Toxicology Research

Accepted Manuscript

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ARTICLE

Vinclozolin Exposure throughout Pregnancy and Its Developmental Toxicity

Cite this: DOI: 10.1039/x0xx00000x

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Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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It is known that vinclozolin which is used commonly in greenhouse cultivation, caused alterations in fetal reproductive system. There were no reports about effects of vinclozolin exposure on placenta during gestation. Therefore, in this study 50 and 100 mg/kg body weight day (bw/d) doses of vinclozolin was administered by oral gavage to pregnant rats during the gestation and they were subjected to caesarean section on gestation day 20. Maternal and fetal liver, kidney, heart, thymus, brain, and placenta were examined histopathologically, placenta and liver tissues were stained immunohistochemically for Vascular Endothelial Growth Factor (VEGF). Morphometric analysis of fetal body lengths, placental measurements, and fetal skeletal staining was performed. The decreases in placental weight and placental index were significant in treatment groups. Degenerations in labirent and spongiotrophoblast were detected in 100 mg/kg bw/d vinclozolin group. Mononuclear cell infiltration, cellular degeneration and edema in maternal liver; increase in the number of megakaryocytes, cellular degeneration and congestion in fetal liver were observed in treatment groups. VEGF staining was dense in trophoblastic giant cells and spongiotrophoblasts, and less dense in the labyrinth region. VEGF staining increased especially around central vein in maternal liver. Minor alterations were also observed in fetal skeleton measurements. These results demonstrated that vinclozolin and/or its metabolites transport to the placenta, and induced histopathological changes in placenta, maternal and fetal tissues.

Introduction

Vinclozolin is a dicarboximide fungicide which is used for protecting fruits and vegetables against various fungal pathogens. This fungicide which inhibits spore germination is used on foods, crops such as grapes, ornamentals and also turf grass[1].Human can expose to this fungicide via digesting the vinclozolin-treated vegetables and fruits. Moreover, greenhouse workers are in danger because vinclozolin is especially used in fruits and vegetables in greenhouses[2]. Besides, vegetarians who eat only fruits and vegetables are potentially under threat. Since vinclozolin can be detected in low concentrations in many plant foodstuffs, farmers may also be exposed to the fungicide during preparation and application, therefore human risk should be well evaluated[1].

Vinclozolin has been identified as an anti-androgenic endocrine disrupter that is related to androgen inhibition in rats[3]. Vinclozolin and metabolites, butenoic acid (M1) and enanilide (M2) derivatives, affect androgen receptor and act as anti-androgens[4]. During developmental stages of animals vinclozolin treatment resulted in serious reproductive effects[5]. The acceptable daily intake (ADI) dose for vinclozolin is 10 µg/kg body weight day (bw/d) based on a no observed adverse effect level (NOAEL) from a chronic and carcinogenicity study in rats[6]. NOAEL of vinclozolin was mentioned as 12 mg/kg bw/d in Wistar rats according to developmental and reproductive alterations[7]. It was reported that 100 mg/kg bw/d vinclozolin treatment to rats during the last trimester of pregnancy, caused sexual differentiation of male offspring[8]. But there were no reports about the effects of vinclozolin treatment throughout pregnancy on fetal tissues, maternal tissues and placenta. VEGF is an important growth factor in vascularization especially for development of placenta (Akercan et al. 2008). Therefore expression of VEGF was investigated in placenta and liver by immunohistochemically. Additionally, morphological investigations such as fetal skeletal and placental measurements are lacking in previously conducted developmental studies on vinclozolin. This study was designed to evaluate the potential maternal and fetal toxicity after treatment with vinclozolin during pregnancy in Wistar rats.

Results and discussion

According to results of all tested parameters, there were no significant differences between the control and vehicle control group. Body weights, food and water consumption of rats in control and treatment groups were shown in Table 1. There were no significant changes in body weights, body weight gain % and appetite of rats. The absolute and relative liver, kidney, thymus, brain and heart weights of dams and fetuses in control and treatment groups were shown in Table 2. According to the result there were no significant changes in organ weights.

Hematological parameters of rats in control and treatment groups were shown in Table 3. According to the results, the alterations in hematological parameters were not statistically significant in vinclozolin treatment groups compared to control group. The results of biochemical parameters were shown in Table 4. There was an increase in AST activity in 100 mg/kg bw/d vinclozolin treatment group but it was not statistically significant. The other parameters including ALP, ALT, urea and creatinine did not change between control and vinclozolin treatment groups.

The results of some placental and fetal parameters were presented at Table 5. The diameter of placenta (x-axis) in 50 mg/kg bw/d vinclozolin treatment group was significantly increased than the control group. Additionally, the weight of placenta and placental index decreased statistically significant in vinclozolin treatment groups than the control group. Other parameters such as trans umbilical diameter, body weight and length of fetus, number of implantation and number of resorption did not change between groups.

