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Effect of Excess Fluoride on Reproductive Potentials in Farm Animals (Ovine)

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ABSTRACT

This study aimed to investigate the effect of the fluorosis on the reproductive and thyroid disorders through its biochemical, histopathological and immunohistochemical findings in one of fluoride endemic area in Egypt. They carried out on forty adult ewes and does which grouped into two groups. The 1st group (20 animals: 10 ewes and 10 does) was control group that brought from the Animal Reproduction Research Institute farm. The 2nd group was a fluoride group (20 animals: 10 ewes and 10 does) that brought from abattoir and farms in Alexandria dessert way, North cost and Alexandria Governorate. Excess water fluorosis (2.9 mg/L) caused a significant ($p<0.05$) decrease in serum progesterone, estrogen, triiodothyronine, calcium, protein, albumin, globulin and total antioxidant capacity (TAC), while urea, creatinine, AST, ALT and MDA levels were increased significantly ($p<0.05$) than normal. Histopathologically, the fluoride group revealed reproductive and thyroid disorders with marked hepato-renal alterations. The examined tissues showed smooth inactive ovaries with atrophied uteri and fibroleiomyoma which were positive immunoreactive to desmine and actine. Also, other cases revealed follicular cystic ovaries associated with chronic cystic endometritis. Moreover, there were degenerative and necrotic changes in the follicular epithelial cells of thyroid glands. Immunohistochemically, marked increased in the apoptosis with caspase3 immunoreactivity in the uterine and thyroid tissues were observed. We concluded that, fluorosis induced reproductive and thyroid disorder as biochemical and pathological alterations are corelated with the significant increase of serum fluoride in fluoride group both in does and ewes (15.01 and 13.03) respectively compared to the control group (3.30 and 2.90) as well as oxidative stress resulted in impaired reproductive function and structure in ovine

1. INTRODUCTION

Minerals are the key component for maintaining health and productivity in farm animals, when increased their levels, leading to toxic effects through their accumulations in meat or milk (Bouaziz et al., 2006). Fluorosis was representing a potential environmental hazard for people and animals (Kumar and Aravindaksha, 2015). Many reproductive problems could be induced by chronic fluorosis as infertility and low birth rates (Zhou et al., 2013). According to WHO guidelines (1994) 1.5 mg/l. is the permissible limit for fluoride in potable drinking water., Fluoride concentration in industrial waste water samples

collected from Abu Zabaal and Ahlia areas around Cairo vary from 1.13 to 7.10 mg/L, significantly exceeding the World Health Organization recommended maximum 1mg F/L (Helal and El Dakdoky , 2006) . Raghib et al. (1994) found that the general emaciation with stunted growth poor performance, reproductive disorders, respiratory and digestive diseases due to fluorosis in dairy Friesian cows nearby aluminum factory in Egypt. Functional and structural damages caused by chronic fluorosis have been reported in many tissues, including kidney (Karaoz et al., 2004), ovary (Chinoy et al., 2004) and liver (Shashi and Bhardwaj, 2011). Fluoride was known to cross the cell membranes and enter the soft tissues (Aydin et al., 2003). Elevated serum urea, nitrogen and creatinine are reported in cows and buffaloes afflicted with fluorosis (Suttie et al., 1987). Proper thyroid function and hormones are essential for healthy and normal reproduction which acting on the cell metabolism regulation (Muderris et al., 2011). They play an essential role in ovarian physiology as the ovarian surface epithelium is the target for thyroid hormone. Therefore, female fertility is inhibited by both hyperthyroidism and hypothyroidism (Kang et al., 2013). Previous studies showed that oxidative stress and generation of free radicals as well as impairment in the natural body antioxidants or reactive oxygen species (ROS) scavenging enzymes (Anuradha et al., 2001).

Apoptosis is an active and regulated program of cell death. It characterized by a series of biochemical and morphological changes, such as caspase family activation, DNA fragmentation, cell volume loss and chromatin condensation. It has been suggested that oxidative stress and ROS which playing the main role to provoke apoptosis and massive cellular damage that associated with lipid peroxidation and alterations of protein and nuclei (Long et al., 2009). However, scanty investigations on fluorosis in domestic animals inhabiting fluoride endemic areas and its effect on fertility

Therefore, this study was conducted to investigate the relationship between the fluorosis and its effect on the reproductive organs and thyroid glands with its reflection on biochemical, histopathological and immunohistochemical investigations in one of fluoride endemic area in Egypt.

