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Prenatal exposure to low-dose diclofenac sodium does not affect total neuron numbers in spinal segment T13 in rats

Murat Çetin Ragbetli[a,*], Mikail Kara[a], Neşe Çölçimen[a], Necat Koyun[b], Gamze Çakmak[c], Veysel Akyol[a], Omur Gulsum Deniz[d], Kıymet Kübra Yurt[d]

[a] Department of Histology and Embryology, Faculty of Medicine, Yuzuncu Yil University, Van 65080, Turkey
[b] Department of Anatomy, Faculty of Medicine, Yuzuncu Yil University, Van 65080, Turkey
[c] Department of Anatomy, Faculty of Veterinary Medicine, Yuzuncu Yil University, Van 65080, Turkey
[d] Department of Histology and Embryology, Faculty of Medicine, Ondokuz Mayıs University, Samsun 55139, Turkey

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ABSTRACT

The main purpose of the present study was to investigate the effects of diclofenac sodium (DS) on the total number of neurons in segment T13 of the spinal cord of offspring of pregnant rats using stereological methods. Eighteen adult female *Wistar albino* rats weighing 150–200 g were used. Pregnant female rats were divided into three groups; a control group, a sham group and a DS (1 mg/kg, intramuscular) exposed group. The DS and sham groups received injection from the 5th day of gestation to the 19th. Twenty eight days after birth, the offspring rats were perfused with 4% buffered formalin. T13, which is one of transverse spinal cord segments, were isolated and processed for routine paraffin histology. 5 μm sections were obtained using a rotary microtome according to systematic random sampling strategies. Every 40th section was taken and sections were stained with modified Giemsa. All types of motor neuron cell were identified according to their morphology. In this study, the *"disector-Cavalieri combination"* method was used in the stereological examination of neurons. The motor neurons were counted in the right gray matter of the ventral horn in the spinal cord segment. The Kruskal–Wallis test was used for comparison the groups. In terms of motoneuron number, no significant difference among the groups was found ($p > 0.05$). In conclusion, our results indicated that prenatal exposure to DS has no effect on the total number of motor neuron of the offspring rats.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed to relieve pain and inflammation through inhibition the syntheses of the prostaglandins, which have major role in the normal and abnormal function in the human body and affect each organ and systems. In addition, NSAIDs act on cyclooxygenase (COX), which is important enzyme in the biosynthesis of prostaglandins from arachidonic acid. Therefore, NSAIDs have many actions on edema, pain, and vasodilatation. Diclofenac sodium (DS) is a one of NSAIDs and has antipyretic, anti-inflammatory, and analgesic effects on tissues and it is absorbed completely after oral administration. It is used in the treatment of many diseases such as osteoarthritis, rheumatoid arthritis, acute gout, and ankylosing spondylitis and subsequent of surgical procedures (Savaser et al., 2005; Siu et al., 2000). However, DS can lead to many gastrointestinal problems such as the development of gastric or duodenal ulceration, hemorrhage or perforation (Liu et al., 2005; Russell, 2001; Savaser et al., 2005; Siu et al., 2000). Besides gastrointestinal adverse effects, the several studies have shown that prenatally exposure to DS can cause to damage in the development of central nervous system (CNS) neurological dysfunctions and neurological anomalies in the animal model (Chan et al., 2001; Kudo et al., 2003). At the same time, there is no enough information concerning the effects of DS on neurological structures. Özyurt et al. (2011) have suggested that prenatal exposure to DS leads to a significant decrease in the numbers of motor and sensory neurons of the rat cervical segment (C1–C4). Additionally administration of DS cause to histopathological alterations on the neuron structure such as chromatin condensation and cytoplasmic shrinkage (Özyurt et al., 2011). Similarly, Gokcimen et al. (2007) have reported that prenatal exposure to

Abbreviations: CV, coefficient of variation; CE, coefficient of error; DS, diclofenac sodium; i.m., intramuscular; NSAID, non-steroidal anti-inflammatory drug.

* Corresponding author at: Department of Histology and Embryology, Faculty of Medicine, Yuzuncu Yil University, Van 65080, Turkey.
E-mail address: ragbetli@hotmail.com.tr (M.Ç. Ragbetli).

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diclofenac sodium can lead to a significant decrease on Purkinje cell numbers in developing rat cerebellum (Gokcimen et al., 2007).

Toxicity caused by diclofenac sodium in the motoneurons at the thoracolumbar junction may cause to upper and lower motoneurons (Chen et al., 2012). This study was planned to determine the effects of DS on numbers of thoracic segment (from T13 to L1) of spinal cord motoneurons in pregnant rat offspring. Due to differential development of the spinal cord and vertebrae, the L4–L5 segments of the spinal cord are located beneath the T13–L1vertebrae. In addition, the numeric alterations in the motoneurons of the thoracic spinal cord were performed in three groups using unbiased stereological methods.