Histopathological changes in maternal and fetal liver and placenta were shown in Figure 1. Increases in the number of megakaryocytes, degeneration of hepatic parenchyma, cytoplasmic lysis, and congestion (Fig 1.B.) were histopathological findings frequently observed in fetal liver tissues of rats in vinclozolin treatment groups. Mononuclear cell infiltration, degeneration of hepatic parenchyma, edema (Fig 1.D.) and congestion were observed in maternal liver especially in 100 mg/kg bw/d vinclozolin group. It was observed that mostly affected region of placenta was the labyrinth region in vinclozolin treatment groups. Degeneration of labyrinth in 100 mg/kg bw/d vinclozolin group (Fig 1.F.) and degeneration of spongiotrophoblasts in 50 mg/kg bw/d and 100 mg/kg bw/d vinclozolin groups (Fig 1.H.) were commonly observed. Incidences of observed histopathological findings were also presented in Table 6.

Table 7 shows the intensity of VEGF in the different regions of the maternal and fetal liver and placenta of rats in the control and treatment groups. The considerable differences were observed in the immunolocalization of VEGF in the liver and placenta sections of rats in the vinclozolin treatment groups in comparison with the control group. There were no significant differences between the control and vehicle control groups in terms of immunolocalization of VEGF antibody in maternal and fetal liver and placenta. For VEGF, while faint and weakly positive staining was observed in central vein and hepatocytes of maternal and fetal liver, weakly staining was seen in these regions of the high dose vinclozolin group (Fig 2.A-D).

When the VEGF immunolocalization in placentas of the rats in control group was investigated, the intensity of VEGF in the trophoblastic giant cells and spongiotrophoblast was seen to be faint or weakly positive but in the labyrinth was seen to be only weakly positive (Fig 2.E, 2.F). However, in placenta of rats especially in the high vinclozolin-treated group, weakly positive and positive staining in trophoblastic giant cells, weakly positive in spongiotrophoblast and faint staining in labyrinth for VEGF were present (Fig 2.G, 2.H).

Results of skeletal measurements of skull, forelimbs, hindlimbs after staining the fetuses with Alcian blue-Alizarine red were shown in Table 8. All measurements were similar in control and treatment groups except the minor change in left hindlimb measurement. In vinclozolin treatment groups there were decreases in the length of left femur, fibula and tibia.

Human can frequently expose to this fungicide via digesting the vinclozolin-treated vegetables and fruits. Consumption of vegetables and fruits are recommended and increased during pregnancy. Therefore, we designed this study to determine the effect of exposure higher doses of vinclozolin than ADI dose throughout pregnancy. The aim of the study was to investigate possible maternal and fetal toxic effects of vinclozolin which was previously defined as reproductive toxicant. In this present study vinclozolin dissolved in corn oil and administered via oral gavage to pregnant dams throughout gestation at dose levels of 50 and 100 mg/kg bw/d.

It was reported that maternal exposure to toxic substances resulted in an impaired detoxification in the offspring and caused histopathological alterations in fetal organs more than adult organs[9]. Xenobiotic detoxification and metabolism can be achieved by liver; therefore liver is sensitive to toxic agents, especially during pregnancy[10]. According to the results, organ weights of liver, kidney, thymus, brain and heart did not change between control and treatment groups of dams and their fetuses. But histopathological changes observed in maternal and fetal liver tissues indicated that the target organ of vinclozolin could be liver or it could be a secondary effect of placental toxicity. Since from placenta to fetus, blood is directly supplied into fetal liver through umbilical vein. It was mentioned in the previous studies that vinclozolin exposure increased the expression of various cytochrome P450-dependent oxigenases which metabolize xenobiotic substances such as drugs and other toxic chemicals toxic in the liver [2,11,12]. In another study with human derived hepatoma cell line HepG2, vinclozolin exposure increased malonaldehyde and free radical content, and decreased glutathione[13]. Van Ravenzwaay et al.[14] pointed out that liver, adrenal gland and androgen sensitive organs are the target identified organs of vinclozolin. Similarly, 200 mg/kg bw/d orally vinclozolin treatment from gestation day 14 to postnatal day 3 induced maternal toxicity including renal system malfunction, hydroureter, hydrophrosis, and urinary bladder stones in Wistar and Long–Evans rats [7,8].

