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Histopathology of the Uterus and Ovary in Experimentally-Induced Lead Poisoning in Rabbit-Does

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Abstract

Many previous studies have established the adverse effect of lead poisoning on many body systems including reproduction. The objective of the present study was to determine the reproductive implication of experimental lead poisoning in rabbit-doe with emphasis on histo-pathology of the ovary and uterus. Nine New Zealand adult rabbit does weighing between 1.5 ± 0.2 to 2.2 ± 0.2Kg were randomly selected into three groups of three animals each. The first group (control) received 2 ml of sterile water per os throughout the period of the study. The second lead group received 5 mg/Kg body weight of lead dissolved in water per as for a period of fourteen days. The third lead group received 10 mg/Kg lead for the same period. The blood samples were taken prior to commencement of lead administration and every 72 hours to the end of the treatment to determine haematological parameters that include packed cell volume (PCV) and haemoglobin concentration while clinical parameters were also measured. Ovaries and uteri were harvested for histo-morphological examination at the end of the study. No significant changes were observed in clinical parameters and haemoglobin concentration between treatment groups compared to the control whereas, anaemia was observed with the 10 mg/Kg lead group. Follicles in the control was healthy morphologically as against atretic follicles observe with lead treated group. Endometrium of the control group showed normal morphology while thinning of the endometrial epithelium was observed with lead treated groups especially the 10 mg/Kg lead group. The reproductive implication of lead treatment in rabbit-doe is possible sub-fertility due to reduced ova at ovulation and thinning of the endometrial epithelium sequel to which is loss of implantation competence of the endometrium. Further studies with more sample size are suggested to validate the results obtained in this study and several mechanisms proposed.

Keywords: Lead, ovary, rabbit-doe & uterus

Running title: Lead poisoning in ovary and uterus

Introduction

Lead poisoning has been recognized as a major public health risk, particularly in developing countries. In spite of various occupational and public health measures undertaken to control lead exposure, cases of lead poisoning are still rampant and reported. At the global level, lead exposure is estimated to account for 0.6% burden of disease, with the highest burden in developing regions(WHO., 2009).Lead acetate is a bio-toxic environmental and industrial pollutant, which accumulates in almost all body

tissues such as the liver, lung, bones, kidneys, reproductive organs and the immune system(WHO., 2009).

Lead is a naturally occurring metal found deep within the ground, however, the widespread occurrence of lead in the environment is largely as a result of human activities, such as mining, smelting, refining and informal recycling of lead; use of leaded petrol (gasoline); production of lead-acid batteries and paints. Particularly in Nigeria, the government effort of diversifying the economy from monolithic oil economy has led to promotion of mining for precious metals and stones. This is not unconnected with associated problem of lead contamination of the environment and water as was observed in Zamfara State in 2010(Lo et al., 2012). Other uses of lead include jewelry making, soldering, ceramics and leaded glass manufacture in informal and cottage (home-based) industries; electronic waste and the use in water pipes and solder(WHO, 2010).Therefore, routes of exposure to lead include contaminated air, water, soil, food, paints and consumer products. Occupational exposure is a common cause of lead poisoning in adults. One of the largest threats to children is lead paint that is seen in many homes, which is the reason for World Health Organisation's (WHO) calling for total avoidance of lead in paint. Animals get exposed to lead through licking of painted wall and metals as well as contaminated water and feed materials.

Among the several factors responsible for infertility and consequential productivity losses in farm animals, malnutrition, ovulatory or hormonal imbalances and infectious agents have been recognized as the major causes. However, information on impact of environmental toxicants like lead on reproduction and how these affect reproduction is almost obscure(Wong and Cheng, 2011). Literatures are filled with lead poisoning in experimental rats with fewer of these studies concentrating on the reproductive system/organ(Omobowale et al., 2014; Sudjarwo et al., 2017). The few on reproductive system/organ have preferred male animals at the expense of the female(Gandhi et al., 2017; Heuser et al., 2013).In female, ovary is primary reproductive organ performing dual roles of oogenesis and steroidogenesis while the uterus harbours the embryo during gestation (Aplin et al., 2008)and also control oestrous cycle by producing the luteolytic prostaglandin $F_{2\alpha}$ in farm animals.

Lead has been associated with intrauterine deaths, prematurity and low birth weight. Mating involving one lead toxic parent has recorded significant decrease in litter size, birth weight and survival rate (Ronis *et al*., 1998). Severe cases of lead poisoning have been reported to be associated with sterility, miscarriage, abortion, premature delivery and infant mortality in female rats as well as in males (el Feki *et al*., 2000; Gorbel *et al*., 2002). These suggests

the possibility of lead crossing the placenta barrier from the maternal environment to the foetus, however, the mechanism still remains unclear.

