The Effect of Dissolved Nitrate Toxicity on Follicologenesis of the Pregnant Mice and Their Offspring

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***Abstract*—Nitrate is one of the most common ground water contaminants in urban and rural areas. These days sodium nitrate has been added to the environment through burning of the fossil fuels and using the chemical fertilizers. In this research the effects of the Sodium Nitrate (doses 450, 900mg / liter in drinking water) during pregnancy of female mice were investigated. The hypothesis of this study was to find out the developmental effects of Sodium Nitrate on ovaries of the pregnant mice and their offspring's ovaries. For these objectives the offspring in 12 and 24 days after birth were examined, the significant changes in the body weight, the quantity and quality of different follicles, ovary diameter and hormones level were investigated in treated groups. These observations indicate the toxicity of excess nitrates can make changes in ovary such as disturbances in development of follicles and ovarian tissue damage. Even more the oral LD_{50} for mice varied from 2480 to 6250 mg of the Sodium Nitrate /kg [1].**

***Index Terms*—Sodium nitrate, pregnancy, ovary, histology**

I. Introduction:

Nitrate (NO_3) is a compound of nitrogen and oxygen, found in nature, which is colorless, odorless and tasteless [2]. The formation of Nitrate is an integral part of the nitrogen cycle in the environment. Generally, the concentration of nitrates in the ground water is low, but human have altered the nitrogen cycle dramatically over the last half century. Thus, nitrate is steadily accumulating in our water resources. In 1945 the first harmful effect of nitrate were reported by Comly after observing cyanosis in infants in Lowa (US) [3]. Since then there has been concern of possible adverse effects related to high Nitrate consumption [4]. The results were obtained from animal studies demonstrates that male rats which they give exposure with both Nitrite and Nitrate via drinking water have shown effect on testosterone production [5]. Experimental animal studies about the effect of Nitrite and Nitrate on reproductive and abnormal development in their offspring provide moderate evidence for an association between exposure to Nitrate and fetal loss, neonatal mortality and maternal toxicity [6]. Nitrate is converted to Nitrite inside the body. Then, Nitrosamines are produced from nitrites [7], [8]. Their formation can occur only under certain circumstances which is including strongly acidic conditions such as ordinary circumstance in human's stomach [9]. It seems the high Nitrate doses *in vivo* can have significant effect on all of the tissues, including ovaries. The aim of the present investigation was to focus on their effects on the ovaries in pregnant mice and their offspring.

II. Material and Methods

Experiments were performed on adult female NMRI mice (30 ±1 g) with the average age of 8 weeks, provided by Kharazmi University (KhU), Tehran, Iran. Before and during the experiments, animals were kept in special cages with a standard space, under controlled light / dark cycle (light on from 06:00 to 20:00). Humidity and temperature were set at 55±15 % and 20 – 24℃, respectively, and free access to water and cornercial food (Behparvar Co, Iran) was provided. All procedures were carried out according to the Guide for the care and use of laboratory animals at KhU [10]. In this experiment, mice after determining estrous, mating and observing of vaginal plug divided into 3 groups (8 individuals for each group): group 1 (control) they drank water without Nitrate, group 2 which they drank 450 mg/lit of nitrate and group 3 which they drank 900 mg/lit of Nitrate during their pregnancy. Then after 19 days half of each group were weighed and anesthetized with ether and the blood samples were collected from the left ventricle and the serum was separated by using a 6,000 rpm Centrifuge for 5 min.

Embryos were removed from mother's uterus and then were weighed and washed in a dish containing PBS solution. Offspring of the rest of those groups were born, and they were examined at the ages of 12 and 24 days old.

Manuscript received June 2, 2013; revised August 1, 2013

doi: 10.12720/jomb.3.1.33-37

Ovaries were collected, weighed and washed in a dish containing PBS solution and the adipose tissue was removed under microscope. Samples were placed in Bouin`s fixative for histological preparation; fixed samples were kept in alcohol solution of 30 to 100% for a period of 45 minutes to dehydrated, and then in alcohol / Toluene (50:50) and Toluene (three times) for clearing, and finally blocked in paraffin. Samples were sectioned about 7 micron thickness and then placed on slides, which is previously coated with gelatin, before hand for staining with Hematoxylin- Eosin for histological observation. In order to determine follicular development, follicles were classified and investigated based on their morphology and diameter into groups of primordial, primary, preantral, antral and graphian follicle.

III. Statistical Analysis

The one – way ANOVA and SPSS software were used to determine the statistical significance of differences between the values for the experimental and control groups. Data was expressed as means ± standard error (SEM), and the results were taken from at least three independent experiments, performed in triplicates. Values of $p \leq 0.05$ were considered statistically significant.