2. MATERIALS AND METHODS

2.1. Animal groups and collection of Samples:

The present study had been carried out on 40 adult ewes and does grouped to two main groups.

1-1st group was as control group (20 animals: 10 ewes and 10 does) which brought from the Animal Reproduction Research Institute farm. They have normal estrus activity and body weight was ranged from 45-50 kg and 25-31kg, respectively.

2- 2nd group was fluoride group (20 animals: 10 ewes and 10 does) which brought from Alexandria dessert way, North cost and Alexandria Governorate (assigned as endemic area). The later was high fluoride level in underground water (2.9 mg/L). This included two subgroups (a &b).

a- Farm samples: blood samples collected from each animal with their reproductive records for the investigation of the reproductive performance.

b- Abattoir samples: blood and tissue samples (ovaries, uteri, thyroid gland, liver and kidneys) were collected from ten ovine female animals.

All the collected blood and tissue samples were applied for hormonal and biochemical examination as well as histopathological, histochemical and immunohistochemical investigations.

2.2. Fluoride in water samples:

Water samples were collected monthly and preserved, analyzed and estimated with using the fluoride ion electrode (Tusl, 1972).

2.3. Evaluation of pregnancy and viability rate:

The breeding data were taken from the farm records; included general health condition, temperature, number of previous parturition, date of the last birth, illness and any previous treatment.

2.4. Biochemical assay:

Blood samples were collected directly from the jugular vein every two weeks (farm samples) as well as before slaughter (abattoir samples) then kept frozen at 20°c till assayed and the serum samples were prepared by centrifugation at 3000 rpm for 10 min.

2.4.1. Fluoride in serum samples: Serum samples were analyzed for fluoride using the fluoride ion electrode (Tusl, 1972).

2.4.2. Serum samples were analyzed for serum transaminases including aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) according to Reitman and Frankel. (1957).

2.4.3. Urea and creatinine (Young, 2000).

2.4.4. Malondialdehyde (MDA) content was measured according to the earlier method reported by Zhang, (1992).

2.4.5. Total antioxidant capacity level (TAC) was measured according to Cortassa *et al*. (2004).

2.4.6. Serum was also assayed for estrogen, progesterone and triiodothyronine hormone which were assayed by direct enzyme linked immunoassay (ELISA) according to Maxey *et al*. (1992).

2.5. **Pathological studies:**

2.5.1. Histopathological examination: ovaries, uteri, thyroid glands, liver and kidneys were dissected and preserved in 10% neutral formalin solution for fixation then dehydrated through ascending grades of alcohol, cleared in xylene, embedded and blocked in paraffin. Sections of 3–5-µm thickness were taken and stained with hematoxylin and eosin as described previously by Suvarna *et al*. (2013) then examined by light microscope.

2.5.2. **Histochemical staining:** Masson's Trichrome stain was used for connective tissue proliferation demonstration and Periodic Acid Schiff (PAS) reaction was applied to demonstrate carbohydrates. All these procedures were applied as previously described by Suvarna et al. (2013)

2.5.3. Immunohistochemical evaluation:

a- Caspase-3: The expression of caspase-3 positive cells was determined immunohistochemically in deparaffinized uteri and thyroid tissue sections for detection of apoptosis as golden or dark brown stained immuno-positive cells, according to Ozmen and Mor (2012) . All the steps for were carried out using image analysis software (Image J, 1.46a, NIH, USA). *b-Desmin and Actin immunohistochemistry***:** They were applied according to Gruchala *et al.* (1997) for recognizing of tumors of myogenic origin.

2.6. **Statistical analysis:**

The mean ± S.D. Valueswere calculated for each group to determine the significance of intergroup difference. Each parameter was analyzed separately using one-way ANOVA analysis of variance. P values <0.05 were considered to be significant (Sendecor and Cochran 1989).