In this study, although alterations in several hematological and biochemical parameters were observed in 50 and 100

mg/kg bw/d vinclozolin treatment groups, they were not statistically significant. In several in vivo studies, rats or mice orally dosed with vinclozolin, moreover human peripheral blood lymphocytes were also investigated in vitro after exposure to vinclozolin[11,15]. According to these studies vinclozolin did not induce any significant increase of chromosomal aberrations and sisterchromatid exchanges and did not lead to the formation of haemoglobin adducts. Besides, no significant micronucleated polychromatic erythrocytes in bone marrow were found indicating that vinclozolin is not genotoxic. Schneider et al.[16] found reduced food consumption, body weight, red blood cell counts, hematocrit, hemoglobin values and elevated plasma triglycerides and cholesterol levels in 100 mg/kg group females, but we did not observe similar results.

In prenatal developmental toxicity study 0-1000 mg/kg bw/d doses of vinclozolin was administered by gavage to 25 female Wistar rats from gestation day 6-19. The fetuses were investigated for external and internal malformations16. According to their F0 parental observations, there were no mortality and clinical signs in dams and offsprings. Parallel to these studies, no mortality, statistically significant resorption ratio, no altered number of implantation, no external malformations were observed after 50 and 100 mg/kg bw/d vinclozolin treatment. The only change in skeletal development of fetus were in left hindlimb measurements in vinclozolin groups, however it was not gross change.

Maternal toxicity, placental transfer and placental-fetal metabolism are important factors that can modify the overall toxicity of a chemical during pregnancy[17]. Placenta regulates the supply of nutrients and oxygen to the fetus and also produces angiogenic factors, vasodilators and the synthesis of steroid and protein hormones. Fetal tissues can accumulate chemical residues because of its inadequate capability for metabolization and elimination. Several cytochromes P450 enzymes found in placenta are largely responsible for the detoxification of drugs and toxins, but their quantity vary with the placental development, length of gestation, and maternal health status[18]. In this study we administered vinclozolin throughout the pregnancy because it is known that expression and activities of P450 enzymes vary depending on the gestation day and vulnerability to xenobiotics can alter.

During pregnancy the placenta is an endocrine organ and both protein and steroid hormones are important in the maintenance of the fetus[19]. Vinclozolin has been described as endocrine disruptors because of its capability of altering the hormonal balance affecting steroidogenesis. Additionally, it was reported that, vinclozolin had adverse effects on offspring, when administered to pregnant mice during GD 13-17 at doses 10 mg/kg bw/d and 50 mg/kg bw/d, caused in feminized males, masculinized females and increased expression of progestrerone receptors[5,20]. Sanderson et al.[21] reported that vinclozolin can cause an increase in aromatase activity; therefore in this study we investigated the changes in the placenta. The maternal influence on fetal growth is reflected by changes in placental size[22]. In our study, placental weight and diameter, and placental index altered in vinclozolin treatment groups compared with the control group.

VEGF which is one of the angiogenic growth factors produced by placenta has important role in mediation of angiogenesis and hypoxia. Increased levels of VEGF may reflect the hypoxic status of the placenta in compromised pregnancies. However, we need further detailed investigations in order to claim that vinclozolin induce hypoxia. Moreover, during implantation and placentation vascularization has fundamental role for successful gestation[23]. In this study, decreased staining for VEGF in the labyrinth and increased staining for VEGF in the trophoblastic giant cells and spongiotrophoblasts in vinclozolin treated rats were observed. Vinclozolin may be a potential inhibitor of angiogenesis, presumably by decreasing VEGF production or blocking VEGF receptor signaling. The decreased staining in the labyrinth zone may be due to the degeneration in this zone after vinclozolin treatment. VEGF staining of maternal and fetal liver in vinclozolin treatment groups was denser than in the control group. The increase in VEGF staining may be explained by the elevation of vascularization in order to compensate the histopathological alterations in liver after vinclozolin administration.

Experimental

Animals

Adult Wistar albino male and female rats were purchased from Company of Lemali Animal Husbundary, Ankara, Turkey. This study was endorsed by the approval of Ethics Committee of Gazi University. Rats were housed in polycarbonate cages and maintained 12:12h light:dark cycle in the laboratory with a temperature of 20.73±0.20 and humidity of 45.50±0.90 %, fed with standard rat diet and water ad libitum. Virgin female rats were mated overnight with male rats. The day in which sperms were detected in the vaginal smear was considered to be the day 0 of pregnancy. The pregnant rats, weighing 160-190 g in the beginning of experiment, were distributed randomly into 4 groups. Each experimental group contained 10 pregnant rats, which were observed two times a day. The study is in full conformity with the National Research Council guidelines for animal experimentation[24].

Experimental Design

Vinclozolin was purchased from Sigma (CAS 50471-44-8). Vinclozolin was dissolved in corn oil and administered orally by gavage to treatment groups throughout pregnancy. There were 4 groups consisted of control group (without vinclozolin treatment), vehicle group (administered corn oil) and two vinclozolin treatment groups 50 mg/kg bw/d and 100 mg/kg bw/d.

