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Prenatal zestoretic exposure ameliorates developmental landmarks in rat offspring

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Abstract

Zestoretic, a combination of lisinopril and hydrochlorothiozide used as anti-hypertension drug prescribed even during pregnancy. Role of zestoretic on the developmental landmars of young ones exposed prenatally are yet to be established. Hence, the present investigation initiated to test in Wistar rats. Three groups of inseminated female rats were oral gavaged with zestoretic (lisinopril varied concentrations + hydrochlorothiozide 12.5mg) 25, 50 and 100 mg/Kg body weight (BW) on gestation days 7, 9, 11 and 13, whereas control group with double distilled water. Clinical toxicity found in few dams from 50 and 100 mg/Kg BW received rat shown aggressive behavior during experimentation. The developmental landmarks, such as pinna attachment, ear opening, fur development, eye opening, upper and lower incisor eruption, crown rump length, vaginal opening and testes descend were measured periodically on their postnatal days in all the young ones. The number of live pups in zestoretic 50, 100 mg/Kg BW administered females are significantly less ($P<0.001$) from control. Comparatively among the groups, sex ratio of male and female offspring is not changed. The BW of young ones on postnatal day (PND) 1, 7, 14 and 21 are significantly ($P<0.001$) reduced in 100 mg zestoretic exposed ones compared to other groups, but weight loss is dose dependent in experimental groups. Decreased survival index of pups exposed to zestoretic 50; 100 mg/Kg BW was reported.Recorded, delay in pubertal onset in females (vaginal opening) and testicular descent in males of experimental groups. Crown rump length in experimental offspring is significantly ($P<0.05$) decreased from controls in a dose dependent manner on PND 1, 7, 14 and 21. No change has been recorded with anogenital distance, pinna detachment, eye and ear opening, fur development, lower and upper incisor eruption, experimental offspring when compared to controls. The present study establishing the interference of prenatal zestoretic exposure on some of the developmental landmarks in the offspring of rats.

1. INTRODUCTION

Conventional procedures including usage of common medicine without prescription at critical stages including during pregnancy may damage the health and sometimes even for life. In developing and third world countries it is very common that medicine has been used by innocent women during pregnancy without consultation with medical practitioner which may leads to congenital defective babies (Banhidy *et al.*, 2005; Punam *et al*., 2009). Drugs to reign blood pressure are used by men and women, even during pregnancy either by intentionally or unintentionally (Lindheimer *et al.*, 2010; Scantlebury *et al.*, 2013). In men the common medicine includes that govern blood pressure may show many side effects like diminish in spermatogenesis and debacle copulation (Eisenberg *et al.*, 2015; David *et al.*, 2017). The embryonic exposure of these chemicals by foetus/new-borns are somehow divergent, may cause malformation in the developing embryo while gestation or after accouchement and sometimes at adult stage. Brezina *et al* (2012) explained the effects caused by pharmaceutical medications on male fertility. Besides these, recreational drugs on male fertility were also well documented (Kulkarni et al., 2014). Similarly, the reason for deteriorated male reproductive health is well presented by Sunil kumar *et al* (2019).

In many cases, malformation may not be identified, baby look like normal and grown up. But at the adult stage malformations including infertility can be identified. However, the reason for increased impotency of males and significant fall in reproduction abilities of women is notable today (Grimm *et al.*, 1997; Peter *et al.*, 2020). This kind of aberrations must be addressed with proper evidence. It is needed especially for woman to prevent the usage of drugs that affect the growth of foetus and young ones during gestation and lactation (Deborah *et al.*, 2005; Sachdeva *et al.*, 2009).

Zestoretic, a drug commonly used to treat high blood pressure (hypertension), and it is amalgamation of

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lisinopril and hydrochlorothiazide (HCTZ). No scientific reports are available on zestoretic induced reproductive aberrations in males and females. Moreover, no reports found for its toxicity, malformations, alteration in the developmental landmarks of infantiles exposed administered to zestoretic prenatally.The present investigation hypothesized that zestoretic alters the developmental landmarks in offspring of Wistar rats upon exposure during pregnancy along with behavioural aspects of dams.