Rabbit is a small mammal in the family Leporidae of the order Lagomorpha found in several parts of the world(Pearce et al., 2007). They have been domesticated by man for many reasons that included companionship, source of meat for human and animal consumption and experimental model of human diseases (Mapara *et al*., 2012).In some societies, rabbits especially with wooling breed are kept for the purpose of the wool to make clothing and leather. Exploration of experimental rabbit in immunology as reliable antibodies producers have translated into its use for production of monoclonal and polyclonal antibodies (Acosta *et al*., 1999). Today, these antibodies have been used for research, diagnosis and treatment of many diseases.

Among animal species, cattle are most susceptible livestock to lead poisoning, with calves the most likely victims(Waldner et al., 2002) However, lead poisoning can occur in all domestic and farm animals including horses, birds/poultry and dogs, while Pigs are the least susceptible(Burger and Gochfeld, 2000; Siddiqui and Rajurkar, 2008). Cage animals like rabbit and rats are also exposed to lead when they are housed in iron cage coated with paints to reduce corrosion. Paint is also the major source of lead poisoning in calves (Sharpe and Livesey, 2006) and lead poisoning is most common among calves because they are curious feeders with both milk and milk substitutes acknowledge to increase the amount of lead absorbed by calves. Therefore, the aim of the present study is to evaluate the reproductive implication of experimental lead poisoning on rabbit-doe with emphasis on histo-morphological structures of the ovary and uterus.

Materials and Method

Experimental Animal and Management

A total number of 9 mature female rabbits were used in this project. The rabbits were sourced from a commercial rabbit farm in Ikot Ekpene, Akwa Ibom state. The rabbits were house individually in a cage of about 1.5 m^3per rabbit and fed with pelletized feed (Vita feed ®) and had access to water *ad libitum.* The acclimatization of three weeks was planned before the commencement of the study. Humane practice for use of these animals for experimental purpose was strictly followed.

Experimental Design

The 9 rabbits were randomly selected into three groups, made of 3 rabbit per groups. The first group (A) served as the control group receiving only water throughout the

period of the study. The second group (B) was giving 5mg/Kg of lead acetate (Sigma, Aldrich, UK) while the third group(C) received 10mg/Kg of lead in drinking water daily for a period of 14 days. Lead acetate was weighed as per individual weight of the rabbit and dissolved in a 2 mL of sterile water. A 5 ml syringe was used to administer the dissolved lead per as to the animal daily for the entire period of the treatment. This was to ensure that the rabbit actually took the lead at the exact dosages (5 mg/Kg and 10 mg/Kg) as per the experimental design. These dosages were chosen based on evidence from previous studies (Assi *et al*., 2016)

Before and after the period of treatment, physiological parameters and haematological parameters were taken. At the end of the treatment, the reproductive organs (ovaries and uteri) were harvested and processed according to standard technique for histological examination (Raheem *et al*., 2016).

Physiological and Haematological Parameters

Physiological parameters measured were body weight, rectal temperature, heart rates, pulse rates and respiratory rates using conventional methods. Blood samples were collected using the ear vein into a heparinized bottle and stored at 4^0C until the determination of Packed Cell Volume (PCV) and Haemoglobin (Hb) concentration. Mission® Haemoglobinometer (Okhla, New Delhi) testing system was used. The machine made use of strip in which a drop of whole blood was added. The results for the PCV and the Hb concentration were then shown on it after about 30 seconds.

Histology of the Ovary and Uterus

At the end of the 2 weeks of lead administration, the rabbits were euthanized and uteri and ovaries were excised and put into Bouin's solution for 24 hours. This was changed to 75% alcohol and further processed into histology blocks. Paraffin-embedded uterine sections (5 µm) mounted on super frost slides were stained using Haematoxylin and Eosin stains (H & E) as was described in earlier study (Raheem, 2013). Briefly, the procedures involved dewaxing the tissue in clearing agent, Histoclear (Fisher Scientific, Loughborough, UK). The tissues were rehydrated by passing through graded alcohol of 100%, 70%, 50%, 30% and 10% for a period of 5 mins each. The sections were then rinsed in distilled water for 5 mins before immersed in H& E stain for another 15 mins. The stains were rinsed off from the slide in a slow running tap into slides container. A drop of a mounting media was dropped on the slide and the cover slip was applied on top of the section and made to dry. Then after, the slides were ready for microscopy.