Maternal and fetal observations

During pregnancy, weights of each dam were recorded on each day of gestation. Maternal food and water consumptions were recorded each day during pregnancy. On gestation day 20 (GD 20), the animals were sacrificed by cervical dislocation. The initial and final maternal group body weights were recorded.

Body weight gains as grams (g) and percentages (%) were calculated. Absolute and relative weights (organ weight/body weight) of maternal liver, kidney, heart, brain, thymus and placenta were recorded at the time of cesarean section.

The uterine horns were exteriorized through a midline abdominal incision and observed for implantations, live and dead fetuses and resorptions. Number of living fetuses were counted and examined for externally visible anomalies. In addition, fetal liver, kidney, heart, and brain were removed and weighed.

Hematological and biochemical analysis

Dams were sacrificed by cervical dislocation, and trunk blood was collected into tubes to make complete blood count with blood counter (Shimadzu Corporation Kyoto, JP). The analyzed parameters were white blood cells, red blood cells, and thrombocytes/mm3; percentage of lymphocyte, monocyte, neutrophil, eosinophil, and basophil; MCV (Mean Corpuscular Volume), HCT (Hematocrit), MCH (Mean Corpuscular Hemoglobin), MCHC (Mean Corpuscular Hemoglobin Concentration), Hb (Hemoglobin), RDW-SD (Red Cell Distribution Width–Standard Deviation, RDW-CV (Red Cell Distribution Width–Coefficient Variation), MPV (Mean Platelet Volume), PDW (Index of Thrombocytes Heterogeneity).

Serum was separated after centrifugation at 3000 rpm for 15 min from the rest of blood samples. Serum samples were used to analyse the amount of urea and creatinine and to measure enzyme activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using a clinical spectrophotometer (CL-770 Shimadzu Kyoto, JP).

Histopathology and immunohistochemistry

All tissue samples were fixed in Bouin's fixative for histopathological examinations. Leica model RM 2125 microtome was adjusted at 5 µm. The sections were placed on slides and stained with hematoxylin eosin (H&E).

For immunohistochemical investigations, tissue samples of maternal and fetal liver and placenta were removed, fixed in 10 % formol, embedded into paraffin, and 5 µm tissue sections were prepared. Placenta and liver (maternal and fetal) slides were stained immunohistochemically for Vascular Endothelial Growth Factor (VEGF) by streptavidin peroxidase method and examined under light microscope. The resulting slides were reviewed independently by three observers. The intensity and distribution of positive stainings were evaluated using standard 4-point scoring system, scale for intensity, with slides being scored as (-) negative, (±) faint, (+) weakly positive, (++) positive, and (+++) clearly positive[25]. All slides were examined using Olympus BX51 system light microscope. The photographs were captured using Bs200prop software.

Morphometric analysis

The rats were euthanized by cervical dislocation on GD 20. The uterus was examined for the presence of dead and live fetuses. Fetuses were removed, separated from the placenta, weighed, and examined. The placental index was calculated as weight of placenta (g)/body weight of fetus (g). The number and morphology of fetuses and the shape of placenta were examined for each rat. The diameter and length of the placenta and the transumbilical length were measured. Fetuses were stained with Alcian Blue (staining the cartilage) and Alizarin Red (staining the bone) to investigate the skeletal development[26,27]. Skull diameter, number of vertebra and ribs, and length of limbs and skull were measured using Leica IMSO Software program under Leica MZ16A light microscope and DFC320 transfer system. Additionally, Nikon D100 camera was used to capture the photos of fetuses.

Statistical analysis

Statistical analysis was performed using SPSS software. Statistical significance was assigned at the $P \leq 0.05$ levels. The homogeneity of variance and normal distribution between groups was evaluated using general linear model procedure and Kolmogorov-Smirnov nonparametric test. Serum parameters were analyzed using one-way ANOVA. To identify the sources of significant main effects, post hoc comparisons (Games-Howell, Tukey) were used. Body and relative organ weights were examined using one-way ANOVA and Games-Howell post hoc test[28]. Incidences of histopathological findings were analyzed by Fischer's exact test ($P \leq 0.05$).

Conclusions

This study was design as lack of sufficient information with respect to potential toxic effects of vinclozolin on placenta, maternal and fetal tissues. We investigated for the first time the effect of vinclozolin administration during pregnancy on morphometric and histopathologic alterations in placenta; fetal tissues and skeletal measurements. According to alterations in placental measurements and histopathological findings, we suggest that vinclozolin and its metabolites could possibly transport via placenta.

Acknowledgements

This study was supported by Gazi University Scientific Research Council (Project no: 21/2008-01).

