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Effects of prenatally exposed diclofenac sodium on rat heart tissue: a stereological and histological study

Fikret GEVREK[1,*], Mikail KARA[2], Murat Çetin RAĞBETLİ[2], Hüseyin ASLAN[1]

[1]Department of Histology and Embryology, Faculty of Medicine, Gaziosmanpaşa University, Tokat, Turkey
[2]Department of Histology and Embryology Faculty of Medicine, Yüzüncü Yıl University, Van, Turkey

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Background/aim: Diclofenac sodium (DS) can cross the placental barrier and affect the fetus, and its consumption during pregnancy may cause developmental malformation of embryos. This study investigates the effect of prenatally applied DS on the quantitative morphology of the adult rat heart.

Materials and methods: Pregnant rats were divided into three groups (control, sham, and test). The rats in the test group were injected with DS; the control group received physiological saline (1 mL; 1 mg/kg, i.m.) from the 5th to the 20th day of pregnancy; and the rats in the sham group were not injected at all. At the 20th postnatal week, all the offspring were euthanized under deep anesthesia and tissue samples were obtained by perfusion fixation. After routine histological procedures, the paraffin sections were stained with hematoxylin and eosin and examined stereologically and histologically.

Results: The volume of the cardiac ventricle wall of each offspring rat was estimated using Cavalieri's principle. The volume of the ventricle walls of the test group was found to be significantly less than that of the controls.

Conclusion: Further studies are required to determine how DS has this effect, by reducing the number of myocytes and decreasing the size of these cells affecting the connective tissue.

Key words: Diclofenac sodium, heart, pregnancy, rat, stereology

1. Introduction

Diclofenac sodium (DS) is a nonsteroidal antiinflammatory drug (NSAID) widely used in the fields of obstetrics and gynecology (1,2). NSAID intake during pregnancy, however, has several negative adverse effects for both the mother and the fetus (2). Experimental animal fetus studies have shown that NSAID treatment causes closure of the ductus arteriosus (DA), pulmonary arterial hypertension, malformation (3), Purkinje cell loss in the cerebellum (4), decreased numbers of pyramidal and granular cells in the hippocampal formation, and suppression of new cell formation in the spinal cords of rats (5). In addition, DS and saline solutions impair sciatic nerve morphology (6).

In humans, maternal effects include prolonged pregnancy and labor (7,8), while reported fetal and neonate teratogenic effects include constriction of the DA, ischemia of the extremities, hydrops fetalis, oligohydramnios, ileal perforation, and cystic lesions of the brain (9–12). Premature closure of the DA after short-term maternal use of DS may result in severe pulmonary hypertension and transient right-sided hypertrophic cardiomyopathy (3). Gastroschisis has also been observed as a possible teratogenic effect of the administration of NSAIDs in early pregnancy (13). As such, DS should generally not be administered during pregnancy (14)

Except for the general work on NSAIDs previously cited, there seems to be a lack of evidence showing that uterine exposure to DS, in particular, increases teratogenicity, especially of the heart (6). Thus, the effects of DS on the development of the heart wall tissues of fetal rats were investigated in this study. Specifically, the effects of DS on the cardiac ventricular tissue of postnatal 20-week-old offspring of rats treated with DS during pregnancy were examined using stereological and histological methods.

2. Materials and methods

2.1. Animals and experimental procedures

This work was approved by the Animal Use Ethics Commission of Yüzüncü Yil University and all procedures were performed according to the Animal Experimentation