2. MATERIALS AND METHODS

2.1. Test animals and their maintenance

The fact-finding animal model i.e., Wistar albino rats (30 rats; 60 days old;195 ± 5g body weight) were selected as test animal in this study and procured from at Sri Venkateswara Traders, Benguluru, India. Test rats domiciled in well ventilated (12 h:12 h light: dark cycle), clean and controlled room temperature (25 ± 2ºC; relative humidity of 50 ± 5%). All experimental rats feed ad libitum with certified rodent feed (Sri Venkateswara Traders, Benguluru, India) and water. The trails executed in accordance with the guidelines of the Indian statutory body (CPCSEA: Committee for the Purpose of Control and Supervision on Experiments on Animals) (CPCSEA, 2003). Further, fulfilled the guidelines of the safe keeping and use of laboratory animals (NRC, 1996) and endorsed by the Institutional Animal Ethical Committee, Yogi Vemana University, Kadapa, A.P., India (No.YVU/IEAC/PRR/13/2017).

2.2. Chemicals and Equipment

The tryout chemical used in the current investigation is zestoretic, it is a combination of both diuretic HCTZ and angiotensin converting enzyme (ACE) inhibitor lisinopril. Lisinopril (zestril)is regular medicine available in pahrama located in, California, USA, HCTZ from life pharma, Kadapa, A.P., India. Lisinopril, a synthetic peptide derivative, chemically it is (S)-1-[N2-(1-carboxy-3-phenylpropyl)-L-lysyl]-L-proline dihydrate (Figure 1A). Lisinopril physically a white to off-white crystalline fine powder with a molecular mass (MW) of 441.53 g/mol and is soluble in water, methanol (moderate) and ethanol (partial). HCTZ is 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1dioxide (Figure 1B). HCTZ a crystalline white or practically white powder with MW of 297.72g/mol and its solubility is partial in water and high in NaOH (sodium hydroxide) solution. Sodium chloride and other chemicals used in this study were purchased from Hi media Pvt. Ltd., India. Orally administration of zestoretic by using oral gavage, type AQRA, HARVARD apparatus, Maharashtra. Measurement of crown rump length by using SSEA stainless iron nickel vernier caliper and body mass of rat is weighed by electronic balance, Type AY220, SHIMADZU Corporation, Japan.

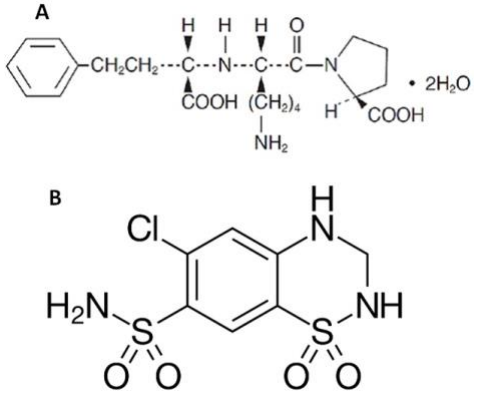

Figure 1. Structure of Lisinopril (A) and Hydrochlorothiazide (B).

2.3 Estrus cycle determination and vaginal smear test

The vaginal smear test method was followed in this study is from Zarrow et al. (1964) and Cooper et al (1993).Briefly, insertion of physiological saline (0.9% NaCl) into vagina was carefully done using pasture pipette and mixed vaginal smear quickly collected back into the pipette. Thin layer of smear was prepared on a sterilized glass slide and observed under phase contrast microscope for identification of estrous stages (Rottenfusser, 2013). This test was done before 7.00 am or after 6.00 pm in a day. Estrus cycle stages were illustrious and dogged based on cytology as proestrus (appears epithelial cells), estrus (cornified cells), metestrus (cornified cells and high number of leukocytes) and diestrus (contains leukocytes) (Mandl, 1951; Westwood, 2008; Cora, 2015). In an individual cages, females in proestrus were cohabited with males in a ratio of 1:1 (Soujanya*et al.*, 2014;Sridevi *et al.*, 2016).