Results

Clinical Parameters

The mean values for rectal temperatures, heart rates, pulse rates and respiratory rates obtained at commencement (day 0) and end of treatment (day 15) for the three groups are presented in Table I. There was no significant (P >0.05) change in these parameters before and after treatment in each of the groups.

Haematological Parameters

The mean PCV values obtained before treatment and then at day 3, 7, 20 and 15 (after treatment) for control, 5 mg/Kg and 10 mg/Kg lead treated groups are presented in Table 2. The PCV for the control was 36.0 ± 3.5%, reduced insignificantly (P>0.05) to 34.0 3.0 at day three and rose again to 38.0 + 0.3% on day 7 while it terminated at 38.0 ± 1.5% at the end of treatment. On the other hand, the PCV for the Group B and C before the commencement of treatment were 35.0 ± 1.5% and 37.0 ± 2.3% respectively. There was a persistent reduction in PCV of Group B to 34.0 ± 0.6, 33.0 + 2.0 and 31.0 + 1.2% on days 3, 7 and 10 respectively while a significant reduced value of 31.0 ± 1.0% was obtained at the end of the treatment. On days 3, 7 and 10, PVC values for Group C significantly (P<0.5) reduced to 31.0 ± 0.9, 30.0 + 1.2 and 27.0 ± 1.5% respectively with a final value of 25.0 ± 1.0% at the end of the treatment Furthermore, there were no significant (P>0.05) changes in the PCV for the three groups prior to treatment while PCV values for Groups B and C at the end of the treatment were significantly (P<0.05) lower than that of Group A control.

The mean haemoglobin concentrations of the three groups are presented in Table 3and haemoglobin concentration for the control groups were 11.1 ± 1.1 g/dl prior to commencement of treatment while those of Group B and C were 11.0 ± 0.5 and 13.8 ± 0.7g/dl respectively. A gradual increase was observed with Group A to 11.6 + 0.1, 11.6 ± 0.1 and 13.8 ± 0.06 g/dl in days 3, 7 and 10 respectively, while the values at the end of the treatment was a significant higher values of 13.9 ± 0.1. The Hb concentration for Groups B and C at days 3, 7, and 19 were continuously lower compared to the initial values prior to commencement of treatment and were finally 10.3 ± 0.5 g/dl and 11.9 ± 0.4g/dl respectively at the end of treatment. Also, there were significant (P > 0.05) lower Hb concentrations for Groups B and C at the end of treatment compared to the control.

Table 1. Other Clinical parameters of rabbits before or after treatment with lead

Groups	Before treatment	After treatment
Rectal Temp (^{O}C)		
Control(Group A)	39.7 ± 0.17	40.0 ± 0.09
5 mg/Kg lead (Group B)	40.2 ± 0.18	40.3 ± 0.22
10 mg/Kg lead (Group C)	40.0 ± 0.06	39.5 ± 0.33
Heart rate (b/m)		
Control(Group A)	165.3 ± 7.70	209.3 ± 8.50
5 mg/Kg lead (Group B)	170.0 ± 12.50	200.7 ± 15.60
10 mg/Kg lead (Group C)	187.3 ± 4.70	194.7 ± 11.80
Pulse Rate (b/m)		
Control(Group A)	216.7 ± 9.76	219.3 ± 5.21
5 mg/Kg lead (Group B)	196.0 ± 12.20	208.0 ± 21.40
10 mg/Kg lead (Group C)	216.7 ± 13.80	194.7 ± 11.80
Resp. Rate (c/m)		
Control(Group A)	49.3 ± 1.30	49.0 ± 1.80
5 mg/Kg lead (Group B)	45.3 ± 4.06	49.3 ± 3.46
10 mg/Kg lead (Group C)	44.0 ± 5.03	42.0 ± 2.31

Table 2. Packed Cell Volume (PCV, %) of rabbit treated with lead acetate

Groups	Day (0)	Day 3	Day 7	Day 10	Day 15
Control (Group A)	36.0 ± 3.5	34.0 ± 3.0	38.0± 0.3	39.0 ± 2.0	38.0 ± 1.5
5 mg/Kg Lead (Group B)	35.0 ± 1.5	34.0 ± 0.6	33.0 ±2.0^{a}	31.0 ± 1.2^{a*}	31.0 ± 1.0^{a*}
10 mg/Kg lead(Group C)	37.0 ± 2.3	31.0 ± 0.9^{x*}	30.0 ±1.2^{x*}	27.0 ± 1.5^{x*}	25.0 ± 1.0^{x*}

Different superscript on the same row show significant difference compared to day 0 (pre-treatment) values, while '*' along same column shows significant difference compared to the control. Normal PCV for rabbit is 30-35 % (Etim, 2012).