* Correspondence: fikretg05@hotmail.com

Ethics Committee. Male and female adult Wistar albino rats weighing 150–200 g were obtained from the Breeding and Research Center for Experimental Animals of the Yüzüncü Yil University Faculty of Medicine, Van, Turkey, and grouped into sham, control, and test groups. Each group was left separately in plastic cages for mating on the same day. The female rats were determined to be pregnant after observing the vaginal plug produced by male vesicular and coagulating gland secretions in the following days (15). The pregnant rats were fed ad libitum and kept in standard plastic cages, with sawdust bedding, in an air-conditioned room (20 °C) under a 12/12 light/dark cycle. Once the rats were determined to be pregnant, DS (1 mg/kg, i.m.) was administered to the test group on gestation days 5–20, as gestation days 9–11 represent the critical period of organogenesis for cardiovascular and midline development in rats, equivalent to 3–6 weeks after fertilization in human embryos (16,17). The rats in the control group received physiological saline (1 mL/kg, i.m.), and those in the sham group received no injected substance. All the pregnant rats were kept under observation for 24 h on the last day of pregnancy; the day the pups were born was deemed day zero postnatal.

Twelve offspring (six males, six females) from each group, for a total of 36 offspring, were chosen randomly after delivery and housed for 20 weeks. At the end of the 20th week after birth, intracardiac perfusion was performed under anesthesia with 0.9% saline and 10% formalin on all the rats in each group. Heart tissue samples were processed with graded alcohol and xylene and then embedded in paraffin blocks (18). In order to conduct successful stereological studies, we first determined a working strategy by conducting a pilot study and then performed the main work. For the pilot study, whole tissue sections (30 μm) were taken transversely from the beginning to the end of one paraffin block with a rotary microtome (Leica RM 2135; Leica Instruments, Germany), and stained with hematoxylin and eosin. Each animal had an average of 12 sections from which a systematic random sampling of slices was taken and stereological and histological analyses were performed.

2.2. Stereological and histological analysis

All of the microscopic examinations for the stereological and histological analyses were performed using a stereo investigator system, which included stereological analysis software (Stereo Investigator; MBF Biosciences, USA) and hardware. Certain processes can be performed easily with this analyzing system, such as determination of tissue boundaries and area, random sampling area, and step-by-step scanning. In order to calculate the volume of the cardiac ventricular wall using the Cavalieri method (19,20), the cross-sections, prepared as determined by the pilot study, were examined under a light microscope. An image of the heart was shown on the screen, and the area to be measured was marked by a line via software under lens 5 of the microscope. After circling the point of measurement, a table was placed on display via software. The space of one point of the point area measurement table (a/p) was calculated as 360,000 μm^2 automatically by the system inputting x- and y-axis values of 600 μm. We analyzed the whole tissue on the cross-sections outside the systematic random sampling area, thereby counting the points falling on the image of the cardiac ventricular tissue on all the sections of each animal examined. Subsequently we calculated the total volume of the heart ventricular wall with the following formula:

$[V = a/p \cdot \Sigma^{n}_{(i=1)} P_i (SSR) \cdot (ASR) \cdot \bar{t}]$, where a/p is the unit area represented by one point, ΣP_i is the total number of points counted from the sections of one animal, SSR is the slice sample rate (1/22), ASR is the area sampling rate (1/1), and $\bar{t}$ is the average cross-sectional thickness. Slice thicknesses in the image areas, determined as systematically random by using all five steps, were measured with a microcator. For test result reliability and impartiality, all data were obtained through the blind study encoding method.

2.3. Statistical analysis

The statistical analyses for this study were performed using SPSS 13.0 for Windows. Evaluation of the results among the groups was performed using a one-way ANOVA test. A t-test was used to compare the average wall volumes of male and female rats.

3. Results

3.1. Stereological results

After obtaining the data from the stereological findings, the volumes of the rats' heart ventricle walls were calculated separately for males and females. The error coefficients of our stereological studies for ΣP_i (total points) were found to be acceptable (CE ≤ 0.05), as shown in the Table. The average volumes of the cardiac ventricular walls of all the groups were calculated as follows: for females, control: 269.14 mm^3, sham: 268.14 mm^3, and test: 204.89 mm^3; and for males, control: 412.97 mm^3, sham: 408.40 mm^3, and test: 328.44 mm^3 (Table; Figures 1–3).