IV. Results

A. Body Weight

In order to evaluate the metabolic changes in mice, animals were divided into three groups of control, the group which were receiving 450 mg / liter of Sodium Nitrate (group 2), and the group which were receiving 900 mg / liter of Sodium Nitrate (group 3), and weighed. The significant decrease in body weight of the pregnant mice and the 12 days – old immature mice was observed. On the other hand, in the 24 days –old immature mice a decrease in body weight was detected which was not significant (Table I).

B. Fetus Weight

A significant decrease in the weight of the fetuses were detected in both treated groups (group2 and group 3) which decrease in group 3 was substantially more than group 2 (Table I).

TABLE I. Body Weight

Experimental Groups	CONTROL MICE	450	900	CONTROL , 450	450,900	CONTROL , 900
pregnant mice(g)	63.5	57.6	49.8	*	NS	*
Fetus Weight(mg)	1.49	1.25	1.23	*	NS	*
12 day – old(g)	6.73	4.57	3.82	*	NS	*
24day – old(g)	16.6	16.2	15	NS	NS	NS

Table I) mice body weight in experimental groups respectively from left to right (pregnant mice,embryo,12day mice, 24 day mice) * = **$p < 0.05$, NS= not significant**

C. Placental Weight

A significant decrease in the weight of the placenta was detected in both treated group (groups 3 and 2) in comparison to the control one (Table II).

D. Histological Studies of Ovary

First of all, the pregnant mice ovaries have shown a significant increased in Atretic follicle which the increase was observed in the number of Atretic follicles in the third group which was considerably more than second and control group (Fig. 1, 3). In addition, a significant decrease was observed in the number of primary follicles, antral follicles, primordial follicles, preantral follicles and corpus luteum. Which decrease in group 3 was somewhat more than group 2. (Fig. 1, 5). Even more the significant alteration was observed in corpus luteum (Fig. 2). On the one hand, the number of graafian follicle was rare in group 3. Also, the significant increase was observed in the number of atretic follicles in 24-days -old immature mice. On the other hand, in groups 3 and 2, a significant decrease in the number of primary follicles, antral follicles, primordial follicles and preantral follicles was observed (Fig4). Finally, the 12-days old mice were shown a considerable decrease in the number of primary follicles and primordial follicles, respectively , in group 3 and 2 (Fig4).

E. Hormone Assay

A significant decrease was observed in the levels of progesterone, estradiol, in both treated groups (group 2 and 3) (Table II). In addition, these changes in group3 were somewhat more than group 2.

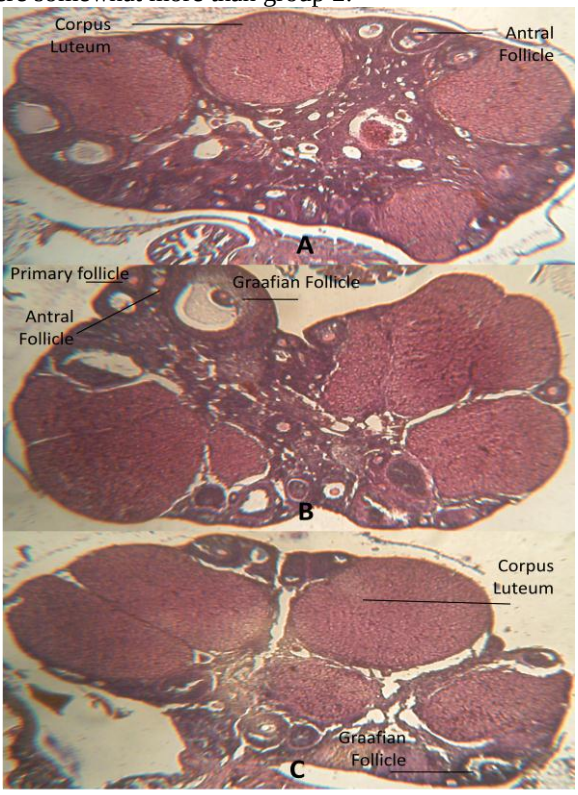

Figure 1. *Histological sections of ovarian* follicles in pregnant mice ovary *(hematoxyline& eosin)* A) pregnant mice ovary which drank 900 mg of nitrate per liter water B) pregnant mice ovary in control group C) pregnant mice ovary which 450 mg of nitrate per liter water.(using microscopy at 40 magnification)

