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Investigation of the potential teratogenic effects of fructose on the embryo using the rat whole embryo culture model

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ABSTRACT

Excessive consumption of fructose-sweetened foods and beverages is a growing concern worldwide. Studies have demonstrated that fructose consumption before and during pregnancy can result in adverse outcomes such as decreased decidualization, increased fetal losses, and low birth weight. The study investigated the teratogenic effects of fructose on rat embryos during organogenesis using whole embryo culture. Within the scope of the study, 4 groups were formed as control, low, medium, and high-dose fructose (HDF) with 10 embryos in each group. The 9.5-day-old rat embryos were cultured with different concentrations of fructose (1, 5 and 10 mM) for 48 h and the possible effects of fructose were examined using morphological scoring, histochemistry, immunofluorescence, and TUNEL methods. According to the analyses, protein synthesis and proliferation were decreased, vascular formation was suppressed, and apoptosis was increased in embryos exposed to fructose, especially at concentrations of 5 mM and above. According to the morphological scoring results, it was determined that heart, hind limb, and somite development were retarded in all experimental groups compared to the control group, developmental retardation increased in direct proportion to fructose concentration, and also significant malformations were observed in all parameters examined in the HDF group. In addition, analysis of yolk sac diameter, head length, crown rump length and somite numbers showed that these parameters were significantly decreased in all experimental groups. End of the study, it was concluded that fructose at concentrations of 1 mM and above may induce embryonic development retardation and other anomalies by decreasing protein synthesis and cell proliferation, suppressing vascular formation, and increasing apoptosis in embryonic tissues.

1. Introduction

Fructose is a monosaccharide carbohydrate found naturally in fruits, vegetables, honey, and in the structure of sucrose (Arslan and Şanlıer, 2016; Johnson et al., 2007; Tran et al., 2009; Turhan and Saka, 2016; Zhang et al., 2017). In recent years, the primary source of dietary fructose has shifted from natural foods to industrial fructose. This shift is undoubtedly due to the significant advances in the food industry, which have led to a substantial increase in the production and use of fructose in ready-to-eat foods (Arslan and Şanlıer, 2016). The food industry commonly uses “high fructose corn syrup” (HFCS) as a sweetener, flavor enhancer, hydrator, osmotic stabilizer, freezing point suppressant, and color and flavor enhancer in food and beverages (Hanover and White, 1993). HFCS is a superior liquid sweetener that can effectively replace sucrose in terms of taste, calories, and content. This is achieved by converting glucose obtained from corn starch into fructose through enzymatic isomerization using α-amylase and glucoamylase enzymes (Kızılaslan, 2017; Örek and Mertoğlu, 2020). In recent years, the average daily intake of fructose has significantly increased to 60–150 gr due to the consumption of foods and beverages containing sucrose and HFCS as sweeteners, which is a substantial rise from the past average daily intake of 16–20 gr through the consumption of natural food

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sources containing fructose (Arslan and Şanlıer, 2016). These values have almost tripled the maximum recommended daily amount of simple sugar, which is 50–60 g. Because the World Health Organization recommends that the daily amount of added sugar should not exceed 10% of the daily energy intake (Giussani et al., 2022; Vickers et al., 2011). If this increasing trend in fructose intake cannot be stopped, the amount of fructose consumed daily may reach 50% or even more of the daily energy needed. The effects of fructose on health have become a topic of increased interest, given the rise in dietary fructose intake. Scientific evidence has consistently shown that a diet high in fructose, combined with low physical activity and high energy intake, significantly increases the risk of developing various diseases. These include hypertension, metabolic syndrome, obesity, insulin resistance, impaired glucose tolerance, type 2 diabetes, hyperlipidemia, cardiovascular diseases, hyperuricemia, gout, and kidney diseases (Johnson et al., 2007; Köseler-Beyaz and Kızıltan, 2019; Turhan and Saka, 2016). Metabolic syndrome is diagnosed with confidence when at least three of the following symptoms are present: abdominal obesity, elevated triglycerides, low HDL, high blood pressure, and high fasting glucose (Ervin, 2009). Metabolic syndrome causes reproductive problems in women of childbearing age, including subfertility, increased pregnancy losses, and preeclampsia. It also reduces the chances of pregnancy and increases the risk of miscarriage in women undergoing infertility treatment (Bellver et al., 2010; Bellver and Pellicer, 2004; Boots et al., 2014; Michalakis et al., 2013; Moragianni et al., 2012). Hypertension during pregnancy causes prenatal hypoxia, leading to fetal growth retardation by disrupting oxygen and nutrient transfer between mother and offspring (Herrera et al., 2014; Hutter et al., 2010). In a study conducted on mice fed with a feed containing 66.8% fructose; It has been shown that excessive fructose consumption decreases steroid hormone synthesis and creates an unfavorable environment in the uterus, resulting in decreased pregnancy rates, deciduation problems, increased fetal losses and low birth weight (Saben et al., 2016). Excessive fructose consumption during pregnancy has been shown in previous animal studies to result in low birth weight, hyperglycemia, hyperinsulinemia, and hypertriglyceridemia in fetuses due to decreased placental vascularization and weight (Giussani et al., 2022). Fructose can cross the placental barrier through diffusion, active transport (Giussani et al., 2022; Sloboda et al., 2014) and GLUT-8 and GLUT-9 hexose transporters (Asghar et al., 2016). Hyperglycemia causes a significant increase in blood fructose levels in diabetic humans, as reported by Kawasaki et al. (2002). Studies conducted on mice fed a diet containing 60% fructose and on rats fed a standard diet and 20% fructose have shown that excessive fructose consumption during pregnancy leads to an increase in fructose levels in maternal and fetal blood, fetal growth retardation, and placental anomalies (Asghar et al., 2016; Vickers et al., 2011).

The results of studies on serum fructose levels in humans and rats are highly variable. Publications report that the blood fructose level varies between 0.0081 and 1.09 mM in people with a normal diet, and between 0.2 and 17.2 mM in people with a diet rich in fructose or sucrose (Douard and Ferraris, 2008; Hui et al., 2009; Kawasaki et al., 2002; Trindade et al., 2011). Studies on rats revealed that rats fed normal feed had blood fructose levels below 0.01 mM, whereas rats fed fructose-rich feed had levels between 0.2 and 0.1 mM (Douard and Ferraris, 2008; Patel et al., 2015). Additionally, Vickers et al. (2011) reported that the blood fructose level in rats fed a feed containing 20% fructose was 0.54 mM, and Douard et al. (2010) reported that the blood fructose level in rats fed a feed containing 60% fructose was 1.4 ± 06 mM.

Fructose has been extensively studied in vitro for its potential as an energy substrate in culture media during the pre-implantation period of embryo development. Multiple studies conducted during this period have consistently reported that fructose significantly improves embryo quality, increases the number of embryos reaching the blastocyst stage, and enhances blastocyst cell count However, its teratogenic effects have not been a primary focus of these studies (Bhuiyan et al., 2007; Hwang et al., 2004; Ludwig et al., 2001).