3. RESULTS & DISCUSSION:

Fluoride interferes with the metabolic processes of carbohydrates, lipids and proteins that induced structural and functional impairment in many organs (Nabavi *et al. 2013)*. Fluorosis may directly or indirectly modulate the enzyme activity by forming complexes with the metal part of enzyme molecules that inhibited enzymes involved in major metabolic pathways as glycolysis and the Krebs cycle. In addition, F inhibits fatty acid oxidation and reduces the activity of pyruvate dehydrogenase which reduced the amount of cellular acetyl-CoA (Chlubek, 2003).

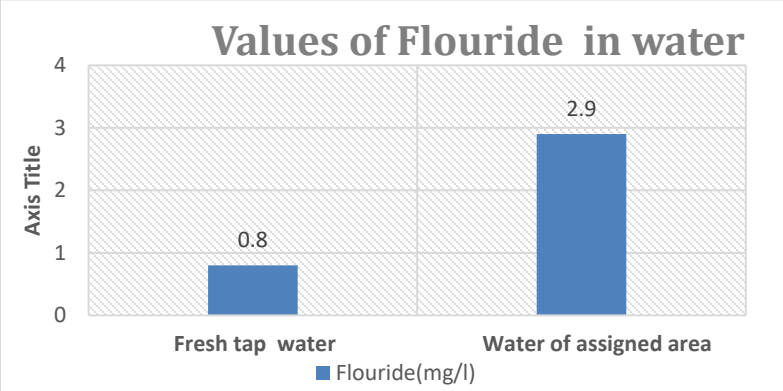
3.1. Values of Fluoride in water:

Examining the water supplied for the control and fluoride groups was described in table (1

Table (1): Values of fluoride level in water samples:

Parameter	Fresh tap water	Water of assigned area
Fluoride(mg/l)	0.8	2.9*

*Values represent mean ± S.E (P<0.05).



The present findings showed a significantly high (P<0.05) values of fluoride in water when compared with the control as shown in table1.The fluoride concentration of 60.12% water samples was 2.9 mg/L which more than the permissible limit (1.5 mg/L) as recorded previously by WHO (1994).

3.2. Effect of excess fluoride on the reproductive performance:

The effect of fluoride on the reproductive performance of both ewes and does showed in table (2 &3).

Table (2): The effect of fluoride on the reproductive performance of ewes:

Items	Group I control (n=10)	Group II fluoride(n=10)
No of ewes	10	10
Body weight	51.7 ± 0.2 [a]	46.3± 0.6 [b]
Pregnancy%	8/10 (80%) [a]	5/10(50%) [b]
Total no of lambs born	11 [a]	5 [b]
Litter weight at birth (kg)	4.3±.16 [a]	3.1±.14 [b]
Prolificacy %	11\10 (110%) [a]	5\10 (50%) [b]
Fertility %	8\10 (80%) [a]	5\10 (50%) [b]

Means with different superscripts in the same row indicate significant difference at (P< 0.05).

Table (3): The effect of excess fluoride on the reproductive performance of does:

Items	Group I control(n=10)	Group II fluoride(n=10)
No of goats	10	10
Body weight	31.8 [a]	24.4 [b]
Pregnancy%	10/10 (100%) [a]	6/10(60%) [b]
Total no of lambs born	13 [a]	6 [b]
Litter weight at birth (kg)	2.81±.17 [a]	3.2±.19 [b]
Prolificacy %	13\10 (130%) [a]	6\10 (60%) [b]
Fertility %	10\10 (100%) [a]	6\10 (60%) [b]

Means with different superscripts in the same row indicate significant different at (P< 0.05).

The effect of the increased fluoride in water on the reproductive performance of the ewes and goats were noted in current study. It was found that the pregnancy % was higher significant (P<0.05) in the control group compared with the fluoride group, the pregnancy % was 80%, 50% in ewes and 100%,60% respectively as shown in tables (2&3). Concerning the birth weight of the newly born lambs was higher in the lambs born from the control ewes as the mean values recorded (4.3±.16) in ewes, meanwhile, it recorded (2.81±.17) in goats. Prolificacy was the lower in the fluoride group (50%) and it was highest in the control group (110%) in ewes, meanwhile, it recorded (130% and 60%) in goats. Fertility was the lower in the fluoride group (50%) and it was highest in the control group (80%) in ewes, meanwhile, it recorded (100% and 60%) in goats. The results indicated that chronic fluorosis was markedly impaired the reproductive function in the

area of investigation in our wok. Those findings came in agreement with Chinoy *et al.* (2004) who recorded that `high prevalence of sterility, still birth, birth of weak offspring, low conception rate and declined in fertility have been reported in domestic animals in the areas with high fluoride contents in water. Moreover, It was reported in cows by Ulemale *et al*. (2010) as a significant increasing in post calving anestrous and decline in fertility as well as repeat breeding were recorded.