These values were compared with the ANOVA statistical test, and a significant difference was found between the males and females according to groups ($P < 0.01$; Figure 3). There were no differences between the sham and control groups in both sexes, but there were statistically significant differences between the test group and the two control groups (sham and control) ($P < 0.05$), as shown in Figures 1 and 2. To compare the males and females, a t-test was carried out, and a significant difference was observed between their mean cardiac ventricular wall volumes ($P < 0.01$). In all three groups, the wall volumes of the male and female rats were different ($P < 0.01$; Figure 3). The volumes

Table. Data used in the calculation of cardiac ventricular wall volume and calculated average heart ventricle wall volume (V) with intragroup coefficients of variation (CV), standard deviations (SD), coefficient of error (CE), and standard error of mean (SEM) values. : average total point, NS: average number of slices, : average cross-sectional thickness; n: number of rats.

Parameters	Females			Males		
	Control	Sham	Test	Control	Sham	Test
	1956	1929	1550	1296	1278	989
NS	14	15	14	12	13	12
CE	0.020	0.023	0.025	0.014	0.017	0.019
(μm)	26.20	26.48	26.14	26.63	26.72	26.74
V (mm^3)	269.14	268.14	204.89	412.97	408.40	328.44
CV	0.033	0.043	0.045	0.055	0.031	0.052
SD	8.901	11.632	9.265	23.045	12.689	17.148
SEM	3.633	4.749	3.782	9.408	5.180	7.00
n	6	6	6	6	6	6

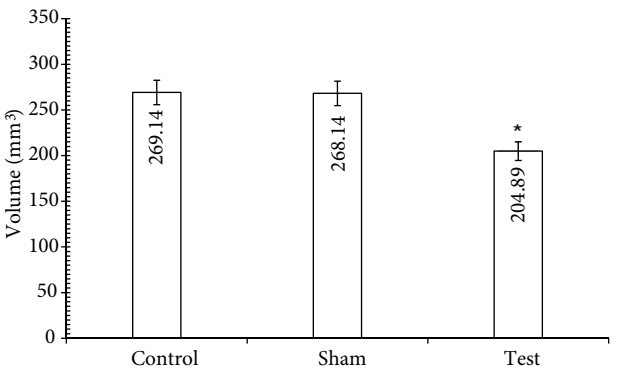

Figure 1. Average volume of heart ventricular wall in each group of female rats (*: P < 0.05).

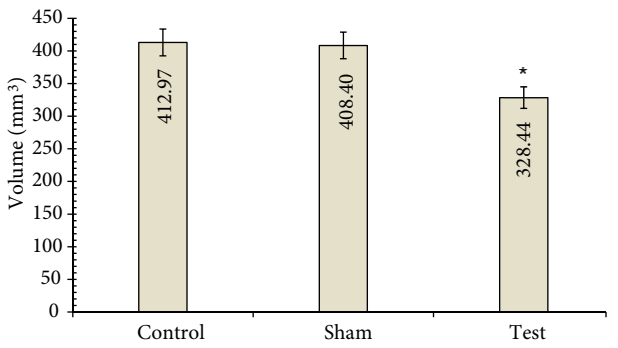

Figure 2. Average volume of heart ventricular wall in each group of male rats (*: P < 0.05).

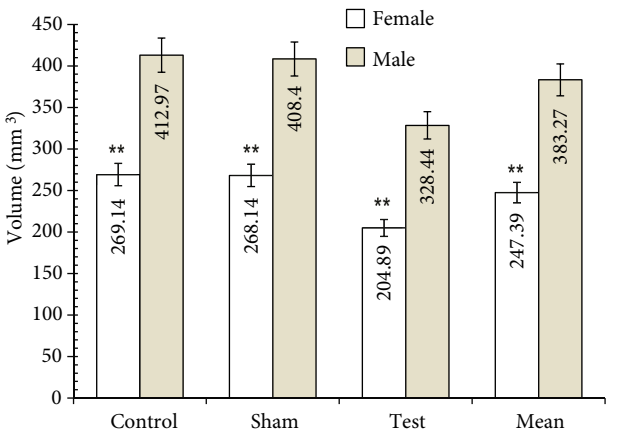

Figure 3. Average volume values for both sexes by group and total mean (**: P < 0.01).

of the heart ventricular walls of both the male and female test groups were significantly lower compared to those of the sham and control groups. This reduced volume in the test group was approximately 20.5% for males and 23.8% for females.

