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Prenatal exposure to diclofenac sodium changes the morphology of the male rat cervical spinal cord: A stereological and histopathological study

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ABSTRACT

Diclofenac sodium is one of the most commonly used non-steroidal anti-inflammatory drugs. It may cause alteration in the nervous system during neuronal development. However, there is no investigation concerning its role in the cervical spinal cord. Pregnant rats were divided into two groups, namely drug-treated and control (saline-injected) groups. To obtain the offspring of the drug-treated group, a dose of 1 mg/kg daily diclofenac sodium (Voltaren, 75 mg/3 ml ampoule, Novartis) was injected into the pregnant rats beginning from the 5th day after mating to the 20th day of the pregnancy. To obtain the control group of offspring, serum physiological at a 1 ml/kg daily dose was injected into the pregnant control rats during the same period. Male offspring were obtained after delivery and each group was divided into two subgroups: 4-week-old and 20-week-old. The total neuron number in diclofenac sodium-treated rats was significantly lower than in the control group animals. The total volume of the cervical spinal cord segments (C1–C4) was also estimated. There was a significant difference between the volumes of the two groups, especially in the 20-week-old subgroup. This may suggest that development of neurons and volume of cervical spinal cord are affected in prenatal animals after administration of diclofenac sodium.

1. Introduction

The spinal cord of all vertebrates possesses "H" or butterfly-shaped central grey matter (GM) which, unlike the cerebellar and cerebral cortex, does not present evident division in layers within the surrounding white matter (WM) (Held et al., 2001; Korzan et al., 2002; Portiansky et al., 2004). It is extremely important to the overall function of the nervous system and the communication link between the brain and the peripheral nervous system inferior to the head, integrating incoming information and producing responses through reflex mechanisms (Elmonem et al., 2007). Cervical spinal cord (CSC) is an area of particular interest since, in humans; different alterations of medical importance such as illness can alter the motor neurons and result in atlanto-axial uncertainty (Malcolm, 2002; Murakami, 1990; Nakajima et al., 1996; Portiansky et al., 2004).

Prostaglandins are major chemical mediators in the human body. They have an importance in both the normal and abnormal function of virtually every organ and system. NSAIDs are prostaglandin synthetase inhibitors and act on cyclooxygenase (COX), which is a crucial enzyme in the biosynthesis of prostaglandins from arachidonic acid (Siu et al., 2000). Diclofenac sodium (sodium-(O-((2,6-dichlorophenyl)-amino)-phenyl)-acetate) (DS) is a non-steroidal anti-inflammatory drug (NSAID) having potent anti-inflammatory, analgesic, and antipyretic effects on tissues (Siu et al., 2000; Savaser et al., 2005). It is used extensively in the treatment of a variety of diseases and conditions including rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and acute gout, and is also used following some surgical procedures (Siu et al., 2000; Beck et al., 2003; Savaser et al., 2005). However, treatment with DS can be associated with many gastro-intestinal problems ranging from mild to severe dyspeptic symptoms, the development of gastric or duodenal ulceration, hemorrhage or perforation, and other events which may lead to hospitalization or death (Russell, 2001; Liu et al., 2005; Andersohn et al., 2006; Capone et al., 2007).

In addition to gastrointestinal adverse effects, it has been shown that exposure to DS during development of the central nervous

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system (CNS) can produce a wide array of neurological dysfunctions and neuroanatomical anomalies in animal models (Chan et al., 2001; Kudo et al., 2003). However, very little information is available concerning DS effects on neurological structures caudal to the brainstem, especially with regard to prenatal development. Therefore, in this study we investigated the effects of prenatal exposure to DS on the total neuron numbers of the GM of the rat cervical spinal cord. Additionally, an analysis of volume alterations in the cervical spinal cord was done, and volume fraction assessment of GM and WM was attempted in both saline-injected and DS-treated rats using quantitative stereological methods.

2. Materials and methods

In this study, male Wistar albino rats were obtained from the Surgical Research Center. Female rats weighing between 150 and 200 g were mated overnight and maintained in our laboratory under controlled environmental conditions (an air-conditioned room, room temperature 20 ± 1 °C and under a 12 h light/dark cycle). They were fed *ad libitum* and kept in separate standard plastic cages. Female rats were accepted as pregnant when a vaginal plug was found, and were divided into drug-treated and control (saline solution-injected) after mating. A dose of 1 mg/kg daily DS (Voltaren, 75 mg/3 ml ampoule, Novartis, Kartal, İstanbul, Turkey) was intraperitoneally injected into the drug-treated group pregnant rats beginning from the 5th day after mating, during pregnancy, for 15 days. Saline solution (0.91% w/v of NaCl, about 300 mOsm/L) at 1 ml/kg dose was intraperitoneally injected into the control group rats during the same period and in the same way. After spontaneous delivery, male pups were separated into two groups (the drug-treated and the control). They were marked by branding on their body and again by coloring with picric acid, and fed for 4 and 20 weeks respectively.