Developmental anomalies often originate during the post-implantation stages of pregnancy. This is a critical period when organs begin to form, and growth and differentiation are highly active (Harris, 2012). The period of organogenesis, which corresponds to 15–60 days of embryonic development in humans and 6–12 or even 15th day of gestation in rats, is a crucial stage of embryonic development (Abebe et al., 2022; Magdy et al., 2023; Manson and Kang, 1994). Agents and conditions that affect organogenesis are the primary cause of most congenital malformations. The direct effects of fructose on embryo or fetal development cannot be isolated from those on the maternal system using in vivo methods. The whole embryo culture (WEC) enables the study of the effects and mechanisms of action of various pharmacological, toxicological, and teratogenic agents on embryo development independently of the maternal system. WEC is a culture technique that allows mammalian embryos in the process of organogenesis to develop in vitro in a manner similar to that in vivo (Hang and Chang, 2012; Nisari and Ülger, 2010; Sadler, 1985). The literature review did not reveal any prior study on the harmful effects of fructose on embryo development using the WEC model. This study aims to investigate the possible teratogenic and other harmful effects of fructose on embryo development by examining the development of in vitro embryos exposed to different concentrations of fructose during organogenesis using the WEC model.

2. Material and methods

2.1. Animals and mating

All experimental procedures in this project (Project no: TYL-2020-10011) supported by Erciyes University Scientific Research Projects Commission were carried out in accordance with the Ethics Committee Approval of Erciyes University Animal Experiments Local Ethics Committee dated 06.11.2019 with decision number 19/218. Twenty 60-day-old female Wistar albino rats were obtained from Erciyes University Faculty of Medicine Hakan Çetinsaya Experimental Research and Application Center (DEKAM). The rats were housed together overnight (between 17.00 and 08.00) with an adult male rat (that is 3 females and 1 male per cage) for mating purposes. Following separation of the male animals, a vaginal smear test was performed to confirm successful mating. Female rats that exhibited sperm during this test were considered to be 0.5 days pregnant. They were then housed for 9 days in cages located in air-conditioned rooms with a temperature maintained at 21 °C. Food and water were provided ad-libitum in a 12-h light/12-h dark environment.

2.2. Serum preparation for culture

After 9.5 days, all animals were anesthetized with a mixture of ketamine (60 mg/kg) and xylazine (7 mg/kg). After anesthesia, the abdominal wall of the female rats was opened and approximately 8–10 cc of blood was taken from the aortic bifurcation using a 10 cc syringe. for culture was performed according to the protocol previously reported by Nisari and Ulger (Nisari and Ülger, 2010).

2.3. Whole embryo culture (WEC)

Embryos lined in the uterine horn were removed and transferred to a sterile petri dish filled with "Hank's Balanced Salt Solution'' (HBSS). The uterine muscle was opened along the anti-mesometrial margin with a pair of sterile forceps under stereomicroscope (Nikon SMZ 800) in a laminar airflow chamber (Telsar AH-100). The resulting decidual tissue mass was then separated from the center and the embryo inside was visualized. The decidual tissue was dissected without damaging the embryo. Reicherts' membrane was removed from the embryonic pole by forceps. Embryos were divided into groups of five and placed in 50 ml sterile glass culture bottles (1 embryo per 1 ml of serum) containing 5 ml

normal rat serum. The embryos in the bottles were initially exposed to a gas mixture of 5% O2, 5% CO2, and 90% N2 for 1 min (Soliman and Madkour, 2021). The bottles were then sealed with cork stoppers and placed on rotating rollers at 30 rpm/min in an incubator at 37 °C. After 24 h of explantation, the bottles were removed from the incubator, exposed to a second gas mixture containing 20% O2, 5% CO2, and 75% N2 for 1 min, and returned to the incubator. After 24 h, the culture bottles were removed from the incubator and re-gassed with a third gas mixture, which consisted of 40% O2, 5% CO2, and 55% N2, for 1 min. The bottles were then returned to the incubator for 4 h. At the end of this period, the embryos were transferred to petri dishes containing HBSS and evaluated under a microscope using the morphological scoring system developed by Van Maele-Fabry et al. (1990).

2.4. Assembly of the study groups

Ten embryos were assigned to each of the four groups (Control, LDF, MDF, and HDF) from the collected 9.5-day-old embryos. The LDF group was supplemented with 1 mM fructose, the MDF group with 5 mM fructose, and the HDF group with 10 mM fructose, while the control group received only rat serum. Following a 48-h culture period, the embryos were examined under a microscope and scored morphologically.

2.5. Histopathological analyzes

The embryos were fixed in 4% paraformaldehyde solution for 24 h and then placed in 30% sucrose solution for 36–48 h. The tissue blocks, which were then blocked with cryomatrix (Thermo Fisher Scientific, Waltham, USA) and made suitable for frozen sectioning, were cut at a thickness of 7 μm with a cryotome (Thermo Fisher Scientific, Waltham, USA). Tissue sections taken on polylysine-coated slides were stained with the methods described in the subheadings and examined with an Olympus BX51 model microscope (Olympus Corp. Tokyo, Japan) and photographed with the camera of the same microscope.

2.5.1. Hematoxylin and eosin staining (H&E)

Frozen sections stored at −80 °C were placed in cassettes and kept at room temperature for 10 min, then fixed in acetone at −20 °C for 10 min and washed in distilled water 2 times for 5 min. The sections were then stained with hematoxylin for 3 min and eosin for 2 min, washed with distilled water after each staining step (2 times for 5 min) and covered with mounting medium.

2.5.2. AgNOR (Silver-stained nucleolus organizer regions) staining

The preparations stored at −80 °C were kept at room temperature for 10 min and then immersed in 90% ethyl alcohol for 15 min. The sections were then washed in distilled water for 5 min and dried at room temperature. 3–4 drops of silver dye were added to the preparations in the Petri dish, covered with coverslips and kept in an oven at 37 °C for 15 min. The slides were removed from the oven, washed with distilled water, dried at room temperature and covered with mounting medium.

2.6. Immunofluorescen staining (vWF)

The tissue sections stored at −80 °C were dried at room temperature for 10 min. Slides were placed in acetone and fixed at −20 °C for 10 min. The slides were washed with Phosphate Buffered Saline (PBS) solution 3 times for 5 min and incubated with normal goat serum for 20 min. Sections were incubated with primary antibody (ab9378, Abcam, Cambridge, MA) diluted 1/100 in a humid environment at 4 °C overnight, washed in PBS (3 times for 5 min), and then secondary antibody (Jackson Immuno Research, Newmarket, UK) diluted 1/200 was applied. Cell nuclei were stained with DAPI and covered with fluoromount-G. Sections used as negative control were treated with PBS instead of primary antibody. Numerical data on “vWF” were calculated using “ImageJ” software.

2.7. TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labelling) analysis

Sections stored at −80 °C were kept at room temperature for 10 min and fixed in absolute ethyl alcohol for 10 min. Apoptosis in embryonic tissues was determined by TUNEL analysis as described in previous publications (Gur and Aktas, 2021; Gur et al., 2021). For this purpose, “Apoptag Fluorescein In Situ Apoptosis Detection kit” (EMD Millipore, Darmstadt, Germany) was used. Cell nuclei were labeled with DAPI-containing mounting medium and the preparations were visualized and photographed with the Olympus BX51 model immunofluorescence microscope.

2.8. Statistical analysis

SPSS 22.0 statistical package program was used in the evaluation of the research data. The Kolmogorov-Smirnov test was used to determine whether the data were normally distributed. Since there were four groups in the variables determined to be normally distributed, One-Way Analysis of Variance (ANOVA) was used for comparisons between groups. The homogeneity of variances was examined with Levene’s test to determine which post hoc test would be performed. Comparison of the groups was performed with Tukey test in the data where the variances were homogeneous and with Tamhane test in the data where the variances were not homogeneous. Data were expressed as mean ± SD and $p < 0.05$ values were considered statistically significant.