Notes and references

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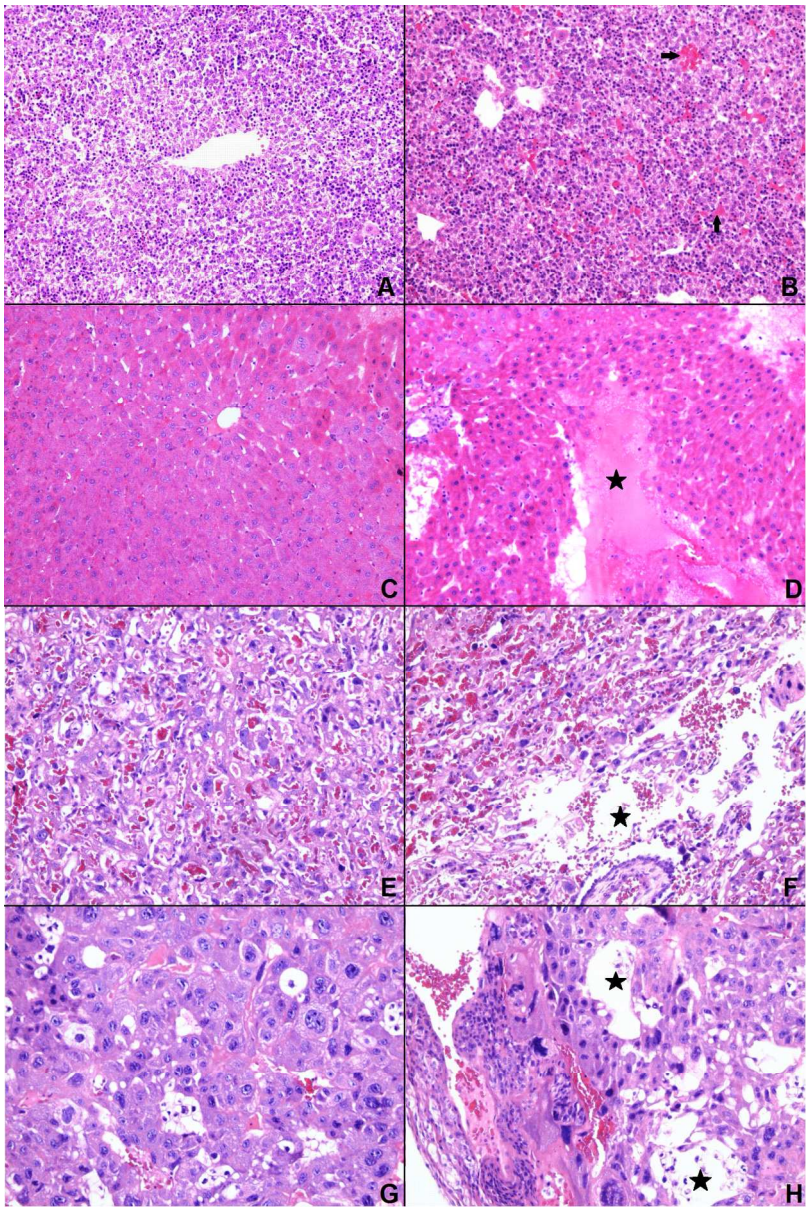

Fig 1. Photomicrographs of fetal liver of the control (A) and treatment group (B): congestion (arrow); maternal liver of the control (C) and treatment groups (D): edema (star). H&E staining, 100X. Photomicrographs of placenta of the control (E,G), and treatment group (F): degeneration of labyrinth trophoblasts (star), (H): degeneration in the spongiotrophoblasts (star). H&E staining, 100X.
423x635mm (96 x 96 DPI)

