow-colored fluorescein turns to green when it diffuses to CSF, so that the fistula is more easily observed (7).

Keerl et al. analyzed the results of 420 intrathecal application of fluorescein sodium solutions and the complications: in Germany, a maximum dose of 50mg/mL fluorescein was used, while in the United States, a more diluted solution of 0.5–2 mL (2.5–10 mg) was used perioperatively (10). They observed two grand mal seizures in Germany, but no apparent side effects with this hypodense fluorescein solution in the United States. In our study, we set the fluorescein doses according to doses

defined in their study. We also found that apoptosis increases depending on the increased dose of fluorescein, and that it also must be diluted before application due to high pH.

NF- κ B is a well known protein structured transcription factor which has an important role in regulating cell activities, such as the expression of genes that promote cell viability, inflammation, apoptosis and cell proliferation (8). The active form of NF- κ B acts like a transcription factor for target genes and can be induced by agents that damage DNA (15). In mammals, NF- κ B has a structurally related proteins. Rel (c-Rel), RelA (p65), RelB, NF- κ B1, and NF- κ B2 are the members of this protein (5). Still there is no consensus about which of the target genes activated by this proteins are critical for the suppression process of apoptosis, while the inhibition of apoptosis by NF- κ B may vary from one cell type to another (7). Studies have shown that inflammatory process is mediated through NF- κ B activation and the agents with anti-inflammatory effect suppress the activation of NF- κ B. Paradoxically, toxic agents, cytokines, and radiation, which induce the apoptosis, also have been shown to activate NF- κ B. This indicates that NF- κ B may be an important part of the cells' auto-defense system and may mediate chemo-radioresistance. The causes that activate the NF- κ B found in tumor cells are misunderstood. In rare cases, NF- κ B has a pro-apoptotic role despite its more common anti-apoptotic role. This opposing effect of NF- κ B might be related to cell type-specific and/or inducing signal-to-cell factors. As a result, NF- κ B activation with different pathways can trigger or inhibit apoptosis (5,15). We observed that administration of fluorescein increased the expression of NF- κ B, while the increased doses of fluorescein showed a moderate strong correlation between NF- κ B-positive stained neuroglial cells and very strong correlation between neuronal cell numbers.

Intrathecal administration of fluorescein sodium may be an important diagnostic method in the localization of CSF leak. Before the procedure, the patient and anesthesiologist should be informed about the side effects and benefits of fluorescein to the surgical team, because intrathecal use of fluorescein is considered as "off-label" use (6).

In our study, comparing histologic changes and apoptosis between the fluorescein groups and the control group, apoptosis occurrence was statistically higher in the fluorescein groups. However, we found no statistically significant difference among the three groups in terms of pyramidal cells,

granular cells, and neuron bodies of fusiform cells, as well as central neuroglial cells in the gray matter of the brain tissue and in the white matter region axons and glial cells. Attention should be given to the use of fluorescein when considering the effects on the cellular level and apoptosis. To our knowledge, this is the first experimental study regarding the toxic effect of fluorescein in the neural tissue.

■ CONCLUSION

Intrathecal fluorescein currently is being used at many centers and there is an emphasis that the safe dose should not be exceeded. Side effects, such as seizures, are known to occur at high doses, but to our knowledge, no animal experiments exist investigating these dose-related neurotoxicity complications. Our study showed that neuron and neuroglial cell staining was statistically different among the three groups in the type of tunneling ($p < 0.001$; Figure 4). Moreover, our results indicated that fluorescein induces apoptosis, resulting in increased NF- κ B expression in a dose-dependent manner.

■ ACKNOWLEDGMENTS

We thank Prof. Huseyin Aktug and Prof. Altug Yavasoglu for their significant contributions and special aids at histology and embryology department laboratories of Ege University.

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