2.4 Design of experiment

The next day of cohabitation before 7.0 am, observed for vaginal plugs in females. Day one of pregnancy (gestation day) was counted fromform the day at which vaginal plugs appear and maintained them in separate cages. About 16 inseminated females' rats were grouped into four of each fourwith equal number. Class of doses 25, 50, 100mg/Kg body weight (BW) of zestoretic were administrated orally on gestation day 7, 9, 11 and 13 for first three groups (1, 2 and 3) respectively. Double distilled water (pseudo) without drug was administered to last group (4th one) which served as control. Normal pellet diet and water supplemented to all the rats ad libitum. The pregnant rats were observed for clinical toxicity and behavioural observations. Pregnant rats of all groups allowed to deliver pups. Schematic representation of experimental design shown in Figure 2.

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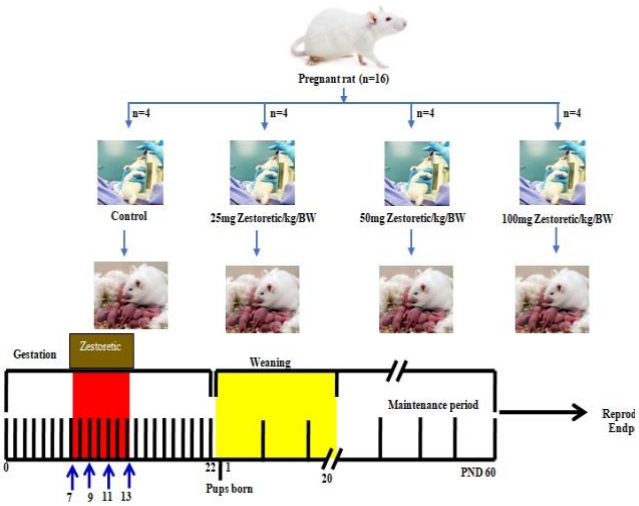

Figure 2. Schematic representation of experimental design shown.

Table 1. Number of live pups/rat and sex ratio of offspring of rats exposed to zestoretic during embryonic development

Parameters	Controls	25mg/Kg BW	50mg/Kg BW	100mg/Kg BW	F- Values
No. of pups/rat	11.75[a] ±0.25	11.75[a]±0.25	10[b]±0.409 (-14.89)	8[c]± 0.409 (-31.91)	P=0.0009 F=14.36
Male: Female pups	26:21	23:24	24:18	17:15	

Values are mean ± SEM of 4 individuals. BW: body weight
Values in parentheses are percent change from control.
Mean values in a row that do not share the same superscript differ significantly at p<0.001.

2.5 Developmental Landmarks

On the postnatal day (PND) 1, 7, 14 and 21 of weaning period the body mass/weight (gm) of young ones was weighed using electronic balance. The number of F1 progeny was measured (live pups/rats) on postnatal day 1 (PND 1). Also measured male and female pups sex ratio, and differences in the develometal land marks in pups of each dam. Crown rump length on day 1 of PND and survival index on 1st and 21st PND was measured (Sridevi et al., 2016). Fur development, detachment of pinna, lower and upper incisor eruption, anogenital distance, preputial separation and testes descent are known developmental landmarks which were detected with unaided eye in F1 young ones. Further eye, ear and vaginal opening were also recorded.

2.6 Data Analysis

The data of the present study statistically analyzed with two tailed ANOVA using GraphPadPrism v5.0.3.477. The resulted data was expressed mean ± SEM with a statistical significance p<0.05.

3. RESULT AND DISCUSSION

Zestoretic exposure have shown no clinical signs in the dams or F1 progeny. Only observed signs that are different from normal are aggressive behavior in few dams which are exposed to 50 and 100 mg zestoretic/Kg BW.The number of live pups per rat significantly (p<0.001) decreased in groups exposed prenatally to zestoretic when compared to controls (Table 1). The litter size is reduced in the 50 and 100 mg zestoretic exposed groups, but the male and female sex ratio is comparable with control group. On PND 1, 7, 14 and 21 the body mass of offspring exposed to zestoretic 100 mg/Kg BW was significantly (p<0.0001) decreased when compared to control group (Table 2). The pups of other two experimental groups (zestoretic 25 and 50 mg/ Kg BW exposed) were shown slight decrease in the body mass/weight on PND 1 and 7, but are in line with controls on PND 14 and 21. However, the weight loss in offspring is dose dependent in the experimental groups over the controls. No significant change has been recorded in anogenital distance, whereas survival index is reduced on PND 4 and 21 in zestoretic 50 and 100 mg/Kg BW exposed pups compare to controls (Table 3).