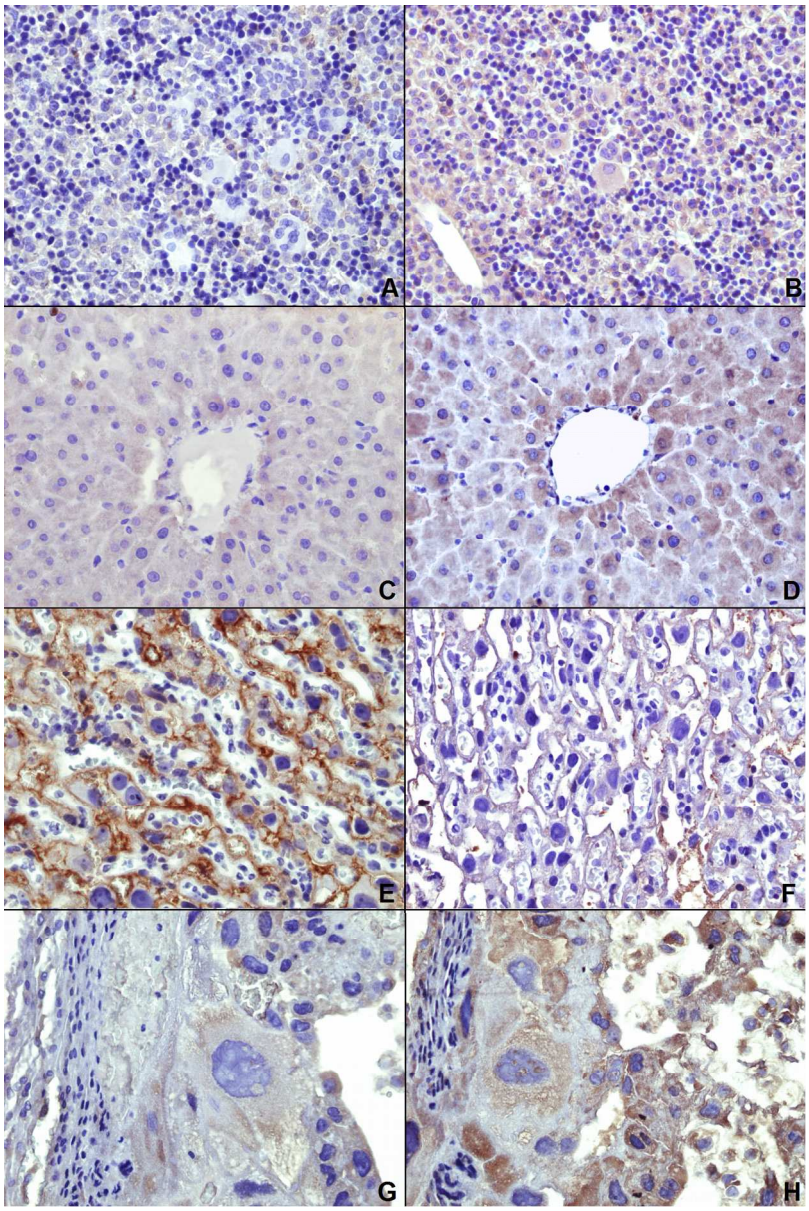

Fig 2. Photomicrographs of fetal liver of control (A), treatment group (B); maternal liver of control (C), treatment group (D); placenta of the control (E,G) and treatment groups (F, H). IHC staining with VEGF (brown), 200X.
423x635mm (96 x 96 DPI)

Table 1. Body weights (g), food (g/day) and water (ml/day) consumption of rats in control and treatment groups

	Groups			
	Control	Vehicle Control	Vinclozolin (50 mg/kg bw/d)	Vinclozolin (100 mg/kg bw/d)
Initial body weight (g)	170.80±5.20	171.80±4.62	172.25±2.49	178.00±3.59
Final body weight (g)	248.80±4.82	237.20±14.81	256.75±2.56	262.83±6.71
Body weight gain (g)	78.00±3.93	65.40±16.97	84.50±2.72	84.83±4.91
Body weight gain (%)	45.96±3.15	38.83±10.70	49.12±2.05	47.70±2.64
Food intake (g/day)	19.41±0.51	18.15±1.06	21.01±0.72	19.46±0.73
Water intake (ml/day)	36.04±1.37	34.03±0.89	37.12±1.31	34.30±0.96

Results are expressed as mean values ± standard error

Table 2. Absolute (g) and relative organ weights of dams and fetuses in control and treatment groups.

	Groups			
	Control	**Vehicle Control**	**Vinclozolin (50 mg/kg bw/d)**	**Vinclozolin (100 mg/kg bw/d)**
Maternal tissues				
Liver				
Absolute weight	9.74±0.40	10.35±0.60	10.95±0.48	10.96±0.68
Relative weight (x10^{-2})	3.92±0.10	4.11±0.20	4.24±0.21	4.17±0.16
Kidney				
Absolute weight	0.75±0.03	0.70±0.03	0.77±0.06	0.70±0.05
Relative weight (x10^{-2})	0.30±0.01	0.28±0.01	0.30±0.03	0.27±0.01
Thymus				
Absolute weight	0.32±0.02	0.28±0.02	0.29±0.02	0.29±0.02
Relative weight (x10^{-2})	0.13±0.01	0.11±0.01	0.18±0.07	0.11±0.01
Brain				
Absolute weight	1.29±0.14	1.19±0.17	1.12±0.23	1.58±0.08
Relative weight (x10^{-2})	0.52±0.06	0.48±0.07	0.43±0.09	0.61±0.03
Heart				
Absolute weight	0.70±0.05	0.66±0.04	0.70±0.03	0.72±0.030
Relative weight (x10^{-2})	0.52±0.06	0.47±0.07	0.43±0.09	0.61±0.03
Fetal tissues				
Liver				
Absolute weight	0.17±0.01	0.18±0.01	0.17±0.01	0.16±0.01
Relative weight (x10^{-2})	0.08±0.00	0.08±0.00	0.11±0.03	0.08±0.00
Kidney				
Absolute weight	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
Relative weight (x10^{-2})	5.58±0.30	5.40±0.20	5.59±0.30	5.08±0.15
Brain				
Absolute weight	0.11±0.01	0.11±0.00	0.11±0.00	0.11±0.00
Relative weight (x10^{-2})	0.07±0.02	0.11±0.04	0.07±0.02	0.05±0.00
Heart				
Absolute weight	0.02±0.00	0.02±0.00	0.01±0.00	0.01±0.00
Relative weight (x10^{-2})	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00