3. Results

3.1. Morphological scoring findings

The developmental levels of the embryos of the experimental and control groups were evaluated using the morphological scoring system developed by Brown and Fabro (1981) and Van Maele-Fabry et al., (1990) (Table 1). In this evaluation method, seventeen parameters including vitellus sac vascularization, flexion, allantois, heart, caudal neural tube, anterior, middle and posterior brain, ear, eye, nose, pharyngeal arches, maxilla, mandible, somites and anterior and posterior limbs were scored between zero and five in 11.5 days old embryos.

In the control group embryos, no malformation was observed, all embryos were viable and their development was normal. It was also found that the development of the yolk sac was normal and there were well-developed, large diameter vessels in the yolk sac that anastomosed with each other and blood flow could be observed in the lumen.

In the morphological analysis of the experimental groups, it was observed that yolk sac development in LDF group embryos showed similar characteristics with the control group, while in MDF and HDF groups, yolk sac diameter (YSD), vascularization in the vitellus sac, and vessel diameters decreased significantly with increasing fructose amounts ($p < 0.05$) (Fig. 1) (Table 1). When heart development was examined, it was observed that dysmorphogenesis increased in parallel with the increase in fructose level in all experimental groups including the LDF group, and the heart tube was S-shaped in the HDF group, where developmental disorders were observed at the highest rate (Fig. 1) (Table 1). The caudal neural tube development in LDF group embryos was almost the same with the control group ($p > 0.05$). In MDF and HDF group embryos, although neural folds and neural grooves were formed and started to form the neural tube by facing each other at the level of 4-5th somites, neural tube formation was not completed. Neural tube defects observed in MDF and HDF groups increased in direct proportion to fructose concentration (Fig. 1) (Table 1).

It was observed that the mesencephalon was formed in the control and LDF group embryos and its border with the diencephalon became clear, and the prosencephalon folds were completely merged. In the

Table 1
Effects of fructose on rat embryonic growth and development in vitro.

	Groups				
	Control	LDF	MDF	HDF	*P*
Mean Yolk Sac Diameter	3,96 ± 0,82[a]	2,56 ± 0,57[b]	2,49 ± 0,52[b]	2,53 ± 0,57[b]	0,00
Crown Rump Length	3,32 ± 0,79[a]	1,90 ± 0,48[b]	1,76 ± 0,52[b]	1,73 ± 0,43[b]	0,00
Somite Number	25,57 ± 4,07[a]	20,61 ± 2,75[a]	16,00 ± 4,51[b]	12,00 ± 6,15[b]	0,00
Head Length Measurement	1,28 ± 0.24[a]	1,18 ± 0,53[b]	1,13 ± 0,32[b]	1,06 ± 0,75[c]	0,00
Morphological score					
Yolk Sac Circulation	4,85 ± 0,37[a]	4,23 ± 0,92[a]	3,20 ± 0,77[b]	2,30 ± 1,03[c]	0,00
Allantois	3,00 ± 0,00[ab]	3,30 ± 0,75[a]	2,86 ± 0,63[ab]	2,38 ± 0,76[b]	0,01
Flexion	4,00 ± 1,00[ab]	4,53 ± 0,77[a]	2,93 ± 1,16[bc]	2,28 ± 0,96[c]	0,00
Heart	4,71 ± 0,48[a]	3,84 ± 0,37[b]	3,13 ± 0,74[c]	2,46 ± 0,87[c]	0,00
Caudal Neural Tube	4,57 ± 0,78[a]	3,84 ± 0,37[a]	2,93 ± 0,59[b]	2,15 ± 0,68[c]	0,00
Hind Brain	4,57 ± 0,78[a]	4,23 ± 0,72[a]	2,80 ± 0,94[b]	2,30 ± 0,85[b]	0,00
Mid Brain	4,71 ± 0,75[a]	4,23 ± 0,72[a]	2,80 ± 0,94[b]	2,30 ± 0,85[b]	0,00
Fore Brain	4,71 ± 0,75[a]	4,23 ± 0,72[a]	2,80 ± 0,94[b]	2,23 ± 0,85[b]	0,00
Otic System	4,57 ± 0,78[a]	4,23 ± 0,72[a]	2,46 ± 1,30[b]	2,00 ± 1,08[b]	0,00
Optic System	4,57 ± 0,78[a]	4,23 ± 0,72[a]	2,46 ± 1,30[b]	2,00 ± 1,08[b]	0,00
Olfactory System	2,00 ± 0,00[a]	1,69 ± 0,48[ab]	1,60 ± 1,05[ab]	1,15 ± 0,37[b]	0,05
Branchial Bars	2,00 ± 0,00[a]	1,53 ± 0,51[a]	1,53 ± 0,51[a]	1,00 ± 0,00[b]	0,00
Maxillary Process	2,00 ± 0,00[a]	1,69 ± 0,48[a]	1,46 ± 0,51[b]	1,07 ± 0,27[b]	0,00
Mandibular Process	2,00 ± 0,00[a]	1,84 ± 0,37[a]	1,66 ± 0,48[ab]	1,38 ± 0,50[b]	0,01
Fore Limb	2,00 ± 0,00[a]	1,69 ± 0,48[a]	1,00 ± 0,00[b]	1,15 ± 0,37[b]	0,00
Hind Limp	1,85 ± 0,37[a]	1,15 ± 0,37[b]	1,00 ± 0,00[bc]	1,00 ± 0,00[b]	0,00
Somites	4,57 ± 0,78[a]	3,53 ± 0,51[b]	2,80 ± 0,94[b]	2,00 ± 0,91[c]	0,00
Total Score	61,57 ± 6,52[a]	54,07 ± 8,05[a]	39,60 ± 11,22[b]	31,53 ± 10,22[b]	0,00

*Same letters on the same line show the similarity between groups, different letters show the difference between groups.

