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RESEARCH ARTICLE

Prenatal and Neonatal Exposure to Glyphosate-Based Herbicide Reduces The Primordial to Primary Follicle Transition in The Newborn Rat Ovary: A Preliminary Study

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ABSTRACT

This study investigated how a glyphosate-based herbicide (GBH) affects the proportional distribution of ovarian follicles that develop from the 18th day of the embryo period (E18) to the 7th postnatal day (PND7) in newborn female rats. A total of 6 pregnant rats that were used in the study were divided into two groups so that there would be 3 pregnant rats in the control group and 3 pregnant rats in the GBH group. Starting from E21 to E18 the pregnant rats in the experimental group were administered at 50 mg/kg/day GBH subcutaneously (s.c.) and the physiological saline was administered as vehicle to the control group. Subsequently, female pups received vehicle or 2 mg/kg GBH from PND1 to PND7. On PND8, all female offspring (neonatal period, 6 newborn female rats from each group) were sacrificed by light ether anesthesia. For the histological examination of the dissected ovaries, the primordial, primary, secondary and preantral follicle numbers were determined using Crossman's modified triple staining method and Periodic Acid-Shiff (PAS) staining methods. The percentage of primordial follicles was significantly higher in the ovaries of female rats in GBH exposed group compare to the control group. However, the percentage of primary, secondary and preantral follicles was lower. Thus, it was observed that prenatal and neonatal GBH exposure decreased the transition of primordial follicle to primary follicle.

Keywords: Endocrine disruptors, follicle composition, glyphosate-based herbicide, newborn rat, ovary

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Glifosat-Bazlı Herbisite Prenatal ve Neonatal Dönemde Maruziyet Yenidoğan Rat Ovaryumunda Primordiyalden Primer Foliküle Geçişi Azaltır: Bir Ön Çalışma

ÖZ

Yapılan bu çalışmada glifosat bazlı herbisit (GBH)'in yenidoğan dişi sıçanlarda embriyo döneminin 18. gününden (E18) doğum sonrası 7.gün (PND7) arasında gelişmekte olan ovaryum foliküllerinin oransal dağılımını nasıl etkilediği araştırılmıştır. Çalışmada kullanılan toplam 6 gebe sıçan; 3 gebe sıçan kontrol grubunda, 3 gebe sıçan GBH grubunda olacak şekilde 2 gruba ayrıldı. Gebe sıçanlara E18'den başlayarak E21. güne kadar günlük subkutan olarak (s.c.) deney grubuna 50 mg/kg/gün GBH ve kontrol grubuna ise taşıt madde FTS (fizyolojik tuzlu su) uygulandı. Daha sonra yenidoğan dişi yavru sıçanlara PND1'den PND7'ye kadar 2 mg/kg dozunda GBH ve taşıt madde uygulamasına devam edildi. Son ilaç uygulamadan bir gün sonra PND8'de (neonatal periyot, 6 dişi yavru/her grupta) yavru dişi sıçanlar hafif eter anestezi ile sakrifiye edildi. Diseke edilen ovaryumların histolojik incelemesi için Crossman'ın modifiye üçlü boyama yöntemi ve Periodic Acid-Shiff (PAS) boyama yöntemleri kullanılarak primordial, primer, sekonder ve preantral folikül sayıları belirlendi. GBH'ye maruz kalan dişi yavru sıçanların ovaryumları kontrol grubuna göre karşılaştırıldığında primordial foliküllerin yüzdesi önemli derecede fazla bulundu. Bununla birlikte primer, sekonder ve preantral folikül yüzdesinin ise azlığı dikkat çekti. Sonuç olarak prenatal ve neonatal GBH maruziyetinin primordial-primer folikül geçişini azalttığı gözlenmiştir.

Anahtar Kelimeler: Endokrin bozucular, folikül kompozisyonu, glifosat-bazlı herbisit, yenidoğan sıçan, ovaryum

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INTRODUCTION

Endocrine disrupting chemicals (EDCs) are a heterogeneous class of environmental pollutants that interfere with the endocrine system by mimicking natural hormones, working antagonistically with natural hormones or by blocking the metabolic pathways of natural hormones that might result temporary or permanent damage to endocrine system (Scognamiglio et al. 2016). Nowadays, it is suspected that more than 800 chemicals interfere with hormone receptors or hormone metabolism and pesticides constitute an important group of these toxic chemicals (Scognamiglio et al. 2016). The term pesticide covers a wide range of chemicals including insecticides, fungicides, herbicides, rodenticides, molluscicides, nematicides, plant growth regulators and others (Aktar et al. 2009). Among these, herbicides, particularly glyphosate-based herbicides (GBHs), have a wide range of use to destroy the weeds in both agricultural and non-agricultural areas (eg, horticulture). After the first introduction to the consumers in 1974, the use of the compound has increased dramatically in parallel to the prevalence of glyphosate-resistant genetically modified crops (Gillezeau et al. 2019). River and surface waters are polluted due to their excessive and unconscious use in agriculture, horticulture, and turfs and hence, humans and other mammals are exposed to this pollutant with food and water (Acquavella et al. 2004). Parvez et al. (2018) found that most of the pregnant women (93%) had glyphosate in their urine. In a recent study, the presence of glyphosate in maternal and umbilical cord serum of pregnant women in three regions of Thailand has been demonstrated (Kongtip et al. 2017). The authors showed that pregnant women working or living with families who are working in agriculture have higher rates of exposure to the herbicide glyphosate compared to pregnant women who are not working in agriculture or non-farmers.