3.2. Histological results

In addition to stereological analysis, the cardiac ventricular tissues were examined under light microscopy for any histological changes in the tissues on thin cross-sections (5 μm) stained with hematoxylin and eosin. The tissue sections of all the offspring were compared among the three groups (test, sham, and control). Microscopic analysis revealed normal cardiac ventricular muscle tissue

in all three groups; that is, the cardiac ventricular muscle tissues all had a centrally located nucleus. Intracellular contractile myofilament (understood striating) branching and anastomosing striated heart muscle cells were observed. However, in some sections, the heart ventricle muscle tissues of the test group had more extracellular matrix and weaker muscle fibers than did those of the sham and control groups, in both males and females. In addition, the heart muscle cells of the females were thinner than those of the males (Figures 4 and 5). A more detailed histopathological study is needed for a final judgment on this issue.

4. Discussion

DS is a member of the NSAID group and is sometimes used during pregnancy in cases of necessity or unawareness of pregnancy, which may have adverse side effects on the developing fetal organs. For this reason, we investigated the effects of DS on the heart ventricle tissues of 20-week-old rats that were exposed to this drug in utero. We found that this drug caused a decrease in the volume of the heart ventricle wall in experimental rats of both sexes.

NSAIDs are widely consumed as analgesics, antipyretics, and antiinflammatories, especially in physical therapy (2,21). Most studies associated with the treatment of rheumatoid arthritis have focused on NSAIDs such as aspirin (acetylsalicylic acid) and indomethacin. The literature is rather sparse regarding the consumption of other NSAIDs, such as ibuprofen, sulindac, ketoprofen, fenamates, oxicams, and diclofenac, during pregnancy. NSAIDs should generally not be administered in early pregnancy, though their use might be advised in some circumstances, such as preeclampsia. In addition, DS is used widely for women of childbearing age to relieve a variety of conditions, including common gynecological problems; although pregnancy is often planned nowadays, pregnant women might still accidentally use NSAIDs such as DS during different stages of gestation (14). There is no absolute proof relating to the teratogenic effects of NSAIDs on the heart (8,22). Because DS is used in the treatment of certain problems observed in pregnancy, such as premature birth and preeclampsia, research is necessary in