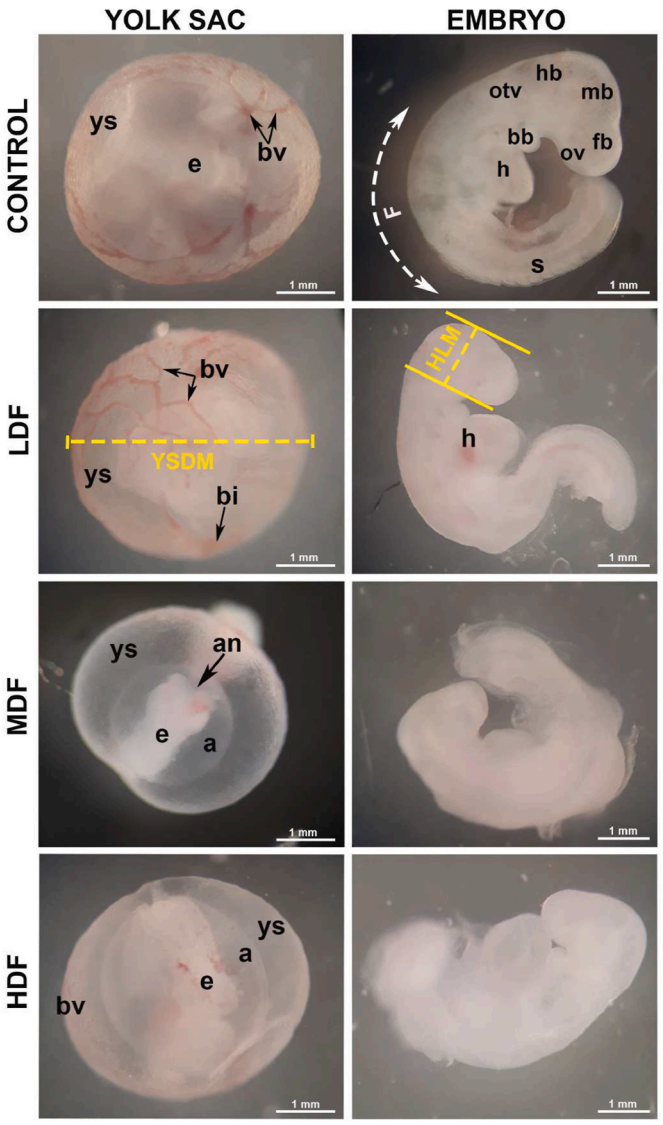


Fig. 1. Control, LDF, MDF and HDF group rat embryos inside and outside the yolk sac (vitellous sac) on day 11.5 of pregnancy after 48 h of culture. **a,** amniotic sac; **an,** anterior neuropore; **bb**, branchial bar; **bi,** blood island; **e,** embryo; **F,** c-shaped flexion; **fb**, forebrain; **h,** heart; **hb**, hindbrain; **HLM,** head length measurement; **mb**, midbrain; **otv**; otic vesicle; **ov**, optic vesicle; **s,** somite; **ys,** yolk sac; **YSDM,** yolk sac diameter measurement.

forebrain and midbrain development of MDF and HDF group embryos, there was a significant developmental retardation compared to the control and LDF groups ($p < 0.05$) (Table 1). When the hindbrain development was examined, it was observed that the development continued in the normal course in the control and LDF groups and the upper edge of the fourth ventricle and the pons were fused. Significant malformations were observed in the hindbrain development of MDF and HDF group embryos ($p < 0.05$) (Table 1).

In addition to these findings, significant malformations were observed in maxillary protrusion, mandibular protrusion, otic system, optic system, and olfactory system parameters along with insufficient flexion in MDF and HDF group embryos ($p < 0.05$) (Table 1).

By comparing the morphological score results of the experimental and control groups, it was determined that the development of the heart, hind limb and somite in the LDF group decreased significantly compared to the control group, and there was no significant difference between the two groups in terms of total score results ($p < 0.05$) (Fig. 1) (Table 1). MDF group embryos showed significant developmental retardation compared to the control group in all other parameters except allantois, nose, pharyngeal arches and mandible development and total score results ($p < 0.05$). HDF group embryos showed significant malformations in all parameters examined compared to the control group ($p < 0.05$). When the experimental groups were compared among themselves; It was determined that there were significant malformations in the yolk sac vascularization, caudal neural tube, pharyngeal arches and somite development in the HDF group embryos compared to the MDF group ($p < 0.05$), while there was no significant difference in other parameters ($p > 0.05$).When the HDF group was compared with the LDF group, a significant developmental retardation was found in the HDF group compared to the LDF group in all other parameters except nasal and hind limb development ($p < 0.05$) (Fig. 1), (Table 1).

In addition to the morphological scoring parameters mentioned above, as a result of statistical examinations on yolk sac diameter (YSD), head length measurement (HLM), Crown Rump Length (CRL) and somite number data, it was determined that there was a statistically significant decrease in YSD, HLM, CRL and somite number parameters in the experimental groups in inverse proportion to the increase in fructose compared to the control group ($p < 0.05$), while the differences

between the experimental groups were found to be insignificant in all parameters except for the HLM group ($p > 0.05$) (Fig. 1) (Table 1). Although there was no statistical difference between LDF and MDF groups in terms of HLM parameter ($p > 0.05$), the difference between these two groups and HDF group was determined to be significant ($p < 0.05$).

3.2. Histochemical findings

3.2.1. H&E findings

In the histological analysis of the control group embryos, it was observed that the histological structures of all other tissues and organs, including the forebrain, midbrain and hindbrain, heart and medulla spinalis, were normal. In addition, the histologic structure of the vitellus sac in this group showed a consistent course and dark staining. The dark staining in the vitellus sac was probably caused by new cells formed with the onset of hematopoiesis (Fig. 2).

Morphologic and histologic development of LDF embryos was similar to the control group. On the other hand, the development of MDF and HDF group embryos was found to be significantly retarded compared to the control group. The HDF group had the most prominent embryonic developmental defect. In the histological and morphological examinations, it was found that the embryos in this group were considerably smaller, the anterior, middle and posterior brain tissues were not sufficiently developed, the cranial and caudal neuropore were not completely closed, and the cellular and extracellular structures in all other tissues and organ structures including the heart were not sufficiently developed compared to the control group. In addition, histologic examinations of MDF and HDF group embryos revealed that the vitellus sac wall was highly thin and irregular (Fig. 2).

3.2.2. Ag-NOR findings

With the increase in fructose concentration in the experimental groups, AgNOR counts in the head, body and tail regions of the embryo gradually decreased compared to the control group. In the head and tail regions, while there was no significant difference between the control group and the LDF group ($p > 0.05$) in terms of the number of AgNORs, the difference between the control group and MDF and HDF was significant ($p < 0.05$). Additionally, the difference between the MDF and HDF groups was also significant ($p < 0.05$) (Table 2) (Fig. 3).

Although the differences in the number of AgNORs between the experimental groups in the trunk region were not significant ($p > 0.05$), the difference between the experimental and control groups was significant ($p < 0.05$) (Table 2) (Fig. 3).

TAA/NA (total AgNOR area/nuclear area) ratios in the head, body and tail regions of the experimental group embryos decreased compared to the control group. The decrease in the head region was significant in all experimental groups, the decrease in the body region was significant