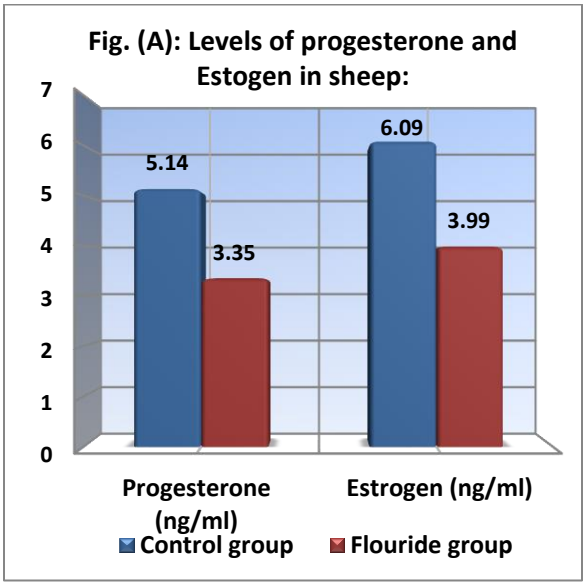
3.3. Effect of fluoride on hormonal profile:

Table (4): Values of serum progesterone, estrogen and T3 in ewes:

Hormones	Control group (n=10)	Flouride group (n=10)
Progesterone (ng/ml)	5.14±0.10[a]	3.35±0.51[b]
Estrogen (pg/ml)	6.09 ± 0.14 [a]	3.99 ± 0.22 [b]
T_3 (ng/dl)	136.7 ± 0.12 [a]	119.2 ± 0.41[b]

*Values represent mean ± S.E.

Means with different alphabetical letters in the same row are significantly different (p<0.05).



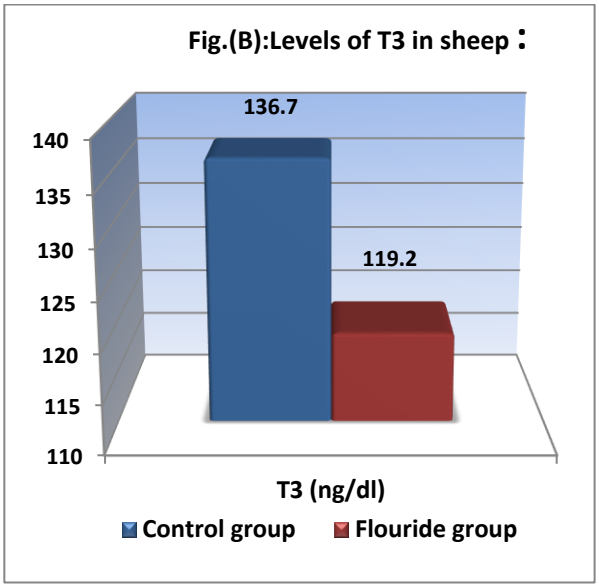
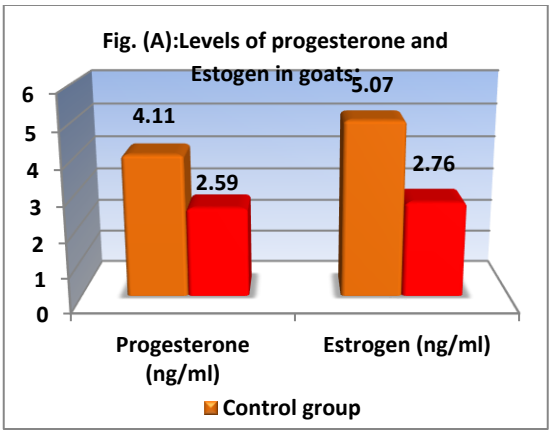