Table 3. Haemoglobin concentrations (g/dl) of rabbit treated with lead acetate

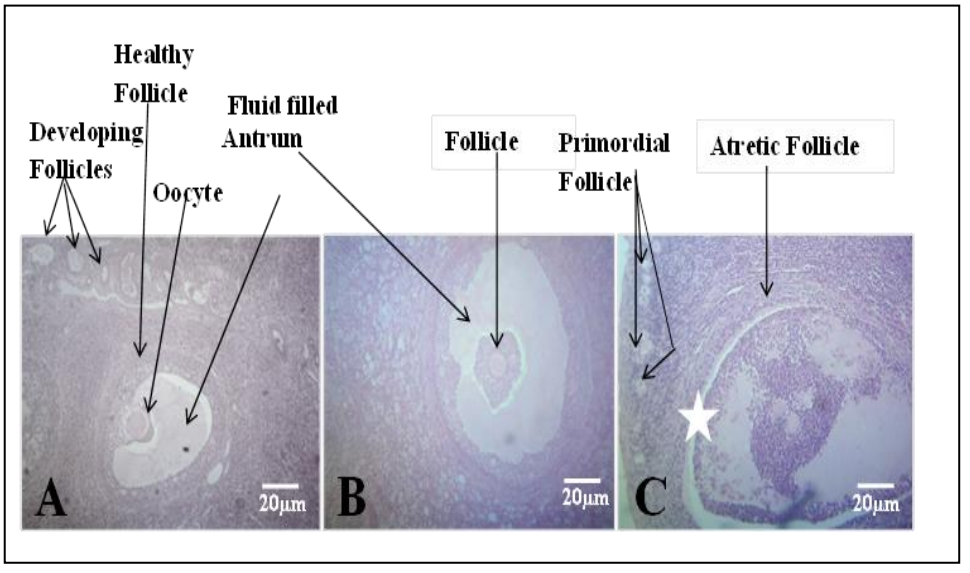
Groups	Day 0	Day 3	Day 7	Day 10	Day 15
Control (Group A)	11.1 ± 1.1^{a}	$11.6 + 0.1^{a}$	11.6 ± 0.1^{a}	13.8 ± 0.06^{b}	13.9 ± 0.1^{b}
5 mg/Kg Lead (Group B)	11.0 ± 0.5	$11.3 + 0.2$	11.3 ± 0.5	10.1 ± 0.7^{a}*	10.3 ± 0.5*
10 mg/Kg lead(Group C)	13.8 ± 0.7^{x}	$12.3 + 0.4^{y*}$	$12.17 \pm 0.42^{y*}$	12.6 ± 3.6^{x}	11.9 ± 0.4^{y}*

Different superscript on the same row show significant difference compared to day 0 (pre-treatment) values, while '*' along same column shows significant difference compared to the control. Normal range of Hb concentration in rabbit is 9.3-19.3 g/dl ((Etim, 2012)

Histology of Ovary and Uterus

In the control, follicles at different stages of development were observed at the medulla of the ovary. Many big follicles looked morphologically healthy with conspicuous antral cavity as well as the oocyte located within one region of the ovary (Figure 1).Group B also showed many follicles at various stages of development (Figure 1B). There are fewer follicles at advanced stage of development, of which few had attained ovulatory size. Group C showed high rate of atresia in the follicles found present in the ovaries (Figure 1C). Most of them did not grow to the ovulatory size. Most of the primordial follicles were not progressive in development. The few found to have reached ovulatory size had started undergoing atresia already. In addition to that, some of the atretic follicles had detachment of the granulosa cells from the theca cells (white arrow in Figure IC).

There were no observable changes in the myometrium and perimetrium of the uterus of groups B and C compared to the control (Figure 2). The effect of lead was observed within the endometrium. There was shrinking of the endometrial glands and thinning of the endometrial epithelia in the lead treated groups compared to the control. These observations are more prominent with the Group B than Group C.



`Figure 1. Histology of ovary in control (Group A), 5 mg/Kg (Group B, B) and 10 mg/Kg lead (Group C; C) treatment groups showing *detachment of the granulosacells from the theca cells of a degenerating follicle (The star in C) H & E*.