For many years, the use of glyphosate is thought to play a role only in plant metabolic pathways. The herbicidal effect of glyphosate is attributed to the cleavage of the Shikimate pathway by inhibiting the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme, which is responsible for the biosynthesis of essential aromatic amino acids, thereby destroying the protein synthesis and ultimately leading the plant death (Mesnage et al. 2015). The Shikimate pathway is not present in humans but is found in bacteria living in human gut (Ratia et al. 2014). Many studies have shown that glyphosate-induced imbalances in intestinal bacteria may be associated with the emergence of different chronic diseases such as celiac disease (Samsel and Seneff 2013), diabetes (Samsel et al. 2013), and neurological disorder (Seneff et al. 2015). Similarly, the endocrine disrupting effects of glyphosate and GBHs are demonstrated in *in vitro* studies with the human cells (Gasnier et al. 2009) and different model species (Richard et al. 2005, Benachour et al. 2007, Benachour and Séralini 2009, Mesnage et al. 2013, Defarge et al. 2016).

Ovary is one of the most important organs for female fertility due to the production of gamete and biosynthesis of hormones. The development and maturation of ovarian follicles is called folliculogenesis (Cox and Takov 2018). The development of ovaries in rats begins before birth and the first and main change is the formation of follicles (Malekinejad et al. 2011). In the early stage of ovarian development, the ovulocytes are paused in the meiosis-I stage and wait for two key developmental processes: (1) primordial follicle formation, (2) transition from primordial to primary follicle (McGee and Hsueh 2000). This process involves (i) embryonic days of 18 through 21 (E18-21) in which the majority of the oocytes in the rats were in the oocyte places and the apoptosis was observed, (ii) peak days 0 to 3 (PND0-3) in which the apoptosis and primordial follicle formation is present, and (iii) the final PND4-7 stage where no oocytes are observed in ovarian sections since all oocyte cavities have been demolished and shaped as primordial follicles and primary follicle transition is present in ovarian sections (Hirshfield and DeSanti 1995, Pepling and Spradling 2001, Kezele and Skinner 2003). In the neonatal period (PND0-7), ovarian follicle development is independent of the pituitary gonadotropins (Luteinizing Hormone (LH) or Follicle Stimulating Hormone (FSH) and the follicles remain as a preantral follicle (Picut et al. 2015).

Various investigators have reported that GBHs cause ovarian damage and dysfunction in pregnant mice (Ren et al. 2018) and rats (Hamdaoui et al. 2018). However, it is not known whether embryonic and neonatal exposure to endocrine disrupters such as GBHs has an effect on ovarian follicle development in juvenile rats. In this study, we aimed to determine the effect of commercial endocrine disrupting chemical GBH on the proportional distribution of developing ovarian follicles from embryonic day 18 (E18) to postnatal 7th day (PND7) in newborn female rats.

MATERIALS and METHODS

Chemical substance

The glyphosate formulation used in this study was Knockdown 48 SL marketed by Safa Agriculture Inc. in Turkey. The active ingredient is a liquid-water soluble formulation containing 48% isopropylamine salt as excipients and inert ingredients. This GBH was selected based on the fact that it is one of the most widely used herbicide against weeds in Turkey and it is a representative of high glyphosate content

formulation that targets the weeds with difficult eradication.

Animals, Experimental Design and Treatment

This study was approved by Afyon Kocatepe University Animal Experiments Local Ethics Committee (No: 67; Date: 03 May, 2017). A total of six female Wistar Albino two months old rats obtained from the Experimental Animals Unit of Afyon Kocatepe University were used in the study. The rats were kept in propylene cages, in 12-hour dark/light cycle, 22±2 °C temperature and humidity (30-70%) was checked in the rooms. Clean tap water and standard rat feed ad libitum was given. The estrous cycle was followed by vaginal cytology of the rats. Three female rats determined to be in the proestus period and one male rat were placed in the same cage. The next day, rats with sperms in the vaginal smear were found to be pregnant and were recorded as 0 days (E0) of pregnancy. The estrus cycles were monitored for seven days and the pregnancy of the rats which were observed in the period of diestrous was confirmed. The experiment consisted of two parts. In the first part of the experiment, six pregnant rats were divided into the experimental and control groups, each including three pregnant rats. Pregnant rats were given 50 mg/kg/day GBH subcutaneously (s.c.) and control group received physiological saline as vehicle on E18 days. GBH was prepared by dissolving it in saline solution. Both vehicle and GBH were administered daily by subcutaneous injections until delivery. In the second part of the experiment, a total of twelve newborn female rats were divided into two groups of six, the experimental and control groups. After birth, an amount of 2 mg/kg/day vehicle (physiological saline) and GBH were administered to the newborn female rats every 48 hours of from PND1 to PND7 subcutaneously. The doses of GBH administered to pregnant and juvenile rats were selected based on the US Environmental Protection Agency, with reference to the level of no side effect of GBH (NOAEL) (USEPA 1993). After the last drug administration in PND8, the female offspring of both groups were sacrificed by light ether anesthesia and the ovarian-uterus-oviduct triad was collectively removed.