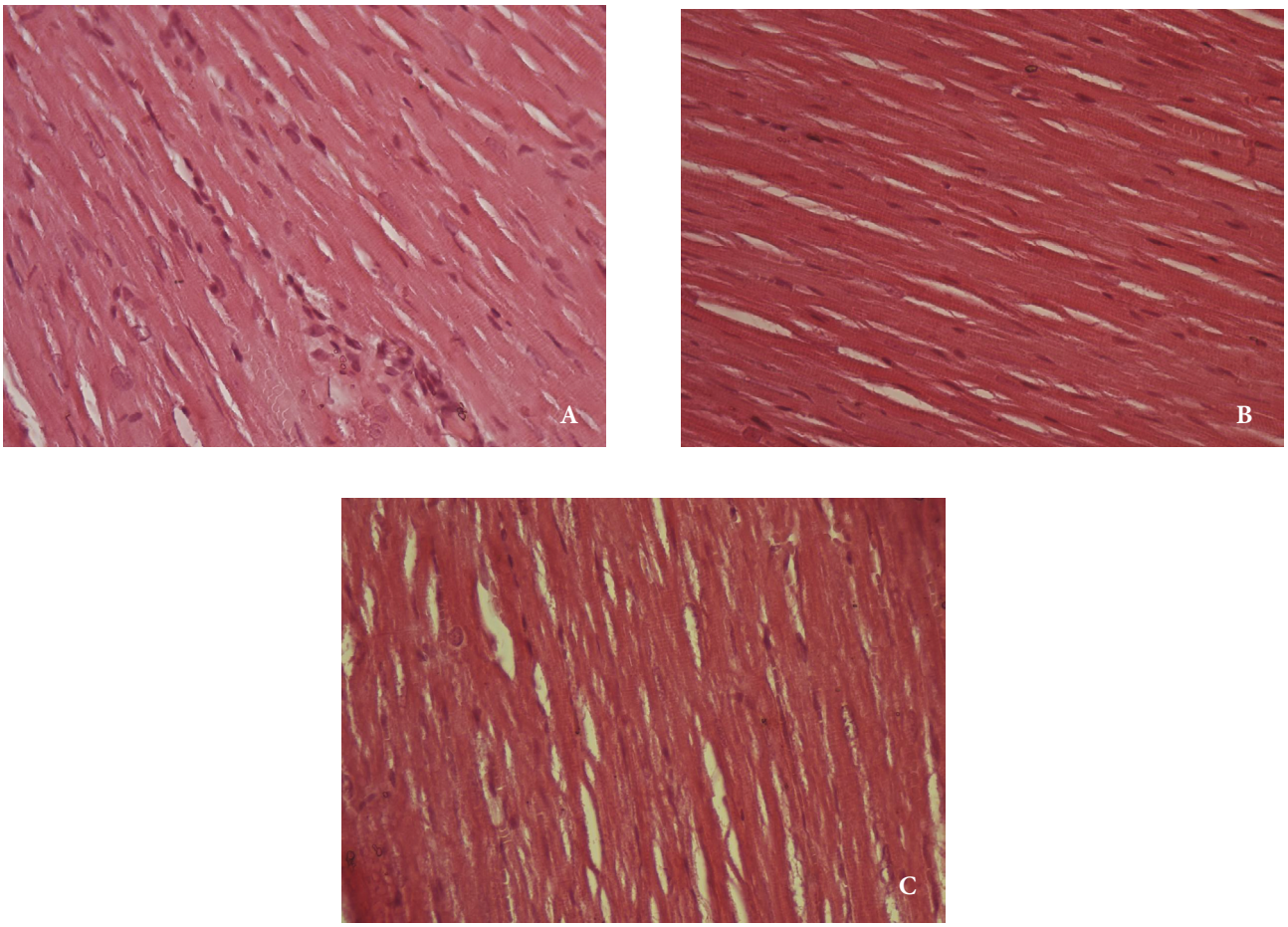

Figure 4. Male rats' heart ventricle tissue. A) Sham, B) control, C) test group. As seen in the pictures, test tissue had more space between myocytes and weak muscle fibers compared to other groups (H&E, 40× obj.).