Results are expressed as mean values ± standard error

Table 3. Hematological parameters of dams in control and treatment groups.

	Groups			
	Control	Vehicle Control	Vinclozolin (50 mg/kg bw/d)	Vinclozolin (100 mg/kg bw/d)
Leukocytes				
White Blood Cell (/mm^3)	9.78±1.80	7.57±0.96	4.49±1.18	6.82±2.74
Lymphocyte (%)	45.98±3.18	47.96±2.61	53.96±2.71	52.60±4.05
Monocyte (%)	11.96±1.62	13.00±0.63	10.90±0.82	11.94±1.69
Neutrophil (%)	38.38±3.12	31.78±3.83	30.16±2.72	30.62±2.29
Eosinophil (%)	1.26±0.56	4.86±2.42	2.32±0.47	2.80±1.51
Basophil (%)	0.88±0.24	1.12±0.06	0.86±0.16	1.02±0.14
Erythrocytes				
Red Blood Cell (/mm^3)	5.83±0.35	5.40±0.68	5.90±0.37	5.93±0.09
MCV	55.30±2.10	63.86±6.40	59.50±1.76	53.58±1.34
HCT	32.42±2.68	32.76±2.13	35.316±2.97	31.86±1.16
MCH	19.48±0.41	25.08±3.67	20.24±0.19	19.44±0.23
MCHC	35.32±0.92	38.80±1.90	34.08±0.80	36.32±0.72
Hb	11.38±0.71	12.54±0.37	11.96±0.83	11.56±0.28
RDW-SD	38.76±0.66	42.20±1.81	38.56±0.26	38.76±0.43
RDW-CV	19.06±0.52	18.34±1.01	17.62±0.42	19.64±0.33
Thrombocytes				
Thrombocyte (/mm^3)	772.6±70.77	610.60±83.01	790.6±56.29	767.4±74.84
MPV	5.88±0.35	6.22±0.42	5.78±0.46	5.42±0.14
PDW	8.72±0.40	8.54±0.25	8.68±0.42	8.20±0.16

Results are expressed as mean values ± standard error

Table 4. Biochemical parameters of dams in control and treatment groups.

	Groups			
	Control	**Vehicle Control**	**Vinclozolin (50 mg/kg bw/d)**	**Vinclozolin (100 mg/kg bw/d)**
ALP (U/L)	386.62±31.88	333.38±36.57	334.48±13.85	241.06±34.74
AST (U/L)	132.94±19.86	166.4±26.43	130.6±17.29	173.3±16.90
ALT (U/L)	54.12±5.12	55.40±8.63	50.26±2.65	52.04±4.73
Urea (mg/dL)	85.24±4.39	78.55±3.85	86.42±8.44	80.58±3.05
Creatinine (mg/dL)	0.86±0.06	0.83±0.05	0.86±0.02	0.72±0.05

Results are expressed as mean values ± standard error

Table 5: Placental and fetal parameter values for the rats in the control and treatment groups.

	Groups			
	Control	Vehicle Control	Vinclozolin (50 mg/kg bw/d)	Vinclozolin (100 mg/kg bw/d)
Diameter of placenta (y-axis)	12.55±0.21	12.74±0.20	13.30±0.16[a]	12.65±0.13[c]
Diameter of placenta (x-axis)	10.30±0.20	10.53±0.15	11.16±0.12[a,b]	10.63±0.11[c]
Thickness of placenta	4.80±0.11	4.94±0.12	5.30±0.14	5.76±0.87
Trans umbilical diameter	21.10±0.45	20.45±0.51	21.93±0.38	21.88±0.32
Weight of placenta	0.52±0.02	0.48±0.01	0.46±0.01	0.40±0.01[a,b,c]
Body weight of fetus	2.39±0.13	2.18±0.08	2.26±0.05	2.04±0.04
Body lenght of fetus	28.5±0.85	28.18±0.44	29.08±0.34	29.12±0.22
Placental index	0.24±0.01	0.26±0.01	0.21±0.00[a,b]	0.20±0.00[a,b]
Number of implantation	10±0.83	11.5±3.10	11.4±3.04	12.2±1.30
Number of resorption	0.2±0.01	0.25±0.02	0.2±0.01	0.0±0.0