Histological Evaluation and Follicle Count

The ovaries from the organs were dissected under the stereo-microscope. The dissected ovaries were stained in Bouin solution for 3 hours at room temperature. After washing in 50% alcohol, dehydration was performed subsequently in 70%, 80%, 95%, and 100% alcohol. In order to clarify the samples prior to paraffin embedding, they were washed in xylene two times. Paraffin-embedded ovarian tissues were cut at 4-5 μm thickness with a microtome and taken on slides. The number and developmental stages of the follicles were determined using samples stained with Crossman's modified triple staining method and Periodic Acid Schiff (PAS). Two consecutive sections covering the largest cross-sectional area of the medulla and cortex sides of both the right and the left ovaries of the experimental and control groups were examined under light microscope to determine the number of follicles. The follicles classified as described in Pedersen and Peters (2007) and were averaged as reported by Nilsson et al. (2007). In this classification, granulosa cell shape and cell layer count are considered. According to this, the follicle that contains the flat single-row granulosa cells surrounding the oocyte is classified as the Primordial follicle (A), the follicle surrounded by single-row cubic granulosa cells as the primary follicle (B), the follicle surrounded by two or more ordered cubic granulosa cells was considered as secondary follicle (C). The follicle that has a diameter considerably larger than that of the other follicles along with small gaps between the granulosa cells and more than 3 granulosa cell lines was called the preantral follicle (D) (Table 1).

Statistical analysis

Chi-square test was used to compare the primordial, primary, secondary and preantral numbers between the two groups. All the analyses were performed with SPSS 22.0 program and $p<0.05$ was considered as statistically significant throughout the study.

RESULTS

The effect of GBH on the follicle composition

The difference of the primordial, primary, secondary, and preantral counts obtained from the two study groups were statistically significant. The percentage of primordial follicles was higher in the glyphosate applied group compared to the control group (***$p<0.01$) whereas the percentage of primary, secondary, and preantral follicles were lower (*$p<0.05$) (Table 2., Figure 1.)

The effects of GBH on follicle types and numbers

All follicle types were evaluated in both GBH and control group preparations. The general state of ovaries of the control group and in the experimental group were first compared. The size of ovary follicles in the experimental group exposed to GBH was smaller than the control group and the number of primordial follicles was higher (Figure 2.)

The ovarian sections of the control and GBH groups were stained with Crossman's modified triple staining method and Periodic Acid Schiff (PAS) staining. Primordial, primary, secondary and preantral follicle counts were performed in the preparations of the medulla and cortex regions of ovarian sections. It was observed that most of the follicles remained in the primordial follicle stage in the GBH group implying a significantly reduced transition from the primordial

follicle to the primary, secondary and preantral follicle (Figures 3, 4, 5, and 6).

Table 1. Classification of follicles

Follicle Classes	Name	Definition
A	Primordial	single layer flat granulosa cell (GC) layer
B	Primary	single layer cubic (GC) layer
C	Secondary	2-3 rows of cubic (GC) layers
D	Preantral	3≤ rows of GC with wide antral clearance, distinct monolayer

Table 2. Comparison of follicle numbers between GBH and control group

			Variable				Total	χ^2	p
			primordial	primary	secondary	preantral			
Variable	GBH	Number (f)	376	90	27	9	502	91.470	0.001
		Percentage(%)	74.9%	17.9%	5.4%	1.8%	100.0%		
	Control	Number (f)	79	40	42	21	182		
		Percentage(%)	43.4%	22.0%	23.1%	11.5%	100.0%		
	Total	Number (f)	455	130	69	30	684		
		Percentage(%)	66.5%	19.0%	10.1%	4.4%	100.0%		