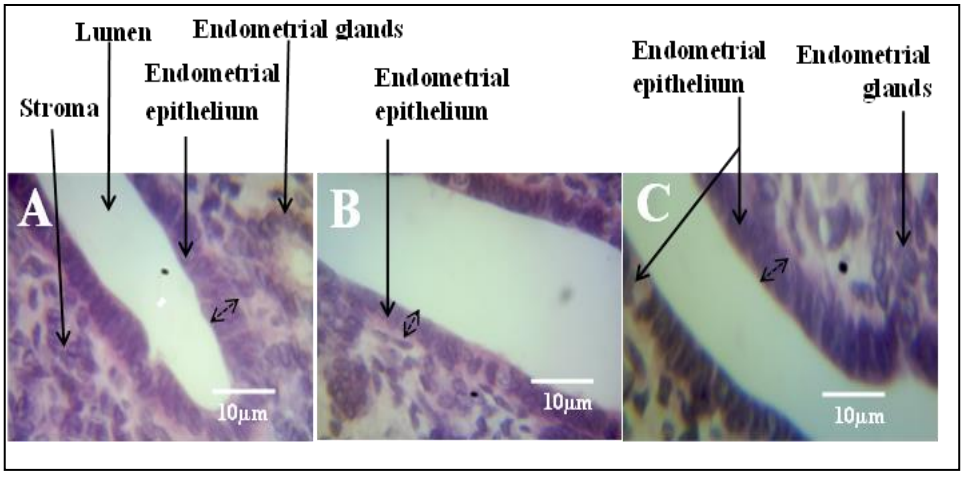


Figure V. Histology of the Uterus of rabbit treated with sterile water (A), 5 mg/Kg (Group B, B) and 10 mg/Kg lead (Group C; C) H& E Stain.*There is shrinkingof the endometrial glands and thinning of the endometrial epithelium (denoted by length of the arrow)of the lead treated groups when compared to the control*.

Discussion

In this study, experimental rabbits were exposed to 5 mg/Kg and 10 mg/Kg of lead acetate dissolved in drinking water and administered orally to determine possible adverse effects on reproductive organs especially the ovary and uterus while other clinical parameters such as body weights, haematological parameters (PCV and haemoglobin concentration) were also measured. Oral administration for 14 days was used since oral route via drinking water especially has been one of the major roots of exposure to lead in humans and animals. According to National Research Council (NRC, 1972), gastrointestinal absorption of lead is highest in children estimated to be about 40% while absorption is 10% in ruminant and 3% in non-ruminants. The duration of 14 days was specific because oestrous cycle in rabbit ranges between 10 to 14 days.

In this study, the basic physiological parameters that include heart rate, respiratory and pulse rate as well as rectal temperature were not affected by the lead treatment when compared to the control. These parameters did not also change significantly with the two lead treatment groups. It was noted that in the rabbit these parameters are greatly affected by handling and hence, this may be responsible for non-significant values across treatments and control groups.

The haematological results in this study showed significant reduction in PCV of lead treated rabbit and relative decline in the haemoglobin concentration as compared to the control group that received water without lead in the drinking water. It is noteworthy that only 10 mg/Kg lead group had an element of anaemia with mean PCV of 25.0 ± 1.0 % at the end of the treatment falling below the normal range of 30-35 % (Etim, 2012)whereas the PCV of the 5 mg/Kg group though reduced but still within the normal range of 30-35 % at the end of treatment. This is similar to various deleterious effects of lead on haematological parameters reported in the literature in both human and animals (Omobowale *et al*., 2014; Ukaejiofo *et al.*, 2009). Specifically, haematological parameters such as haemoglobin, haematocrit, red blood cell count, and mean corpuscular volume were reportedly low in lead-exposed individuals than controls (Feksa et al., 2012). Lead causes reduction in PCV and haemoglobin concentration (as parts of haemopoetic system) through two main mechanisms (Flora et al., 2012). The first is inhibition of the synthesis of haemoglobin by inhibiting various key enzymes involved in the haeme synthesis pathway and secondly via reduction in the life span of circulating erythrocytes by increasing the fragility of cell membranes. In addition, after absorption, almost 99% lead binds to erythrocytes and the remaining diffuses into soft tissues and bones, where it equilibrates with blood lead culminating into the lead

accumulation in erythrocytes, soft tissues and rapidly growing bones(Gwiazda et al., 2005; Kempe et al., 2005; Simons, 1993). Therefore, lead directly affects the hematopoietic system through blocking the synthesis of haemoglobin by inhibiting various key enzymes involved in the haeme synthesis pathway. It also increases the destruction of the cell membranes of the erythrocytes which leads to reduction in their lifespan and the erythrocytes in circulation. These two blood-associated disorders culminate into anaemia.