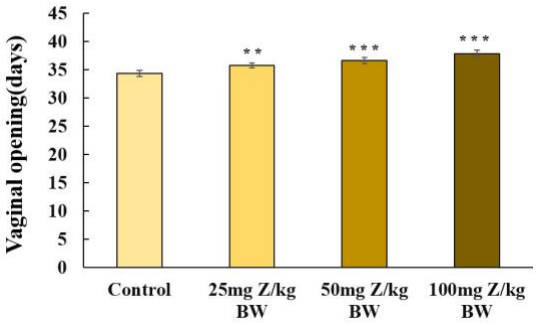

Figure 3. Testes descent of the pups exposed to zestoretic prenatally on postnatal day 1, 7, 14 and 21. Z= zestoretic; BW=body weight.

Table 2. Body weight (gm) of pups exposed to zestoretic during transplacental and perinatal development on PND 1, 7, 14, and 21.

PND	Control	25mg/Kg BW	50 mg/Kg BW	100mg/Kg BW	F-Values
1	6.17 ± 0.097	6.10± 0.062 (-1.13) P=0.4406	5.79± 0.027 (-6.15) P=0.0153	5.69± 0.034 (-7.15) P=0.0015	P<0.0001 F=15.5
7	11.64± 0.15	11.45±0.07 (-1.63) P=0.1599	11.13 ± 0.125 (-4.38) P=0.0953	10.41± 0.048 (-10.56) P<0.0001	P<0.0001 F=20.87
14	18.63± 0.051	18.57± 0.074 (-0.32) P=0.4524	18.47 ± 0.164 (-0.85) P=0.3358	17.56± 0.036 (-5.74) P<0.0001	P<0.0001 F=28.7
21	23.67± 0.135	23.57± 0.054 (-0.42) P=0.5893	23.52± 0.059 (-0.63) P=0.2887	22.68± 0.072 (-4.18) P=0.0006	P<0.0001 F=28.84

Values are mean ± SEM of 8 individuals. BW: body weight
Values in parentheses are percent change from control. P<0.05 was considered as significant

The low survival index was recorded in 100 mg zestoretic/Kg BW exposed pups on PND 4 (75%) and 21

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(62.5%). Crown rump length is significantly decreased (P<0.0001) in zestoretic exposed pups when compared to control group on PND 1, 7, 14 and 21 (Table 4).

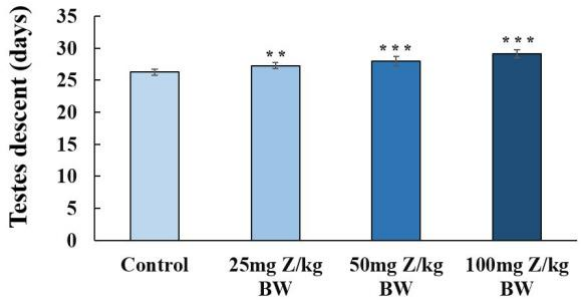
Table 3. Trans-placental exposure of zestoretic on anogenital distance and survival index of rat pups.

Parameter	Control	25mg/Kg BW	50 mg/Kg BW	100mg/Kg BW
Anogenital distance (cm) of male pups on PND 1	0.42±0.016	0.41± 0.012 (-2.38) P=0.5983	0.41± 0.018 (-2.38) P=0.5983	0.4± 0.019 (-4.76) P=0.3506
Anogenital distance (cm) of female pups on PND 1	0.35±0.018	0.33± 0.018 (-5.71) P=0.6845	0.32± 0.016 (-8.57) P=0.6845	0.32± 0.016 (-8.57) P=0.3506
Survival index of pups on PND 4 (%)	47/47 (100)	47/47 (100)	35/42 (82.50)	24/32 (75.0)
Survival index of pups on PND 21 (%)	45/47 (95.74)	42/47 (89.36)	32/42 (71.42)	20/32 (62.50)

Values are mean ± SEM of 8 young rats. BW: Body Weight.
Values in parenthesis are percent change from controls. P<0.05 was considered as significant.