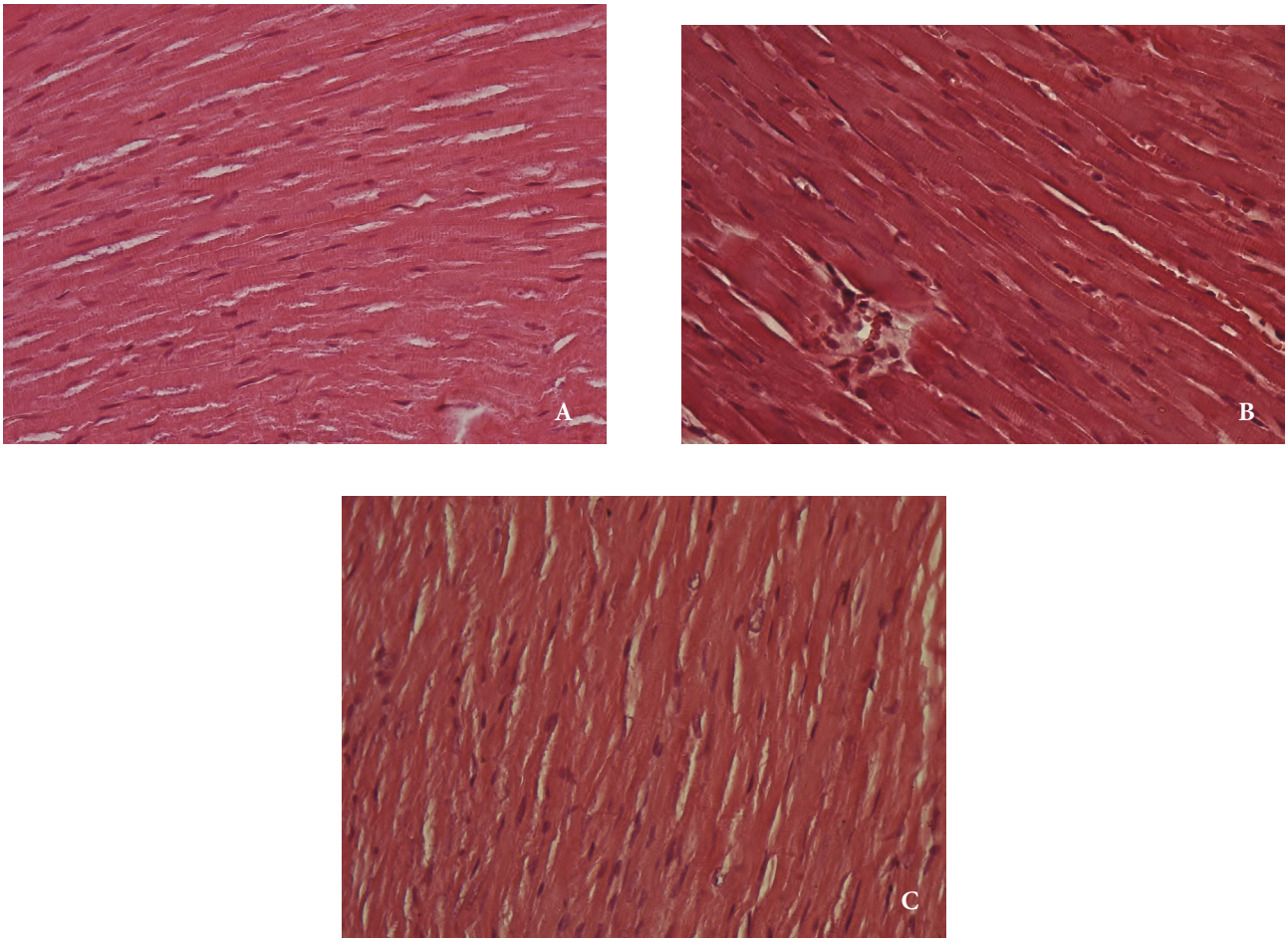

Figure 5. Female rats' heart ventricle tissue. A) Sham, B) control, C) test group. As seen in the pictures, test tissue had more space between myocytes and weak muscle fibers compared to other groups (H&E, 40× obj.).

order to determine its possible teratogenic effects (23,24).

Among the common features of NSAIDs are their prevention of several mechanisms, such as phospholipase C enzyme activation, ion transport, mitochondrial oxidative phosphorylation, and cell interaction (2,23). They also inhibit the cyclooxygenase enzyme, which catalyzes the formation of prostaglandin and some prostanoids from arachidonic acid in tissues (2,25). All NSAIDs are thus prostaglandin inhibitors, which explains their fetal toxicity, as prostaglandins produced in many tissues and membranes have very important roles in fetal development (2,26,27). NSAIDs inhibit prostanoid biosynthesis and cross the placental barrier by joining the fetal circulation, upon which they cause teratogenic effects in the fetus (2,12,28).