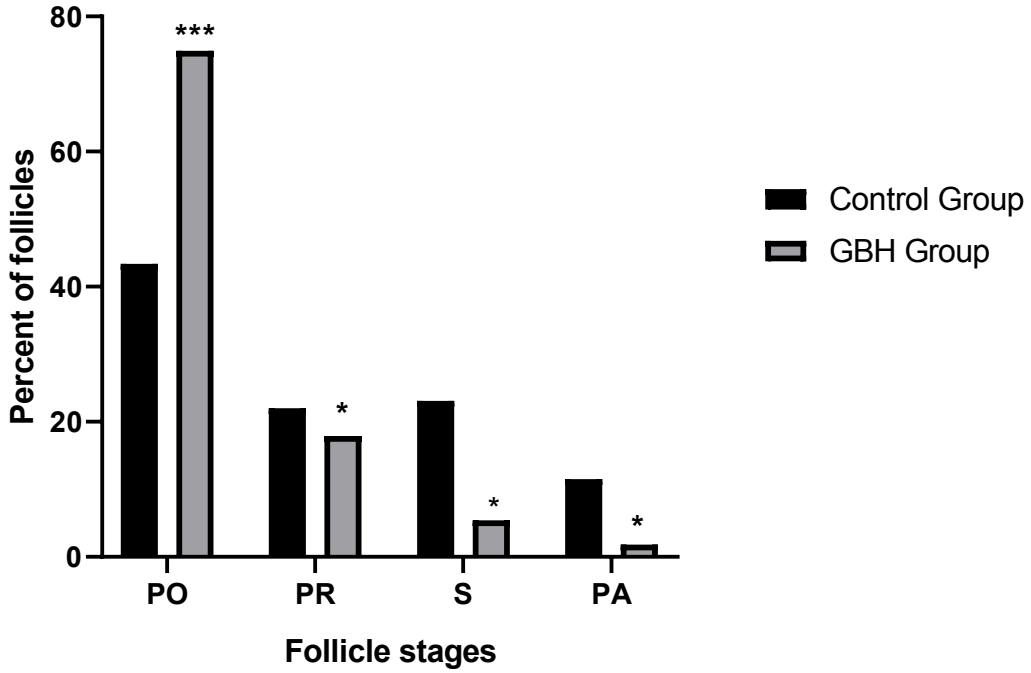

Figure 1. Effects of prenatal and neonatal exposure to GBH on follicle composition in newborn rat ovaries. The percentage of primordial follicles increased in GBH treated ovaries, while percentage of primary, secondary and preantral follicles decreased compared to controls. PO, primordial follicles; PR, primary follicles; S, secondary follicles; PA, preantral follicles. *$p < 0.05$, **$p < 0.001$, $n = 6$.

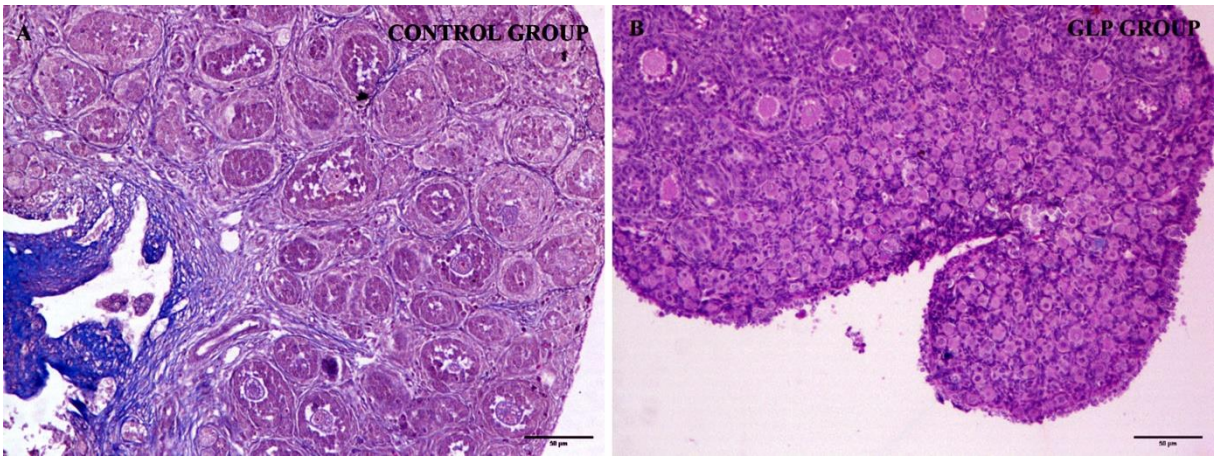

Figure 2. Representative photomicrograph of Crossman Modified Triple and Periodic acid–Schiff (PAS)-stained sections of ovary of control and GBH groups of rats. A. Control group, Crossman Modified Triple (20x), B. GBH group, PAS (20x), Bar = 50 μm.