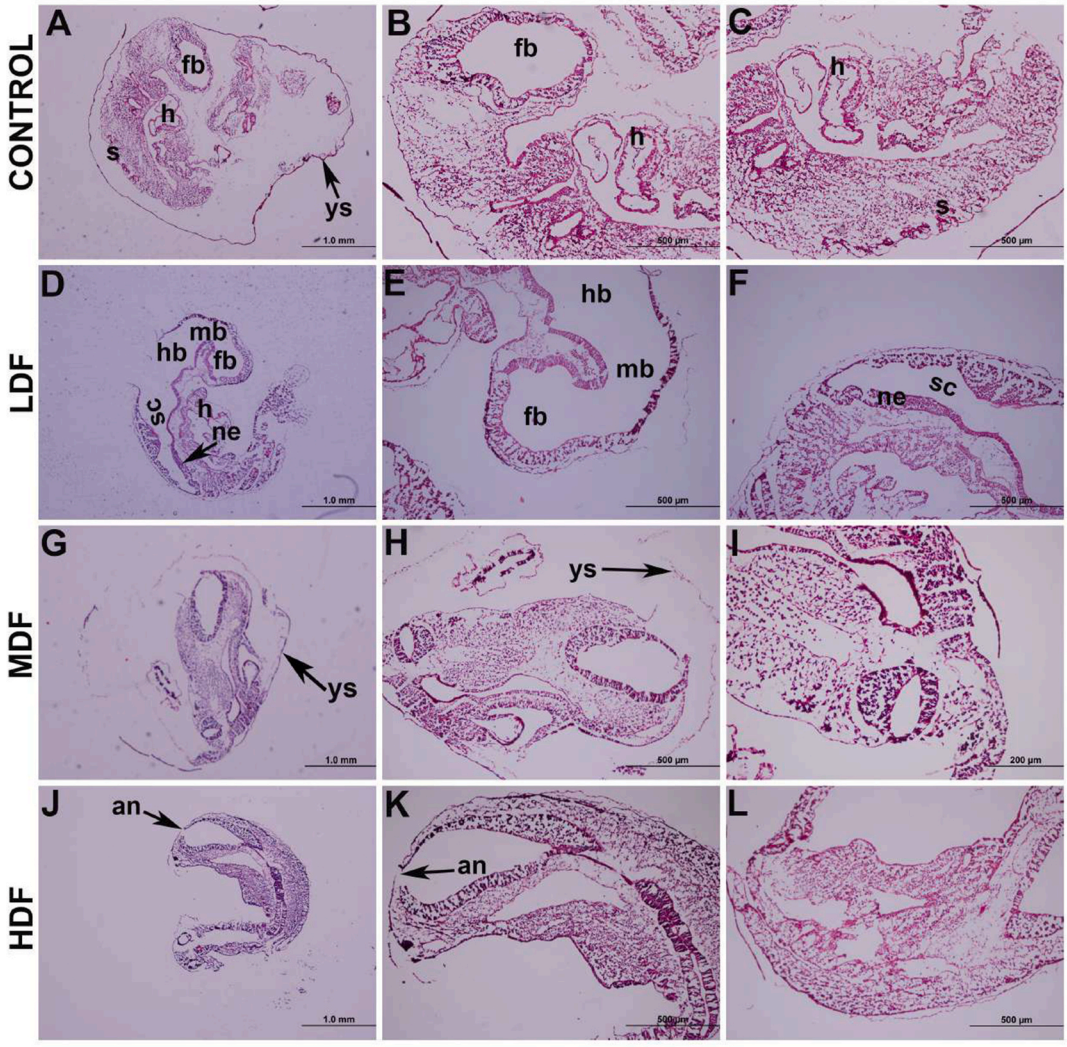


Fig. 2. Light microscopic view (H&E) of control, LDF, MDF and HDF group rat embryos at 11.5 days of pregnancy. **an,** anterior neuropore; **fb,** forebrain; **h,** heart; **hb,** hindbrain; **mb,** midbrain; **ne,** neuroepithelium; **s,** somite; **sc**, spinal cord; **ys,** yolk sac (vitellous sac) (A, D, J x40; B, C, E, F, G, K, L x100; H, I x200).

Table 2
Mean AgNOR number, TAA/NA, and vWF + and TUNEL + cell numbers of control and experimental groups.

		Groups				p
		Control	LDF	MDF	HDF	
HEAD	Mean AgNOR Number	3,18 ± 1,48[a]	3,05 ± 1,29[ab]	2,88 ± 1,46[b]	2,58 ± 1,08[c]	0,001
	TAA	1132,61 ± 563,44[a]	657,01 ± 352,70[b]	733,29 ± 421,37[b]	726,70 ± 369,81[b]	0,001
	NA	5781,16 ± 2097,97[a]	4026,31 ± 1767,31[b]	4646,61 ± 1914,08[c]	5097,91 ± 4912,11[c]	0,001
	TAA/NA	0,20 ± 0,10[a]	0,17 ± 0,07[b]	0,16 ± 0,07[b]	0,16 ± 0,14[b]	0,001
TRUNK	Mean AgNOR Number	3,30 ± 1,50[a]	2,93 ± 1,19[b]	2,80 ± 1,26[b]	2,70 ± 1,13[b]	0,001
	TAA	1158,07 ± 549,56[a]	899.33 ± 396,22[bd]	781,86 ± 381,80[c]	848,07 ± 21,62[dc]	0,001
	NA	6154,32 ± 2442,22[a]	5219,76 ± 1994,84[b]	4554,41 ± 1893,17[c]	4947,76 ± 1856,60[bc]	0,001
	TAA/NA	0,19 ± 0,08[a]	0,18 ± 0,08[ab]	0,17 ± 0,07[b]	0,17 ± 0,06[b]	0,002
TAIL	Mean AgNOR Number	3,41 ± 1,77[a]	3,33 ± 1,55[a]	2,94 ± 1,36[b]	2,36 ± 0,98[c]	0,001
	TA	997,56 ± 436,06[a]	933,99 ± 468,49[a]	796,49 ± 389,82[b]	896,48 ± 310,19[ab]	0,001
	NA	6174,40 ± 687,78[a]	5582,01 ± 2098,95[ab]	4948,72 ± 2088,85[ab]	6477,04 ± 2227,15[b]	0,001
	TAA/NA	0,18 ± 0,18[a]	0,16 ± 0,06[ab]	0,16 ± 0,06[ab]	0,14 ± 0,04[b]	0,026
TUNEL + cell number		32,60 ± 14,69[a]	49,00 ± 13,96[b]	49,65 ± 21,94[b]	73,70 ± 14,81[c]	0,000
vWF + cell number		20 ± 2,71[a]	15,90 ± 3,09[b]	12,60 ± 2,16[c]	10,80 ± 1,93[c]	0,000

*Same letters on the same line show the similarity between groups, different letters show the difference between groups.