DS also has some important, specific, and adverse effects on the DA, the most important being the contraction of the fetal DA (29). Arachidonic acid metabolites can be highly concentrated in fetal blood circulation; they regulate umbilical cord blood circulation and enable the DA to remain open during fetal life (30). In uterine life, deoxygenated blood flows away from the pulmonary artery and goes to the fetal placental circulation, where gas exchange occurs (31). As a result of their complex biochemical and physiological entanglement, these vessels quickly contract and constrict after birth; the direction of deoxygenated blood flow thus changes from the placenta to the lungs for gas exchange. Premature closure of the DA may cause pulmonary hypertension and even death in utero (32,33).

Closed approximately 10–15 h after birth, the DA must remain open for fetal tissue oxygenation throughout fetal life (13). Contraction and shrinkage of the DA is inevitable as a result of treatment with cyclooxygenase inhibitors to prevent premature birth (10,12,34). DA contraction may lead to an increase in pulmonary vascular resistance, and, thus, right ventricular dilatation and tricuspid valve insufficiency may occur in the heart. Right ventricular failure leads to hyperemia and stasis, which affects the liver, spleen, kidney, and brain tissues, causing congestion

of these organs (33). Right ventricular failure, prominent in congestive heart failure, leads to congestion of the liver; therefore, developing liver stasis may result in dilatation of the sinusoids, hepatocyte degeneration, and even the death of parenchymal cells (35). It has also been observed that administration of NSAIDs to pregnant rats on gestation days 9–10 reduced maternal body weight and feed consumption, fetal weight, and increased ventricular septal defects (17). Logically, the results of our study support these findings. As mentioned, the most pronounced effect of DS is contraction of the fetal DA, which may result in negative events such as escape back to the tricuspid, right-sided ventricular heart failure, and shunting between the atriums in the heart. Eventually, a reduction in the amount of systemic blood reaching the liver tissues in particular may result, leading to a number of likely degenerations in the parenchymal cells of these tissues. Our study indicates that a reduction in cardiac ventricular wall volume and the histological changes observed in the cardiac ventricular muscle are not directly due to the contraction of DA. They may be the combined effects of DS and its metabolites and the side effects of DS-deregulated umbilical cord blood circulation (36).

Two different examinations were made of the 20-week-old rats, which were exposed to the same DS doses during the same period and days of uterine life. The total number of Purkinje cells in the cerebellum was calculated using stereological methods, and the differences between the control and test groups were determined (4). The results showed that this drug decreases the number of cells. Similar results of the side effects of prenatal exposure to DS on the central and peripheral nervous tissues were also found by our group (4–6). On the other hand, even prenatal exposure of DS increased total Purkinje cell number in the female rats (37), but no significant differences were found between the granular cell numbers of male juvenile and adult rats exposed to DS prenatally (38). These results may be sex-dependent and require a detailed explanation. Besides the investigation of the nervous tissue, we also examined the lung tissues of the rats exposed prenatally to DS, and no histological difference was observed between the control and test groups (39).

As understood from the detailed description here, prenatal exposure to DS can cause a number of challenges in fetal tissues. Our present study results indicate that DS causes a decrease in the cardiac ventricular wall mass of 20-week-old rats exposed to DS in utero. All of these findings point to the importance of avoiding DS during pregnancy, regardless of the recommended dose (14). While the possibility of treatment with antirheumatic drugs in pregnancy has become inevitable, NSAIDs should be considered in terms of the teratogenic risks to human embryos.

We observed significantly reduced cardiac ventricle wall volume in 20-week-old offspring rats prenatally exposed to DS (1 mg kg^{-1} day^{-1}) for 15 days, from gestational days 5 to 20, which is the critical period of organogenesis in the rat, equivalent to 3–6 weeks after fertilization in human embryos (16). However, in this study, no significant histological changes were found between the controls and the test group. How the volume of the heart ventricular wall decreases with administration of DS is not fully understood. Further studies should investigate how the effect of DS on the heart tissue occurs, by reducing the number of myocytes, decreasing the size of the cells, or affecting connective tissue between the cells. Considering all of these complications, women should refrain from taking DS, especially during pregnancy.

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