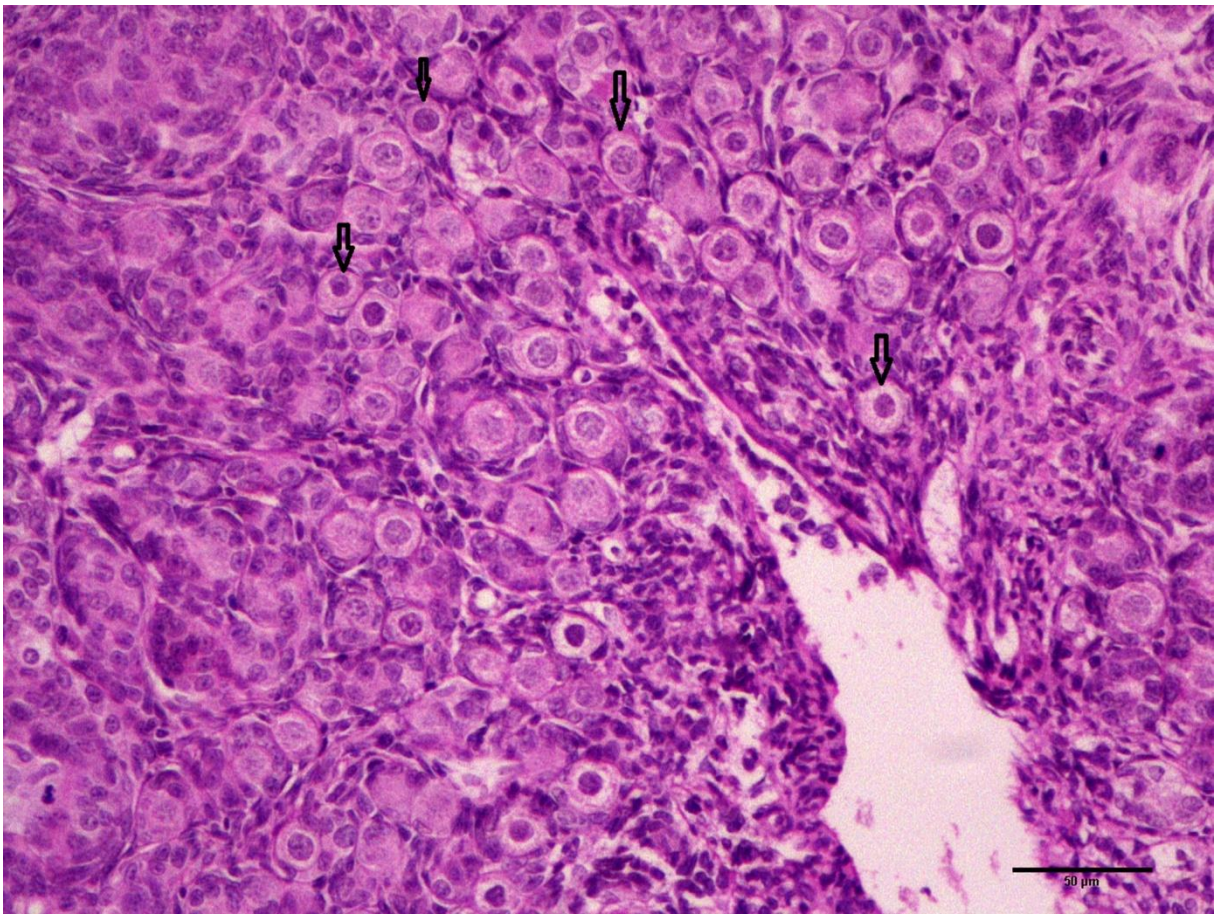

Figure 3. Primordial follicles in GBH group ovarian medulla. Arrow: primordial follicles. PAS (20x), Bar = 50 μm.