[a]: Significantly different from control group (P≤0.05), [b]: Significantly different from vehicle control group (P≤0.05), [c]: Significantly different from Vinclozolin (50 mg/kg bw/d) group (P≤0.05). Results are expressed as mean values ± standard error

Table 6: Incidence of observed histopathological findings in maternal and fetal liver and placenta of rats in the control and treatment groups.

	Groups			
Histopathological findings	**Control**	**Vehicle Control**	**Vinclozolin (50 mg/kg) bw/d)**	**Vinclozolin (100 mg/kg)**
Maternal liver				
Mononuclear cell infiltration	-		3/5	4/5[a]
Degeneration of hepatic parenchyma			4/5[a]	5/5[a]
Edema			1/5	4/5[a]
Congestion	-		-	2/5
Fetal liver				
Increases in the number of megakaryocytes	-	-	3/11	6/11[a]
Degeneration of hepatic parenchyma		-	3/11	6/11[a]
Cytoplasmic lysis	-	-	2/11	2/11
Congestion	-	-	3/11	5/11[a]
Placenta				
Degeneration in the Giant cell and nucleus polymorphism	-	-	5/20[a]	5/20[a]
Degeneration in spongiotrophoblasts	-	-	9/20 [a]	10/20[a]
Hemorrhage				
Basal zone	-	-	4/20	8/20[a]
Labyrinth	-	-	4/20	16/20[a]

[a]: Significantly different from control group (P≤0.05)

Table 7. The immunolocalization of VEGF in the liver and placenta of rats in the control and treatment groups.

	Groups			
	Control	**Vehicle Control**	**Vinclozolin (50 mg/kg) bw/d)**	**Vinclozolin (100 mg/kg) bw/d)**
Maternal liver				
Central vein	± / +	± / +	± / +	+
Hepatocytes	± / +	± / +	+	+ / +
Fetal liver				
Central vein	± / +	± / +	± / +	± / +
Hepatocytes	± / +	± / +	+	+
Placenta				
Trophoblastic giant cells	± / +	± / +	+	+ / ++
Spongiotrophoblast	± / +	± / +	± / +	+
Labyrinth	+	+	± / +	±

*Intensity of labeling, defined as (±) faint, (+) weakly positive and (++) positive.

Table 8: Skeletal measurements of fetuses in the control and treatment groups.

		Groups			
		Control	**Vehicle Control**	**Vinclozolin (50 mg/kg bw/d)**	**Vinclozolin (100 mg/kg bw/d)**
Skull (mm)	*Diameter of x- axis*	7.53±0.37	7.39±0.28	7.53±0.22	7.28±0.28
	Diameter of y-axis	10.93±0.55	10.76±0.36	10.92±0.18	10.64±0.55
	Area (mm2)	67.17±6.23	63.80±3.25	67.50±3.17	69.00±4.00
Right forelimb	*Humerus*	13.26±0.45	12.76±0.24	12.85±0.39	11.95±0.32
(x10^{-2} mm)	*Radius*	12.37±0.42	11.56±0.46	11.86±0.22	11.63±0.36
	Ulna	10.60±0.21	10.16±0.23	10.23±0.24	10.43±0.29
Left forelimb	*Humerus*	13.80±0.97	11.96±0.66	12.25±0.35	11.68±0.47
(x10^{-2} mm)	*Radius*	12.63±0.49	11.35±0.43	11.89±0.30	11.67±0.33
	Ulna	10.85±0.56	10.22±0.36	10.05±0.25	10.29±0.23
Right hindlimb	*Femur*	11.89±0.48	11.04±0.30	10.62±0.21	11.20±0.23
(x10^{-2} mm)	*Fibula*	11.95±0.42	11.28±0.37	10.82±0.28	11.28±0.29
	Tibia	10.58±0.32	10.32±0.42	9.50±0.29	9.87±0.20
Left hindlimb	*Femur*	13.27±0.64	12.89±0.26	11.16±0.41[a]	11.19±0.29[a]
(x10^{-2} mm)	*Fibula*	11.17±0.47	9.44±0.30	9.31±0.26[a]	10.10±0.32
	Tibia	12.33±0.48	11.01±0.18	10.90±0.26[a]	11.41±0.18

[a]: Significantly different from control group ($P \leq 0.05$). Results are expressed as mean values ± standard error