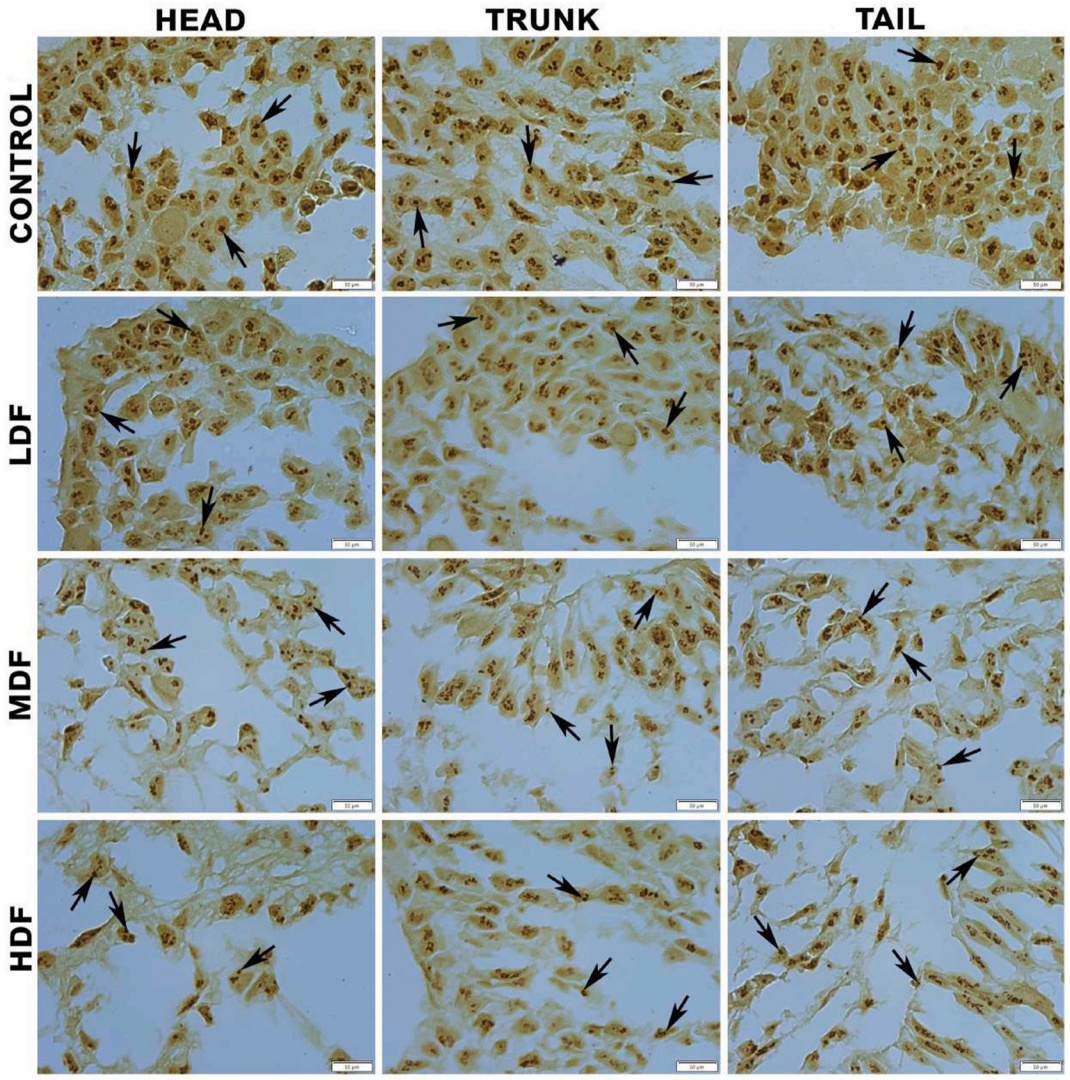


Fig. 3. Tissue sections of the head, trunk and tail regions of control, LDF, MDF and HDF group embryos (AgNOR staining; x200).

in MDF and HDF groups, and the decrease in the tail region was significant only in the HDF group ($p < 0.05$).

3.3. Immunofluorescent findings

The findings of immunofluorescence staining to detect vWF-positive cells in experimental and control group embryos are shown in Fig. 4 and the data on the number of vWF-positive cells are shown in Table 2. Immunofluorescence staining results showed that the number of vWF-positive cells in embryos decreased with the increase in fructose concentration in the experimental groups. statistical results showed that the number of vWF-positive cells in all experimental groups was significantly reduced check against to the control group ($p < 0.05$). There was no significant difference between the HDF and MDF groups, which was the group with the least number of vWF-positive cells ($p > 0.05$). However, the difference between these two groups and the LDF group was statistically significant ($p < 0.05$).

3.4. TUNEL findings

TUNEL-positive cells were observed in the embryo and vitellus sac in all of the groups examined (Fig. 5), and the data on the number of apoptotic cells of the groups are given in Table 2. The number of TUNEL-positive cells in the LDF, MDF and HDF groups was significantly increased compared to the control group ($p < 0.05$). While there was no significant difference between the LDF and MDF groups ($p > 0.05$), the number of TUNEL-positive cells in the HDF group embryos increased significantly ($p < 0.05$).

4. Discussion

Without using the WEC technique, it is impossible to examine the direct effects of fructose on an embryo or fetal development independently of the maternal system using in vivo methods. Therefore, in the present study using this technique, the possible effects of different doses of fructose on embryo development were investigated using morphological, histochemical, immunofluorescence, and TUNEL analysis methods.

Almost all in vitro studies on the effects of fructose on embryo development cover the developmental processes up to the blastocyst stage, also called the pre-implantation stage (Bhuiyan et al., 2007; Hwang et al., 2004; Ludwig et al., 2001). Furthermore, these in vitro studies aimed to investigate the potential of fructose as an energy substrate in culture media rather than its teratogenic effects on embryo development (Bhuiyan et al., 2007; Hwang et al., 2004; Ludwig et al., 2001). In a study, it was reported that 1 g/L fructose added to the culture

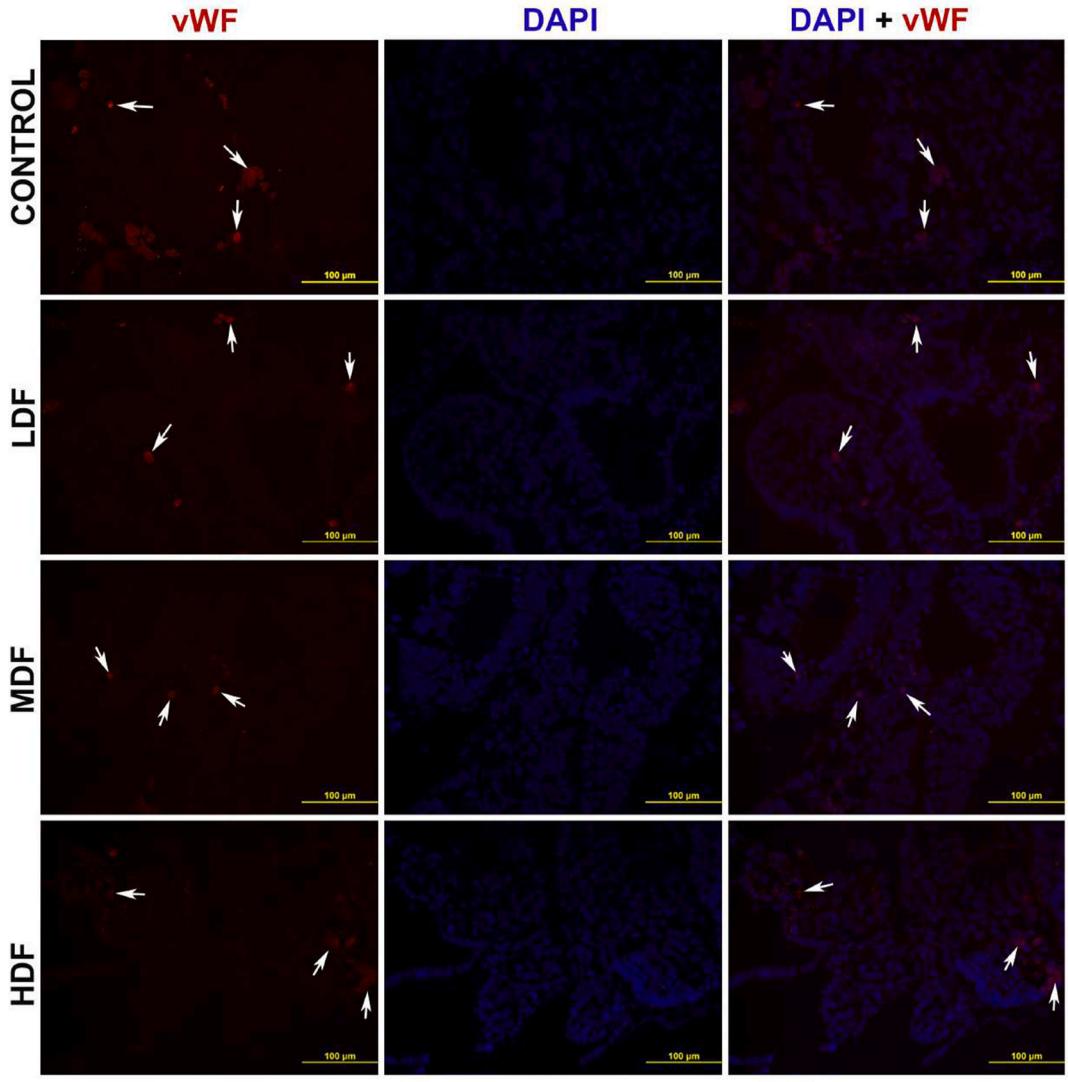


Fig. 4. Immunofluorescent expression of vWF in control, LDF, MDF and HDF group embryos. arrows indicate vWF + cells (x200).