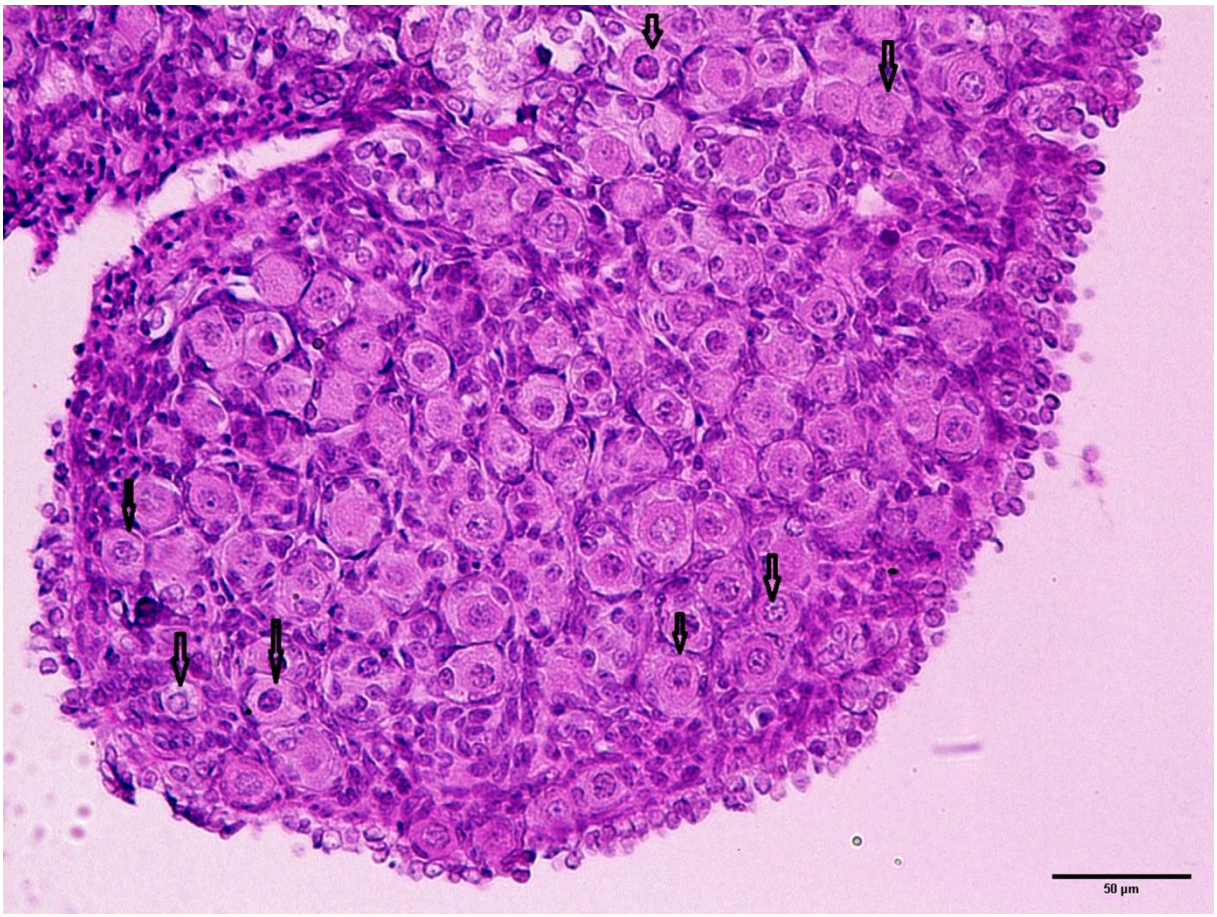

Figure 4. Primordial follicles in ovarian cortex of GBH group. Arrow: primordial follicles. PAS (20x), Bar = 50 µm.

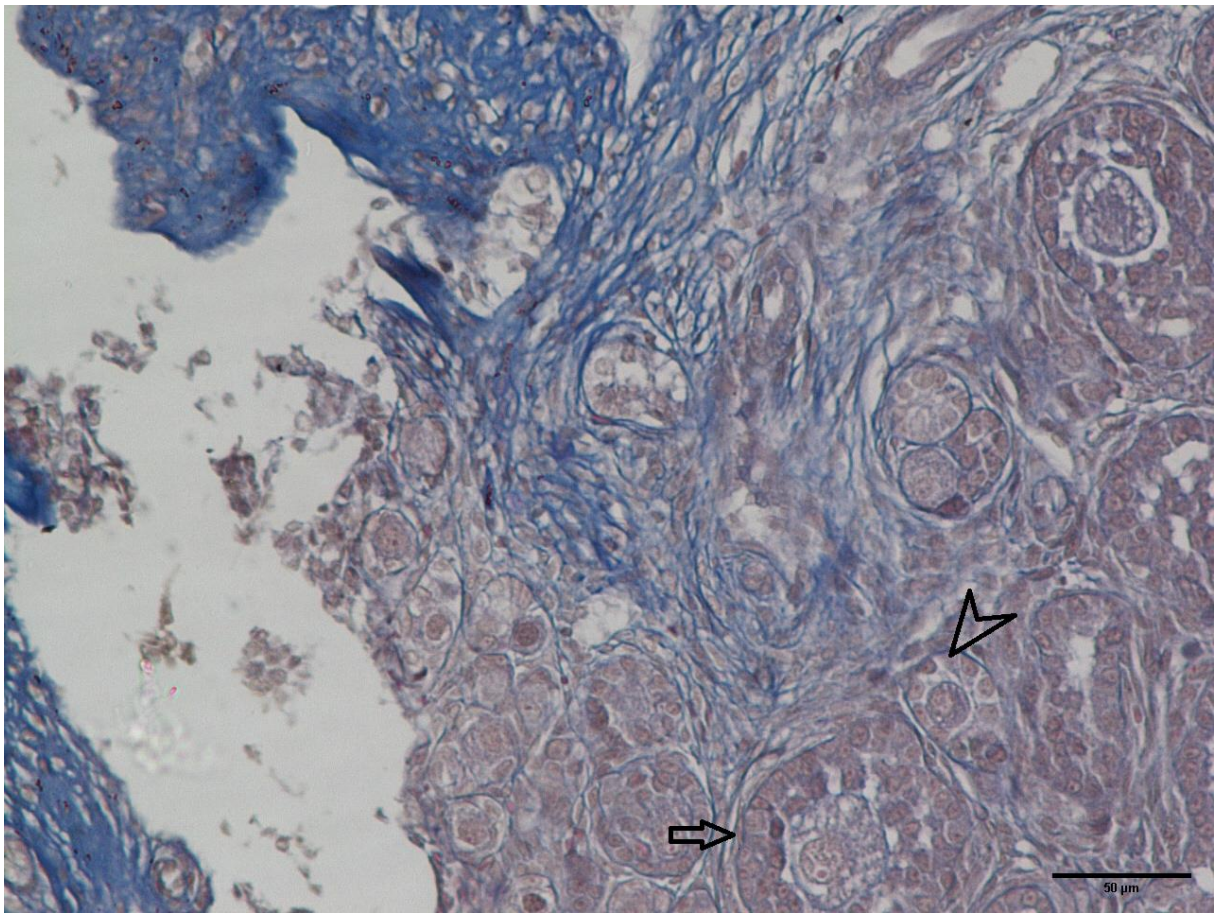

Figure 5. Primary and secondary follicle in the control group ovarian medulla. Arrowhead: primer follicul; Arrow: secondary follicul. Crossman Modified Triple (20x), Bar = 50 µm.