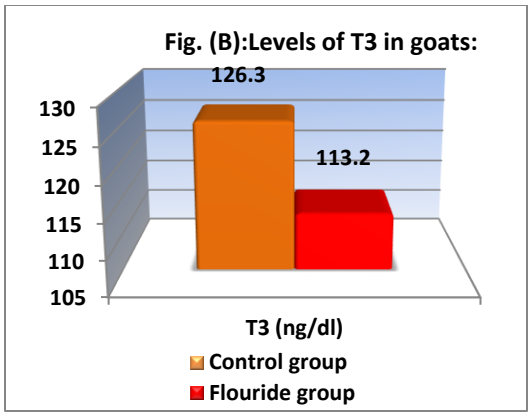
Table (5): Values of serum progesterone, estrogen and T3 in goats :

Parameters	Control group (n=10)	Fluoride group(n=10)
Progesterone (ng/ml)	4.11±0.13[a]	2.59±0.71[b]
Estrogen (pg/ml)	5.07 ± 0.03 [a]	2.76± 0.05 [b]
T_3 (ng/dl)	126.3 ± 0.12 [a]	113.2 ± 0.31[b]

*Values represent mean ± S.E.

Means with different alphabetical letters in the same row are significantly different (p<0.05).





This study revealed a significant decrease ($p<0.05$) in progesterone, estrogen and triiodothyronine in GP II against the control group in all animals (ewes and goats) as shown in tables (4&5), respectively.

These findings might be due to fluorosis that induced structural and physiological changes which reducing the fertility and disturbing the reproductive hormones levels through inhibiting the ovarian hormonal function and cellular integrity (Wang *et al.,* 2009 and Dhurvey *et al.,* 2017) .Several studies indicated that fluoride can directly damage thyroid follicles which interfering with the biosynthesis of thyroid hormones as well as impairing the reproductive functions and structure of female system. This interference mainly through three mechanisms: impairing normal structures of the thyroid gland, disrupting iodine metabolism in thyroid glands, and interfering with tissue-specific metabolism of thyroid hormones. In this respect Thyroid function ultimately depends on the iodine supply to the gland. The structure of F is similar to iodine because both belong to halogen group and fluoride is more chemically active than iodine. Therefore, F binds to the iodine receptor on thyroid gland, inhibits Na/K-ATPase activity, (Wang *et al.,* 2009). This mechanism disturbs the conversion of T4 into active T3 and decrease in the circulating thyroid hormones. More over F able to elevate the level of catecholamines through the stimulation their release which cause marked changes in the levels of reproductive hormones (Zhan *et al.,* 2006). Development of hypo-thyroidism had been observed after ingestion of fluoride in calves and rats by Wang *et al*. (2009).

3.4. Serum chemistry:

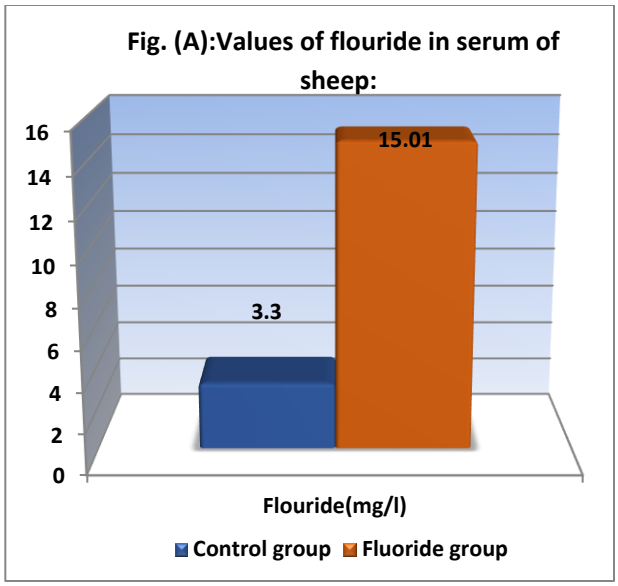
3.4.1. Fluoride, Protein profile, urea, *creatinine, AST and ALT:*