Ovary is the primary reproductive organ in the female, producing the ova and hormones. The results of this study showed that treatment with lead impaired with follicular growth and enhanced atresia of the follicles. This result is at par with lead-induced reduction in number of primordial follicle and increase in number of atretic follicles in ovaries of mice (Sharma, 2012). The mechanism may be attributed to lack or inadequacy of gonadotropins support for the follicle. The growth of follicle from secondary to ovulatory follicle is gonadotropin dependent. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are essentially required for follicular growth and development, follicular maturation and ovulation(Scaramuzzi et al., 2011).

In the present study, the effect of lead on the uterus was minimal and limited to the endometrium while the myometrium and perimetrium were not affected. The effect on the endometrium observed had to do with thinning of the endometrial epithelium and shrinking of the endometrial glands when compared to the control. Endometrial glands roles in implantation was brought into limelight by Spencer through development of uterine gland knock out ewe model (UGKO ewe) (Spencer et al., 1999).The reduction in the endometrial lining may impair implantation of the blastocyst to the endometrium in a conceptive cycle. Similar pathologies of the uterus have been reported in earlier studies. In contrary to the thinning of the endometrial epithelia observed in this study, Nakade et al. (2015)reported thickening of endometrium leading to narrowing of the lumen and vacuolar degeneration in endometrial epithelial cells. This disparity may be due to different species (rat versus rabbit) and duration of lead administration (14 days versus 28 days). Lead was reported to damage endometrium, myometrium and perimetrium, along with reduction in uterine gland and decrease in height of columnar cells in mice (Qureshi and Sharma, 2012). The non-significant effect of lead poisoning on the morphology of the myometrium and perimetrium reported in this study is in consonance with previous study in rat (Tchernitchin et al., 2003).

The mechanism of lead inducing thinning of the endometrial epithelium as found in this study is

possibly through the reduction in ovarian steroids especially oestrogen. The ovarian steroids progesterone and oestrogen control the architectural physiology of the endometrium and the whole of the reproductive tract during various stages of oestrous cycle(Baby and Bartlewski, 2011). During oestrous, the endometrium is under the influence of oestrogen which brings about the turgidity of the reproductive tract, increase secretion of the tract and behavioural oestrus (Noakes et al., 2009). Hormonal assay for oestrogen was not done in this study due to a number of constrains, however, lead was reported to have caused reduction in progesterone, oestrogen, LH and FSH in primate even though ovulation was not impaired (Franks et al., 1989). In addition it is also possible that lead action on the uterus might be through inhibition of the uterus response to oestrogen as was earlier reported in rat(Tchernitchin et al., 2003). On the contrary, pre-pubertal exposure to lead in mice may neutralize the effects of chronic exposure to lead, providing partial protection of cell function against the adverse effects of chronic exposure to lead in postnatal period and as a non-genetic adaptive protection against long-term environmental variations (Tchernitchin et al., 2011).

The overall implication of lead exposure especially at 10 mg/Kg body weight in drinking water to rabbit is reduced folliculogenesis and alteration in the endometrial epithelia and endometrial glands and hence subfertility. In agreement with the findings in this study, several other studies have shown deleterious effects of lead on reproduction. In experimental animals, chronic exposure to lead may cause inhibition of menstruation, ovulation and follicular growth in monkeys (Vermande-Van Eck and Meigs, 1960), delay in vaginal opening in pubertal rats ((Kimmel et al., 1980) and decrease in frequency of implanted ova and of pregnancies in mice(Odenbro, 1977). Taupeau et al. (2001) demonstrated dysfunction of folliculogenesis, with fewer primordial follicles and an increase in atretic antral follicles in lead treated mice as was found in the present study.

In conclusion, it is evident from the results of the present study that exposure of rabbit-doe to lead (5 mg/Kg and 10 mg/Kg respectively) caused reduction of PCV and haemoglobin concentration. The 10 mg/Kg lead caused significant adverse effect on follicles and endometrial epithelia. The summation of these is possibly subfertility in rabbit exposed to 10 mg/Kg lead of lead in drinking water. Further studies are suggested using higher sample size to validate these findings and several physiological mechanisms proposed in this study.

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