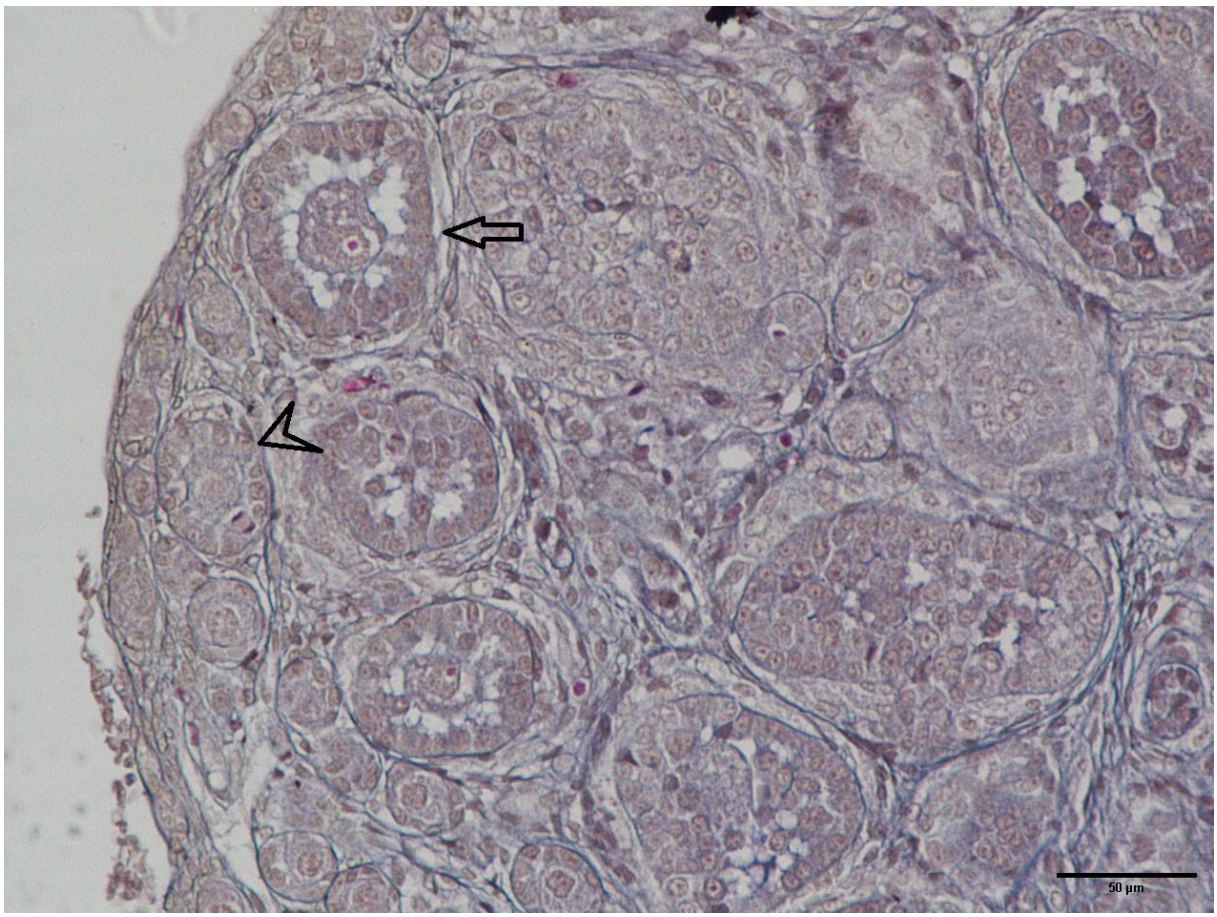

Figure 6. Primary and preantral follicles in the ovarian cortex of the control group. Arrowhead: primer follicul; Arrow: preantral follicul. Crossman Modified Triple (20x), Bar = 50 µm.