In this study, laboratory procedures were used as described in detail elsewhere (Gokcimen et al., 2007; Odaci et al., 2003; Unal et al., 2004). Briefly, at the end of the 4th and 20th weeks, the rats were anesthetized with urethane (1.25 g/kg) and perfused through the left cardiac ventricle with 10% neutral-buffered formalin. After extraction of the vertebral column, the cervical spinal cords were dissected out and stored in 10% formalin for 10 days at 4 °C. After rinsing in tap water for 12 h, spinal cords were dehydrated in a series of alcohols and embedded in paraffin. The segment of cervical spinal cord consisting of C1–C4 segments was cut into serial sections of 45-μm thickness in the transverse plane using a rotary microtome (Leica RM 2135, Leica Instruments, Nussloch, Germany).

The sections were mounted onto gelatin-formaldehyde mixture-coated glass slides and stored for 24 h at 37 °C in a thermostatically controlled oven and later stained with cresyl violet (0.1%). All experiments described in the study were conducted according to institutional guidelines. The Animal Experiments and Ethics Committee of Yüzüncü Yil University approved the protocol, and appropriate measures were taken to minimize pain or discomfort. In addition, a special effort was made to minimize suffering and the number of animals used in this study.

2.1. Stereological analysis

2.1.1. Stereology workstation

For stereological analyses, a semi-automated stereology workstation comprised of a CCD digital camera (Nikon Coolpix E 4500, Tokyo, Japan), image capture card (Flash Point 3D, Integral Technologies, Indianapolis, IN, USA), personal computer and computer controlled motorized specimen stage (Prior, Rockland, MA, USA), a microcator (Heidenhein, Traunreut, Germany), and a light microscope (Nikon, Eclipse E 600, Tokyo, Japan) was used. Neurons were counted using a 100× Nikon Plan Apo objective (NA = 1.40) and total magnification was 1680, which allowed for accurate recognition.

2.1.2. Estimation of the total neuron number

Total neuron numbers were estimated using the optical fractionator counting method. About 15 to 20 sections required for an unbiased estimation of the total neuronal cell number were obtained from the cervical spinal cord by choosing every 15th section according to the systematic random sampling procedure (Gundersen and Jensen, 1987). In this study, sampled section numbers were changed between 14 and 23. A sampling area of 3600/10,000 μm^2 was found to be optimal for this study. Dissector height was 20 μm and a 5-μm zone at the uppermost part of the section was excluded from the analysis at every step as the upper guard zone. Thus, a thickness-sampling fraction of 20 μm/t was used (t = represents the mean section thickness). Each neuron was counted according to the unbiased counting rules that means if a neuron do not touch the exclusion line of the counting frame but inside into the frame or superimposed with the inclusion line of the frame, it is counted. Besides this rule, another rule is that during counting if the widest part of a nucleus of neuron comes into focus in the dissector volume; it is counted as a dissector neuron. Both rules should be met by each neuron that is assumed to be a dissector neuron (Howard, 1998; Gundersen, 1986). The total neuron number of GM in the segment of spinal cord was estimated according to the formula given below:

$$N = \sum Q \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{1}{tsf}$$

where $\sum Q$ represents the total number of neurons counted in all optically sampled fields of the spinal cord; *ssf* is the section sampling fraction (1/15); *asf* is the area sampling fraction (3600/10,000) and *tsf* is the thickness sampling fraction (defined by dissector height (20 μm) divided by estimated mean section thickness) (West et al., 1991).

2.1.3. Estimation of the volume of cervical spinal cord segment

The Cavalieri principle was used to estimate the volume of the C1–C4 segments of the spinal cord using the same stereology workstation. The same sets of consecutive sections of the cervical spinal cords were used for volume estimation. A combined point counting grid (CPCG) composed of circled and non-circled points with 1/3 area fraction (i.e., d = 100 and 300 μm) was used for volume estimation to obtain maximum efficiency. The representative areas per points (a/p) were 10.00 (for GM) and 90.00 (for WM and whole spinal cord) μm^2 for encircled and non-encircled points, respectively. The encircled points were used for the WM and the whole spinal cord measurements and the non-encircled points were used for GM (Fig. 1). After applying the CPCG on the sampled sections in a systematic random fashion, we counted the number of points hitting each spinal cord compartment. These point counts were used for the estimation of the volume of each compartment using the following formula:

$$Volume_{(sesment\ of\ SC)} = (a/p) \times \sum P \times ssf \times t$$

where "a/p" represents the area of each point on the point counting grid; "$\sum$P" is the total number of points hitting the layer; "t" is the mean section thickness, and *ssf* (1/15) is the section sampling fraction (Howard, 1998; Gundersen, 1986; Odaci et al., 2003).

2.1.4. Estimation of the volume fraction of GM and WM

The volume fraction of a component within a reference volume is a simple and very widely used parameter in biomedical science (Howard, 1998; Gundersen, 1986). Thus, it is used to express the proportion of a phase or component within the whole structure. The volume fraction of an X phase within a Y reference volume is simply expressed as follows:

$$Vv(X,Y) = \frac{Volume\ of\ X\ phase\ in\ Y\ reference\ space}{Volume\ of\ Y\ reference\ space}.$$