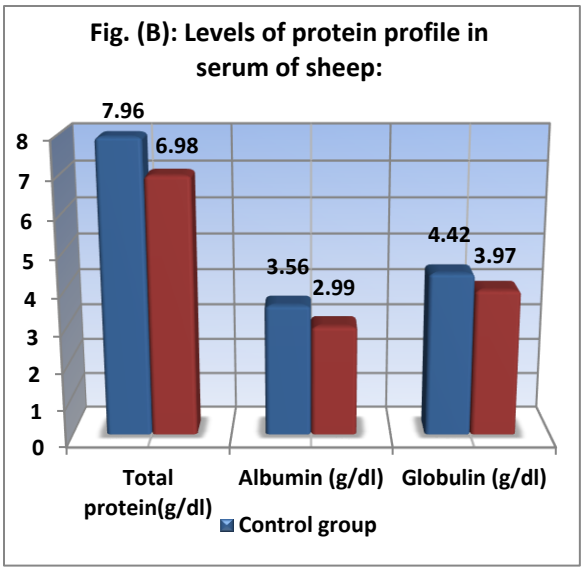
Table (6): Values of Serum biochemical parameters in sheep:

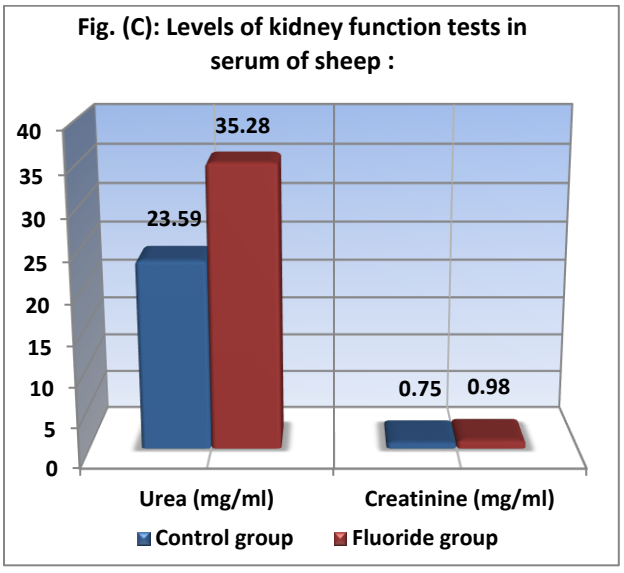
Parameters	Control group (n=10)	*Fluoride group* (n=10)
Fluoride(mg/l)	3.30[a]	15.01[b]
Total protein(g/dl)	7.96±0.15[a]	6.98±0.07[b]
Albumin (g/dl)	3.56±0.12[a]	2.99±0.12[b]
Globulin (g/dl)	4.42±0.09[a]	3.97±0.09[b]
Urea (mg/ml)	23.59±0.86[a]	35.28±0.60[b]
Creatinine (mg/ml)	0.75±0.02[a]	0.98±0.05[b]
Calcium (mg/dl)	8.98±0.10[a]	6.46±0.26 [b]
Inorganic phosphorous (mg/dl)	5.54±0.15[a]	4.96±0.28[a]
A.S.T(Iu/L)	34.80±0.56[a]	60.82±1.93[b]
A.L.T(Iu/L)	28.09±0.16[a]	59.05±2.14[b]
MDA (nmol/mL)	2.98 ±0.56[a]	3.83 ±0.47[b]
TAC (mmol /L)	1.54 ±0.22[a]	1.28±0.25[b]

*Values represent mean ± S.E.

Means with different alphabetical letters in the same row are significantly different ($p<0.05$).