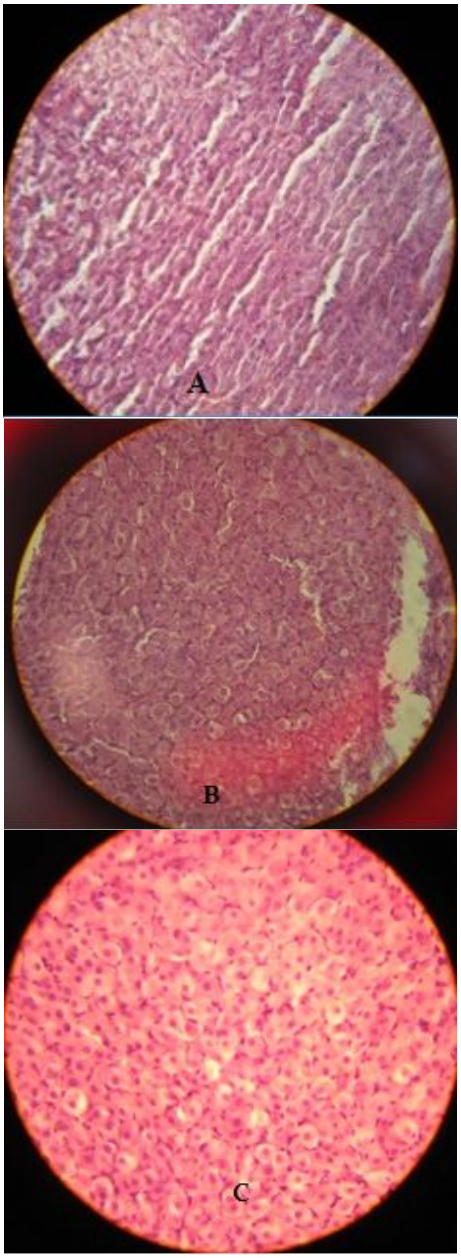

Figure 2. *Histological sections of* corpus luteum in pregnant mice ovary *(hematoxyline& eosin)* A) corpus luteum in 900 group B) corpus luteum in control group C) corpus luteum in 450 group.(using microscopy at 400 magnification)

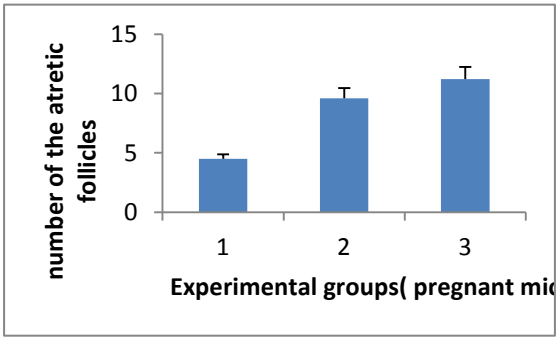

Figure 3. The chart has demonestrated the atretic folicule(group 1:control,group 2:450mg/lit nitrate,group 3 :900mg/lit nitrate) which is shown the significant increased in both treated groups(group 2 and 3)

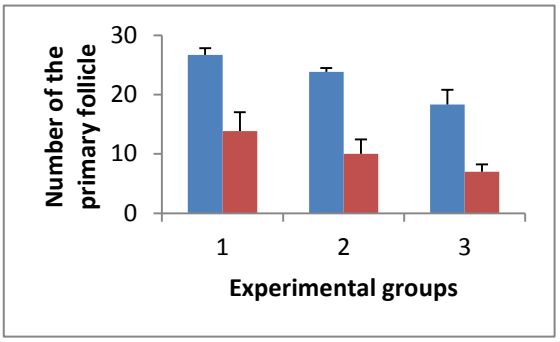

Figure 4. The chart has demonstrated the primary follicles in 12-day-old mice (blue columns) and in 24-day-old mice(red columns) .(Fig1).

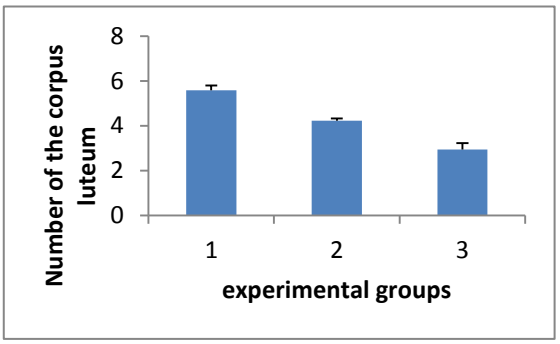

Figure 5. Figure has demonstrated the number of corpus luteum in pregnant mice.the significant reduction was observed in the number of corpus luteum in both treated groups

V. Discussion:

Nitrate can be converted to nitrite in the gastrointestinal tract [10], [11] .Then, nitrite by oxidation of iron hemoglobin can prevent of oxygen transport which this is methemoglobinemia [10]-[12]. Methaemoglobinnmia is because of oxidation of the heme iron is occurred from the normal ferrous (2+) to the met (ferric or 3+) state, in which it cannot combine with oxygen. This disease mostly occurs in the fetus and newborn [12]. Investigation correlated to the fetus and offspring's weight showed a significant decrease in fetal and infancy mice body weight. However, we cannot see a significant change in 24 days old mice likely, this is due to the interaction between nitrite and hemoglobin which interferes with the oxygen-carrying capacity of hemoglobin [12]-[15] which weight decrease can be due to lack of receiving oxygen by tissues. Since, 24-days - old mice are independent and do not uses breast milk. As a result there is a possibility of which these problems can be reversible.