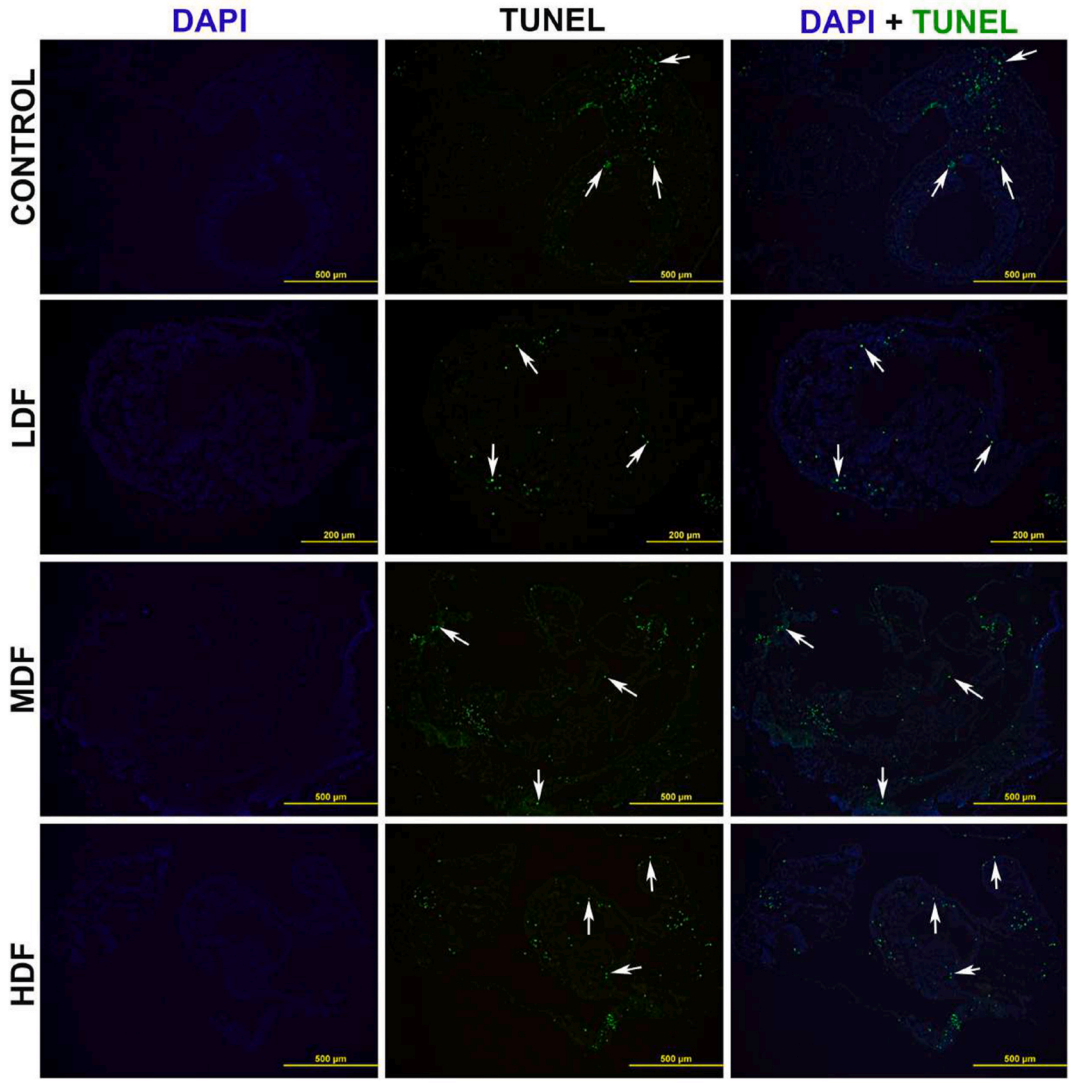


Fig. 5. TUNEL results in control, LDF, MDF and HDF group embryos. Green fluorescence (arrows) indicates TUNEL positive cells (x200). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

medium increased the number of cells in mouse embryos at the morula and blastocyst stage (Sakkas et al., 1993). It has been shown that adding 0.25 mM fructose to the culture medium in hamster embryos increases the number of embryos reaching the morula and blastocyst stages and the number of cells in them. It was determined that adding up to 5 mM fructose to the culture medium caused an insignificant decrease in the number of embryos at the morula and blastocyst stage and the number of cells in them (Ludwig et al., 2001). In a study conducted on bovine embryos, it was observed that adding fructose to the culture medium at concentrations of 0.75, 1.5, 3.0, or 5.6 mM increased the number of embryos at the morula and blastocyst stages compared to those not added. Additionally, it was determined that the embryo increase at 5.6 mM was reduced compared to 1.5 mM (Kwun et al., 2003). It was reported by Tsujii (2017) that adding fructose to the culture medium at concentrations of 0.5, 1.0, 2.5 and 5.0 mM significantly increased the number of blastocysts and hatched blastocysts in mouse embryos compared to the medium without fructose. In addition, it was determined that this increase occurred most at 0.5 and 1 mM concentrations, while the rate of increase at 5 mM concentrations decreased compared to 0.5 and 1 mM. A careful examination of the results of the mentioned studies on the pre-implantation period shows that fructose added to the culture medium as an energy source at concentrations below 5 mM increases the cleavage rate, the number of embryos going to morula and blastocyst, the number of cells in morula and blastocyst stage embryos and the number of hatching blastocysts, while this positive effect starts to decrease at concentrations of 5 mM and above. Contrary to these studies, in the current study, it was observed that the embryotoxic effects of fructose began in the LDF group embryos administered 1 mM fructose. This difference may be due to embryos in different developmental periods meeting their energy needs from other sources. It can be said that the effects of fructose at doses of 5 mM and above on the embryo are compatible with the results of the above-mentioned studies.