2. Material and methods

2.1. Specimens

Adult female eighteen *Wistar albino* rats weighing approximately 150–200 g were mated overnight. The day following observation of a vaginal plug was designated gestational day zero. The rats were housed in our Animal Laboratory at a room temperature of 20 ± 1 °C in a perpetual cycle of 12 h light and 12 h dark, located in a room with air conditioning. Food and water were available *ad libitum*. Pregnant female rats were divided into three groups, control, sham and DS (1 mg/kg, i.m.). Dams received i.m. injection of 1 mg/kg DS (Voltaren® CIBA GEICY, Istanbul, Turkey). The DS group received injections from the 5th day of gestation to the 19th. Members of the sham group received 1 mg/kg saline solution (0.91% w/v of NaCl) by i.m. injection. No procedure was performed on the control group. On postnatal 28th day, 18 female offspring were fixed by transcardial perfusion under urethane anesthesia (1.25 g/kg) with heparinized saline solution followed by 10% neutral-buffered formalin. Transverse sections of spinal cord segments T13 were dissected out and then post-fixed for two weeks in the same fixative and embedded in paraffin for use in routine histological techniques.

2.2. Tissue selection and staining

Transverse sections were taken using a rotary microtome (Leica RM 2125, Leica instrument, Nussloch, Germany) at a thickness of 5 μm. Every 40th section was taken for a total of approximately 10 sections, which were then stained with modified Giemsa. All types of motoneuron cells were identified on the basis of their morphology, especially those with well-defined nuclei and clear euchromatic nucleoplasm (Fig. 1).

2.3. Stereological analysis

2.3.1. Stereology

In stereology, random and systematic sampling is utilized to gain unbiased and quantitative information. Stereology is a crucial and effective instrument in various microscopic applications (Gundersen and Jensen, 1987; Pakkenberg and Gundersen, 1989; Walløe et al., 2011). The unbiased stereological dissector-Cavalieri combination method, also known as the physical dissector counting method (Fig. 2), was used in our study (Gundersen, 1986; Pakkenberg and Gundersen, 1988; Howard and Reed, 1998). SHTEREOM 1.0 software was used for volume estimation. Estimation was performed using the formula $V_{(obj)} = t \times a/p \times \sum P$, where "$V_{(obj)}$" is the volume of the objective and "t" is the section thickness. The total number of motoneurons in the right ventral horn in a spinal cord segment was estimated using the formula:

$$N = Q^{-} \times \sum P \times k \times (a/p)/a_{(frame)}$$

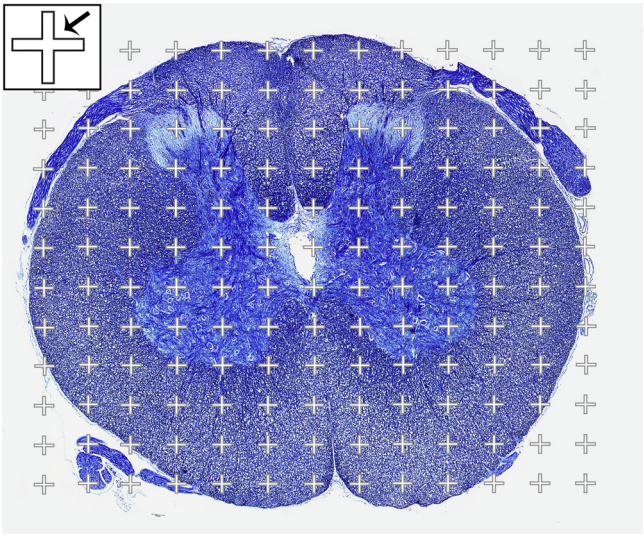

Fig. 1. Transverse section of spinal cord photographed at x4 magnification and a point counting grid superimposed onto it, for the volume estimation. The arrow shows the right upper corner of the cross as a point. The number of the points hitting the structure was counted for the volume estimation of the spinal cord.

where "N" t = represents the total neuron number, Q^{-} (0.376) the mean number of neurons counted in all sampled neuron fields in the ventral horn of the spinal cord, "$\sum P$" (574) is the total number of points hitting the motoneuron areas, "k" is the section sampling fraction (1/40), "a/p" (16 900 μm^2) represents the area of each point on the point counting grid and "a" (2704 μm^2) is the counting frame. Each count was performed using the unbiased method. In order to prevent double counting, the software employed an unbiased counting frame. The mean numbers of motoneurons in rat spinal cord T13 segments (right ventral horn) were counted for all experimental groups. All neurons in the spinal segment were counted at a magnification of x63 (Plan-neufluar [NA = 1.25 Oil]). The operations were conducted in a blind fashion throughout all microscopic observations. A hypothetical line was drawn for reference through the central canal of the spinal cord, from top to bottom and from left to right on the segment. The right ventral horn was selected for counting.

2.3.2. Coefficient of variation and coefficient of error

Coefficient of variation (CV) and coefficient of error (CE) are considered in order to determine the optimum sample size in each group in the standard stereological approach (Gundersen and Jensen, 1987). Stereological estimation values based on our results are given in Table 1.

2.4. Statistical analysis

The statistics were given under the headings of mean, standard deviation, minimum and maximum values. The Kruskal–Wallis test was performed for the comparison of the diclofenac, saline and control groups. Significance level in statistics was set at 5%, and SPSS (version 13) software was used for all statistics.

3. Results

Numbers of motoneurons in the right lower quadrant of the thoracic segment were estimated for the three groups. At this point; there were no statistically significant differences among the groups in terms of total number of motoneurons ($p > 0.05$). In the histopathological examination no changes were observed in all sections of the spinal segment T13 in rats that were prenatal

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