DISCUSSION

In contemporary agriculture, many technological advances are employed to increase the productivity of crops. Weeds in particular can cause large scale crop losses and consequently to a reduction in yield. Nowadays, herbicides are the most commonly used pesticide class in the fight against weeds. Today, commercial glyphosate product is used in over 140 countries in various chemical forms such as isopropylamine salt, ammonium salt, diammonium salt, dimethylammonium salt, and potassium salt (Landrigan and Belpoggi 2018). In Turkey, a total of 3208 products containing the active ingredient glyphosate has received a license from the Ministry of Agriculture and Forestry (T.C. Tarım ve Orman Bakanlığı 2019). It is still debatable whether glyphosate is harmful to human health, especially in relation to exposure levels. Albeit controversial, cancer is the forefront of concern regarding the glyphosate exposure. However, most regulatory authorities consider that glyphosate is not carcinogenic, as highlighted in a recent article by the US Environmental Protection Agency (USEPA) (USEPA 2017). Other claimed health problems include kidney diseases, pregnancy complications and reproductive dysfunction, but none have been empirically confirmed (Bai and Ogbourne 2016, Myers et al. 2016, Van Bruggen et al. 2018).

Many contaminants used in both agricultural and non-agricultural activities are known to have endocrine disrupting effects, and glyphosate-based herbicides are one of the most prominent contaminant (Drašar et al. 2018). In particular, a fetus may be exposed to glyphosate in the uterus by contamination of the mother. Regarding the effect of glyphosate on fetus in the uterus, Richard et al. (2005) and Benachour et al. (2007) reported that glyphosate has a toxic effect on human placental cells. Benachour et al. (2007) investigated the effects of glyphosate on human embryonic and placental cells and addressed how these effects are increased with the dose and exposure time and strongly concluded that exposure to glyphosate affect fetal development. Benachour and Séralini (Benachour and Séralini 2009) also show that even in low concentrations, GBH can induce apoptosis and necrosis (hence toxic effects) in human embryonic, navel and placenta cells. Since glyphosate can pass through the placenta as indicated by Poulsen et al. (2009), the baby may be exposed to glyphosate even in the uterus. This may lead to deterioration of the estrogen balance with endocrine disrupting activity of glyphosate that affects the development of testicular cells and testosterone production (Richard et al. 2005, Haverfield et al. 2011, Clair et al. 2012). In addition, the endocrine disrupting effects of GBHs in males were documented in several experimental studies (Dallegrave et al. 2007, Romano et al. 2012, Cassault-Meyer et al. 2014, Avdatek et al. 2018a, 2018b). The ovary is a female reproductive gland and is the main source of the female hormones estrogen and progesterone, as it shows a cyclic rhythm of germ cell maturation, including the proliferation, synthesis and accumulation of egg yolk in oocytes (Stefansdottir et al. 2014). Hamdaoui et al. (2018) reported that GBH caused ovarian damage and induced a decrease in absolute and relative ovarian organ weight. Although there are some studies examining the effects of GBH on female reproductive system, to date no studies have examined the effects on the ovarian follicular composition of the newborn female rats. Considering the fact that the ovarian follicles are formed in fetal and neonatal periods in rats, the nature of the effects of GBH exposure at E18 and PN7 days on the follicular activation (transition from primordial to primary follicle) in female ovary rat ovary was presented for the first time in this study.

In the present study, we found that GBH administration changed the follicular composition in the ovary. We also found that the transition from primordial follicle to primary follicle in the ovary of animals decreased with the GBH and therefore the number of secondary and preantral follicles was low. Ren et al. (2018) applied pure glyphosate and the trademark Roundup to pregnant mice from day 1 to day 19 (E1-E19) of the pregnancy and did not detect any change in primary and secondary follicle numbers compared to the control group. This may be attributed to the difference in dose and duration of glyphosate administration or the animals used in the study. Wistar race rats were used in our study were previously shown to be more sensitive to endocrine disruptors (Diel et al. 2004). Furthermore, GBH was applied between E18 and PND7 days including primordial and primary follicle development stages in embryonic and neonatal period in our study. In a study designed in a similar way to our study, an organic chlorinated insecticide, methoxychlor, was applied to Wistar breed rats and the results revealed that methoxychlorine reduced the number of primordial and primary follicles in the ovaries and increased the number of secondary follicles (Ozden-Akkaya et al. 2017). The findings of Ozden-Akkaya et al. (2017) are not in congruence with our results. This may be due to the fact that methoxychlor had prevented the formation of the primordial follicle in the fetal period by destroying the oocyte bases and induction of primary and secondary follicle from the primordial follicle. In addition, Ozden-Akkaya et al. (2017) collected ovaries between PND 50-60 days in their study whereas we collected and analyzed ovaries PND8 where follicle development reached to preantral follicle stage

CONCLUSION

The critical stage in ovarian biology is the transition from primordial follicle where development stopped to primary follicle where development persists. With the results of our study, changes the female rats' ovarian follicle composition and reduction of primary follicle transition from primordial follicle due to the exposure to GBH at the fetal and neonatal periods were reported for the first time in the literature. However, more specific advanced studies are needed to demonstrate the role of GBH in the ovarian follicles development processes. The findings will shed light on future research and more effective approaches.

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Ruhi Turkmen and Turkan Turkmen contributed equally to this work.

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This study was approved by Afyon Kocatepe University Animal Experiments Local Ethics Committee (No: 67; Date: 03 May, 2017).

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