No considerable change has been reported in developmental landmarks like pinna detachment, fur development, lower and upper incisor eruption, eye and ear opening in the young ones exposed prenatally to zestoretic when compare to control (Table 5). Significant increase (P<0.0001) in the mean numbers of preputial separation was observed in 50 (43.5 ± 0.534) and 100 (45 ± 0.756) mg zestoretic/Kg BW exposed pups when compare to control and 25 mg zestoretic/Kg BW exposed groups (Table 5). The delay in mean days for testes descent of experimental male pups (25, 50 and 100 mg/Kg BW exposed; 27.25 ± 0.463, 28 ± 0.756 and 29.12 ± 0.641 respectively) is significant (P<0.001) when compared to control rats (26.25 ± 0.463) (Table 5, Figure 3).Compared to controls (34.37 ± 0.517) female pups exposed prenatally to zestoretic (25, 50 and 100 mg/Kg BW; 35.75 ± 0.463, 36.62 ± 0.517 and 37.87 ± 0.641 respectively) showedsignificant (p<0.001) delay in mean days ofvaginal opening (Table 5, Figure 4).

Figure 4: Vaginal opening of the pups exposed to



zestoretic prenatally on postnatal day 1, 7, 14 and 21. Z= zestoretic; BW=body weight.

Zestoretic is a antihypertensive drug used to treat blood pressure. In the current study we experimented the developmental abnormalities and toxicity of zestoretic at 25, 50 and 100 mg/Kg BW prenatal exposure in Wistar rats.

Reduced litter size and decline in number of male and female sex ratio was reported in the present study. Reduced male and female ratio from control compared to increase the dose concentration, correlating with decrease live pups number/chance, increased trans-placental fetal toxicity of zestoretic towards rise of resorptions and less number of implantations in a dose dependent manner and which is in concurrence with other

Table 4. Prenatal exposure of zestoretic on crown rump length of young ones.

Values are mean ± SEM of 8 young rat. BW: body weight; PND: postnatal day.
Values in parenthesis are percent change from controls. P<0.05 was considered as significant.

PND	Control	25mg/Kg BW	5 0 mg/Kg BW	100mg/Kg BW	P & F-Values
1	4.61 ± 0.023	4.53± 0.018 (-1.73) P=0.0479	4.51± 0.023 (-2.16) P=0.0185	4.42± 0.025 (-4.12) P=0.0004	P<0.0001 F=11.27
7	6.3± 0.019	5.82±0.0163 (-7.61) P<0.0001	5.81 ± 0.0742 (-7.77) P=0.0005	5.71± 0.035 (-9.3) P<0.0001	P<0.0001 F=41.94
14	7.75± 0.0462	7.47± 0.0163 (-3.61) P<0.0001	7.23 ± 0.102 (-6.7) P=0.0005	6.96± 0.0943 (-10.19) P<0.0001	P<0.0001 F=24.8
21	8.6± 0.0.038	8.46± 0.026 (-1.62) P=0.008	8.38± 0.048 (-2.55) P=0.0002	8.22± 0.059 (-4.41) P=0.0002	P<0.0001 F=23.62

viability of pups, male and female ratio in transplacental ACE inhibitors orally administered groups compared to the control (Shrim*et al.*, 2005; Bhatia *et al.*, 2013; Hetterich *et al.*, 2014). Annette *et al* (2009) explained about lisinopril (ACE inhibitor) nature and Olesen*et al* (2001) described diuretics in decreasing the body weight of new borns. In current study, prenatal exposure of zestoretic showed significant (p<0.0001) decrease in the mean body mass/weight of zestoretic 100 mg/Kg BW exposed pups on 1st, 7th, 14th and 21st PND. No modification found in the body mass of zestoretic 25 and 50 mg/Kg BW exposed offspring on PND 1, 7, 14 and 21. Survival index of pups on 4th and 21st PND, and in the survival rate of young ones exposed to zestoretic prenatally found lower.

No change has been recored in anogenital distance by prenatal zestoteric exposure and are similar to the controls in both genders of offspring. Compared to control offspring, significant decline (p<0.0001) in crown lump length and survival index on 1st, 4th, 7th and 21st PND is reported in this study. Aslo found significant decrease (p<0.0001) in the survival rate of offspring exposed prenatally to zestoretic. Change is not significant in pinna detachment, ear opening, fur development, upper and

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lower incisor eruption of experimental groups from the control young ones. Kwak *et al* (2012) reported ACE inhibitors had significantly decrease serum dehydroepiandrosterone (DHEA) level and lower level of serum androstenedione (Westwood, 2008). Transplacental exposure of zestoretic 25, 50 and 100 mg/Kg BW caused significant decrease ($p<0.0001$) in delaying the testicular descent of male offspring when compared to the control. Similar results are reported in rat offspring exposed prenatally to biochanin-A and baicalein (Sridevi *et al.*, 2016).