There is evidence that nitro compounds may be generated from nitrites or nitrates *in vivo* [16]-[18]. Also, nitro sating agent (N_2O_3) can be formed from nitrites (NO_2), nitrates (NO_3) and nitro compounds ($C.NO_2$) [16], [18]. N_2O_3 can react with other molecules of oxygen such as super oxides (o2) and produce peroxynitrite ($ONOO^-$) which it can cause tissue damage [17], [19]. It appears that, the ovarian tissue damages were created by these compounds. On the other hand, ovaries are the organs which have changed a lot during the reproductive cycle.

Luteolysis is natural and essential mechanism, which is a sign for decreasing in the number of cellular function. Likely, reduction in the number of the corpus luteum in experimental groups can be created by water nitrate.

TABLE II. EXCESS NITRATE EFFECTS IN MICE PREGNANT AND THEIR OFFSPRING .

Measured parameters	Control	450	900	Control ,450	Control , 900	450,900
atretic folliclesin mice 24day old (n)	3.9	9.91	10.38	*	*	NS
graaf follicle(n)	0.88	0.3	0.15	*	*	NS
Diameter of the ovary in pregnant mice(μm)	327.57	277.55	208.62	*	*	*
Diameter of the ovary in mice 24 day old (μm)	158.33	124.16	119.28	*	*	NS
serum estradiol(pg/ml)	20.88	20.03	16.90	*	*	*
Serum progesterone(ng/ml)	15.79	12.21	11.08	*	*	*
Placental Weight(mg)	0.133	0.108	0.101	NS	*	NS
Diameter of the complexes oocyte - cumulus(μm)	26	19.14	15.42	NS	*	NS
Pregnant ovary weight	0.015	0.013	0.0105	NS	*	NS
Number of the embryo	12	11	9	NS	NS	NS
Preantral follicle theca layer thickness	31	29	19	NS	*	NS
Early antral follicle theca layer thickness	60.43	50.11	46.66	NS	*	NS
Late antral follicle theca layer thickness	111	98.87	65.71	NS	*	NS
Preantral follicle Granulosa layer thickness	37.69	33.84	25.38	NS	NS	NS
Early antral follicle Granulosa layer thickness	67	54	53	NS	NS	NS
Late antral follicle Granulosa layer thickness	54.28	33.33	31.42	NS	NS	NS

* = $p < 0.05$, NS= not significant

In addition, hormone assay in treated mice has showed a significant decrease in serum LH, FSH, Estrogen and Progesterone levels. Reduction in the level of Progesterone has a direct relation to decrease in the number of the corpus luteum. So likely, ovarian tissue damage occurred as a result of Secretory Disorders. Evidence showed which one of the nitrate effect was decreasing the number of the corpus luteum. According to the fact we can say that the reduction of Granulosa thickness of the interna and corpus luteum can be respectively caused by the decrease in the level of estrogen and progesterone in blood serum. Nitric oxide (NO), a highly reactive and short-lived radical, is considered as an important trigger molecule for several physiological mechanisms including gonadotrophin releasing hormone (GnRH) secretion in mammals [20]. And, also No plays important roles in ovulation [21]. Nitrite and nitrate are altered to NO, which binds heme in cytochrome P450 enzymes, thereby inhibiting Steroidogenesis which these events can be related in hormone changes too [22].

Even more there is evidence which is shown the ability of proxy nitrates to cause DNA strand breaks [23], [24]. So, these changes lead to prevent of follicular development in ovary. Therefore, deduction was observed in the number of primary follicles, antral follicles, primordial follicles, Graafian follicles, which are associated with these events. These changes was observed in all treated pregnant mice, 24 days-old mice and 12 days-old mice (Table II).

VI. CONCLUSION

Due to the excess nitrate harmful effect on the ovarian tissue structure it can be concluded that use of nitrates may be causing infertility in long term. Since, increase in the level of progesterone is essential to prevent miscarriage during pregnancy. Therefore, decrease in the number of corpus luteum and hormone level have direct relation and perhaps lead to reduce in the number of embryos. Even more, this observation indicate the toxicity of dissolved nitrates in water can make changes in ovarian tissue such as disturbances in development of follicles and ovarian tissue damage.

ACKNOWLEDMENTS

This project was performed in the laboratory biology at Kharazmi University and the authors would like to thank all lab members, Dr. Kochesfahani, Dr. Azarnia , Ms. Farzaneh Sohoolian and Mr. Arash Salmani for their support.

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