In the literature review, very few studies investigating the effects of fructose on embryo development using the WEC model were encountered. As previously mentioned, these studies focused on fructose's potential as an energy substrate in culture media rather than its teratogenic effects. Eriksson et al. (1989) reported that embryos cultured in 6.6 mM D-fructose showed normal growth and morphology despite increased sorbitol accumulation (Eriksson et al., 1989). Contrary to this result, in the current study, MDF group embryos in which fructose was added to the culture medium at a concentration of 5 mM showed significant growth retardation compared to the control group. Gunberg (1976) investigated the effects of using rat serum, dialyzed rat serum, dialyzed rat serum + 5 mM glucose, dialyzed rat serum + 10 mM fructose, and similar culture mediums on embryo development in the WEC technique. It was observed that heartbeat occurred, the otic vesicle formed and the

anterior neuropore closed in all embryos in which rat serum was used as a medium. In addition, it was determined that circulation was observed in the yolk sac in 80 of these embryos, flexion was formed in 75%, and the caudal neuropore was closed in 60%. While it was determined that there was no development in any of the embryos in which dialyzed serum was used as the culture medium, the development level in the embryos in which dialyzed rat serum +5 mM glucose was used was similar to the rat serum group. It was reported that only 20% of the rat embryos in which dialyzed rat serum +10 mM fructose was used as culture medium developed a heartbeat, there was no development in these embryos in terms of other parameters examined and the embryos necrosed. In addition, the same researcher reported that rodent hearts at the 16 somite stage of development were not capable of utilizing fructose for contractile activity in vitro (Gunberg, 1976). In the current study, significant developmental retardation occurred in HDF group embryos, in which fructose was added to the culture medium at a concentration of 10 mM, compared to the control group. The malformations observed are not as severe as those in the related study, although the results are compatible with the findings of the study conducted by Gunberg (1976). This difference between the two studies is probably due to the culture medium. It is thought that the use of normal rat serum instead of dialyzed rat serum as a culture medium in the present study compensated for the adverse effects of fructose and reduced the teratogenic effects.

In the literature review, it was seen that in many studies using the WEC technique, the effects of many chemicals other than fructose on embryo development were evaluated using parameters such as the morphological scoring system, YSD, HLM, CRL, and somite numbers. (Brown and Fabro, 1981; Gwon et al., 2020; Lin et al., 2014; Xing et al., 2010). This study found that the parameters listed above were considerably decreased in the experimental groups compared to the control groups. While there is no existing literature specifically examining the impact of fructose on the parameters we studied, our findings align with previous research, supporting the validity of these criteria in evaluating embryo development.

Ribosomal subunits are synthesized in the nucleus in regions called nucleolus. Genes that synthesize ribosomal RNAs are carried on specific chromosomes in species. In a cell in interphase, the regions of DNA that contain rRNA synthesizing genes and proteins and form the nucleolus are called NORs (Akbulut, 2010; Masabanda et al., 2004). NORs transcribe rRNA, which will form ribosomes in the future (Yilmaz et al., 2020). Actively transcribing NORs, i.e. rDNA regions, have the ability to be stained by silvering. The staining of NORs by silvering method is called AgNOR (Akbulut, 2010; Lee et al., 1999). The increase in the number of AgNORs and TAA/NA ratio in cells is considered an indicator of increased protein synthesis and cell proliferation (Ertekin et al., 2016; Nisari et al., 2016; Yilmaz et al., 2020). In the current study, it was determined that the number of AgNORs in the head, trunk, and tail parts of the embryos in the experimental group decreased compared to the control group. Similarly, it was determined that the TAA/CA ratio in the experimental groups decreased compared to the control group. The results obtained showed that protein synthesis and cell proliferation, and therefore the level of embryonic development, in the embryos of the experimental group, decreased as the amount of fructose increased.

vWF is a large and sticky glycoprotein produced by endothelial cells and used as a marker of endothelial cells (Coffin et al., 1991; Randi et al., 2018). This factor is expressed in megakaryocytes and platelets as well as endothelial cells, and it has been reported that vWF-positive endothelial cells are observed in mouse embryos as early as day 8 of embryonic development (Coffin et al., 1991). The presence of vWF-positive endothelial cells has also been demonstrated in chicken embryos (Yablonka-Reuveni, 1989). In parallel with these studies, vWF-positive cells were observed in all experimental and control group rat embryos in the present study. In this study, it was also observed that the number of vWF-positive cells in the experimental groups decreased proportionally with the applied fructose concentration. This result shows that fructose suppresses new vessel formation by negatively affecting angiogenesis. In the literature review, no similar study was encountered. However, a study reported that ionizing radiation caused a decrease in the number of vWF-positive cells in the yolk sac (Balcioglu et al., 2021). When the results of both studies are evaluated together, it can be said that embryo-toxic factors negatively affect angiogenesis and embryo development by reducing endothelial cell proliferation.

As is known, apoptosis is a programmed and controlled form of cell death that can be initiated by both physiological and pathological stimuli (Elmore, 2007; Gil et al., 1999; Yilmaz et al., 2020). In this study, it was found that the number of cells undergoing apoptosis was significantly higher in the experimental groups than in the control group. The HDF group showed the highest increase in the number of apoptotic cells. Although there are no studies on the apoptotic effects of fructose on the embryo, many embryo-toxic agents have been reported to cause developmental retardation by increasing apoptosis in the embryo and yolk sac (Gwon et al., 2020; Lin et al., 2014). In this study, it can be said that the increase in developmental retardation in embryos directly proportional to fructose concentration may be due to the apoptotic effects of excess fructose on embryonic tissues.

A multitude of in vivo research has been carried out to examine the impact of fructose on embryonic development throughout pregnancy. In these studies, it was found that excessive fructose consumption during pregnancy reduces the pregnancy rate (Saben et al., 2016), increases the fructose level in maternal and fetal blood (Asghar et al., 2016; Vickers et al., 2011), has harmful effects on the placenta (Alzamendi et al., 2012; Asghar et al., 2016; Saben et al., 2016; Vickers et al., 2011), causes fetal growth retardation and causes decreased birth weights (Asghar et al., 2016; Saben et al., 2016). It has been suggested that the developmental defects occurring in the offspring of mice fed with excessive fructose-containing feed are caused by fructose-induced indirect causes such as decreased progesterone synthesis, deterioration of uterine functions and increased oxidative stress in the uterus (Asghar et al., 2016; Saben et al., 2016). Several investigations have indicated that rats ingesting fructose and sucrose-rich meal can have serum fructose levels as high as 1 mM or even 2 mM (Douard et al., 2010; Patel et al., 2015). According to Trindade et al. (2011), the fructose level in the umbilical cord blood of humans is approximately 37.5% greater than in the peripheral circulation. If the identical scenario occurs in rats, it is probable that the fructose concentration in the rat umbilical cord will rise to 2.75 mM or above. The present study found that heart, hind limb, and somite development, vWF-positive cell numbers, YSD, HLM, and CRL values in embryos treated with 1 mM fructose in the LDF group significantly decreased compared to the control group. Additionally, the number of TUNEL-positive cells increased in the LDF group compared to the control group. In this study, the histopathological, morphometric and other changes observed in the yolk sac and embryo in the LDF, MDF, and HDF groups indicate that exposure to fructose at concentrations of 1 mM and above may directly cause developmental retardation in the embryo.

5. Conclusion

The study's results indicate that exposure to fructose at concentrations of 1 mM and higher can cause notable delays in embryo development. This is achieved via decreasing protein synthesis and cell proliferation, inhibiting angiogenesis, and promoting apoptosis This finding indicates that the negative impacts on fetal development and reproductive health produced by excessive consumption of sucrose and fructose during pregnancy may be attributed to both the indirect and direct effects of fructose. This study represents the first documented evidence of the direct harmful impact of fructose on embryonic cells and tissues, as demonstrated through the use of the WEC model. Further investigations are necessary to uncover the precise molecular processes responsible for the embryotoxic effects caused by fructose.

CRediT authorship contribution statement

Hatice Emel Gür: Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Esra Balcıoğlu:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Dilara Patat:** Investigation. **Sümeyye Uçar:** Investigation, Formal analysis. **Fatih Mehmet Gür:** Writing – review & editing, Supervision, Software, Project administration, Methodology, Investigation, Data curation. **Betül Yalçın:** Investigation. **Mehtap Nisari:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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