Table 5. Effect of trans-placental and weaning exposure of zestoretic on developmental landmarks of rat pups**.**

Values are mean ± SEM of 8 young rats. BW: body weight.
Values in parenthesis are percent change from controls. P<0.05 was considered as significant

Parameter	Controls	25 mg/Kg BW	50 mg/Kg BW	100 mg/Kg BW	F-Values
Pinna detachment	3.25± 0.163	3.25± 0.163 P=1.000	3.37± 0.183 (3.6) P=0.5983	3.25±0.163 P=1.000	p=0.9497 F=0.116
Lower incisor eruption	3.37±0.183	3.37±0.183 P=1.000	3.37± 0.183 P=1.000	3.5± 0.189 (3.85) P=0.5983	p=0.9234 F=0.1579
Fur development	7.5±0.189	7.5±0.189 P=1.000	7.62± 0.183 (1.6) P=0.5983	7.5±0.189 P=1.000	p=0.9599 F=0.09859
Upper incisor eruption	10.25±0.167	10.37± 0.183 (1.17) P=1.000	10.25±0.163 P=0.5983	10.25± 0.163 P=1.000	p=0.9497 F=0.116
Ear opening	13.12±0.353	13.12± 0.353 P=1.000	13.12±0.353 P=1.000	13.12± 0.462 (0.99) 0.5983	p=0.8625 F=0.2471
Eye opening	14.25±0.463	14.62±0.517 (2.59) P=0.197	14.75±0.463 (3.5) P=0.1036	15.37±0.517 (7.85) P=0.5983	p=0.0011 F=7.737
Testes descent	26.25±0.463	27.25± 0.463 (3.8) P=0.0011	28± 0.756 (6.66) P=0.0008	29.12±0.641 (10.95) P<0.0001	p<0.0001 F=7.737
Vaginal opening	34.37±0.517	35.75±0.463 (4) P=0.0012	36.62± 0.517 (6.54) P<0.0001	37.87±0.641 (10.18) P<0.0001	p=<0.0001 F=64.47
Preputial separation	42.38±0.517	42.63±0.517 (0.589) P=0.175	43.5±0.534 (2.64) P<0.0001	45±0.756 (6.18) P<0.0001	P<0.0001 F=105

Prenatal exposure with the concentrations of 25, 50, 100 mg zestoretic/Kg BW displays significant rise ($p<0.0001$) in the number of days for vaginal opening in female rat pups. In process of factfinding results supports that the increased days for vagina opening in zestoretic exposed female young rats may be due to early release of pubertal hormones promoted by zestoretic. Moreover, there are no major change observed in the body mass/weight of offspring exposed to 25 mg zestoteric prenatally. But, zestoteric 50 and 100 mg/kg BW prenatal exposure resulted to significant change ($p<0.0001$) in preputial separation in young ones. Joel *et al* (2007) explained additional risk of ACE inhibitor cause malformation of cardiovascular system and central nervus system leads to foetal toxicity. Using of diuretics during gestation period were at increased risk of premature delivery and rise risk of malformations in the progeny (Olesen *et al.,* 2001).

4. CONCLUSION

The effect of zestoretic on growth and development of the gonadal tissue in both sex offspring of rats which may alters the levels of gonadal hormones. For all the observed abnormalities, though there is no direct evidence of zestoretic induced abnormalities in humans at the recommended dosage, developmental abnormalities are reported in rats in the present study. So, the current data may not be extrapolated to humans. However, the pregnant mothers should be aware of these orally administration of zestoretic in order to overcome the infertility and impotence complication in offspring. Future studies with longer duration of zestoretic exposure is necessary to better account for its effects and possible mechanisms as an endocrine disruptor to the reproductive system in both male and female. Thus, in spite the uncritical recommendation on the use of zestoretic and it is advised to avoid the usage of complicated medicine during pregnancy.

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