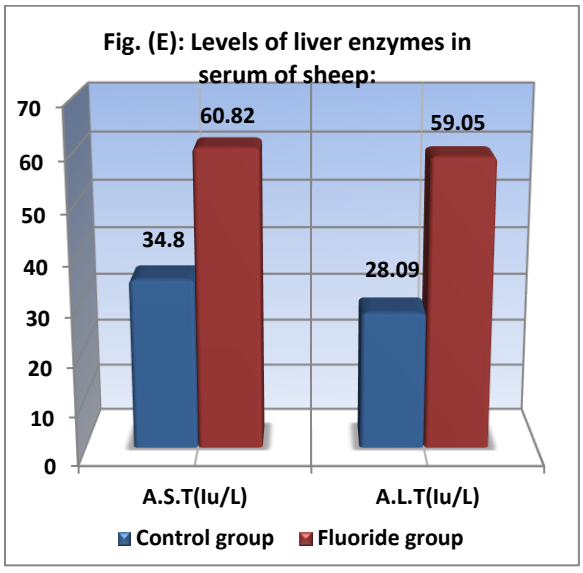
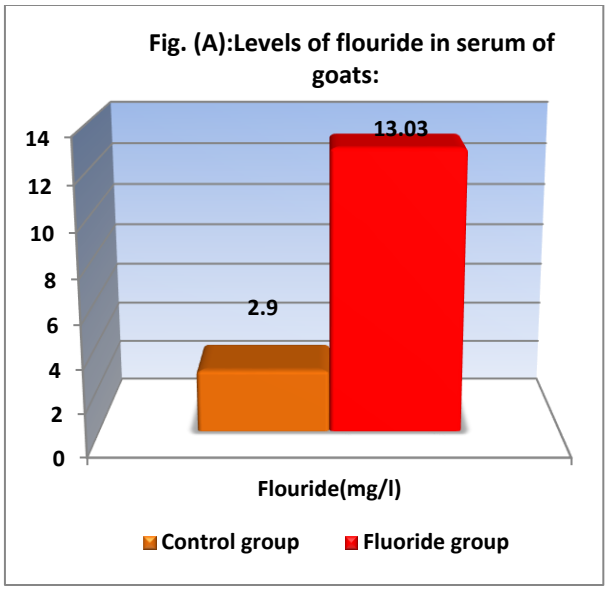


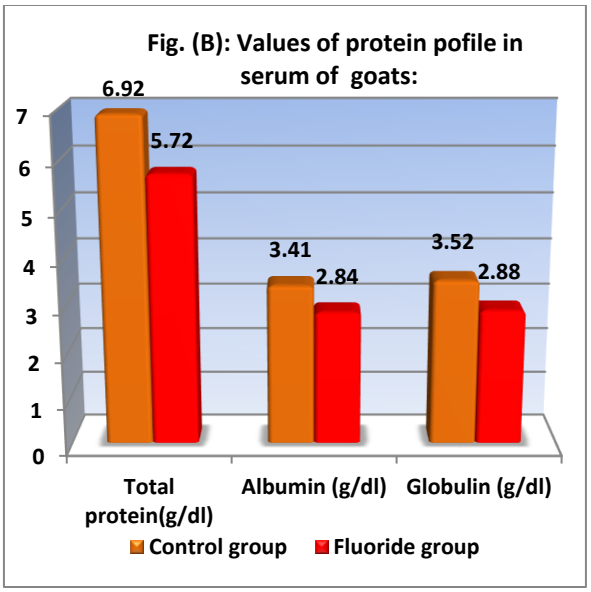
Table (7) values of some biochemical parameters in goats:

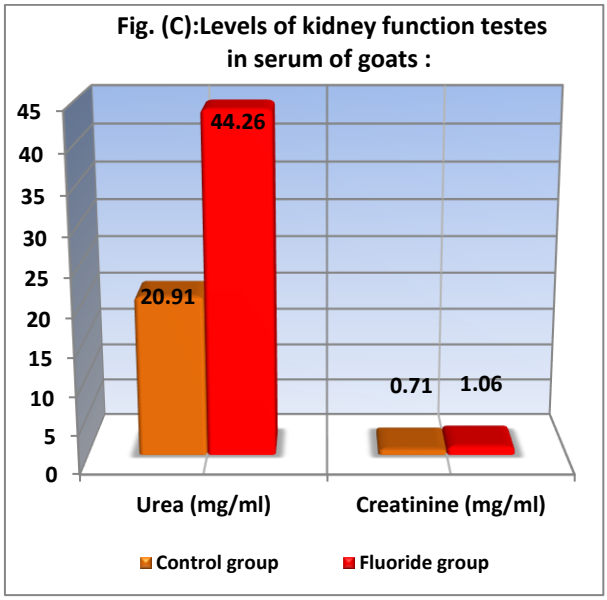
Parameters	Control group (n=10)	Fluoride group (n=10)
Flouride(mg/l)	2.90[a]	13.03[b]
Total protein(g/dl)	6.92±0.07[a]	5.72±0.10[b]
Albumin (g/dl)	3.41±0.10[a]	2.84±0.09[b]
Globulin (g/dl)	3.52±0.14[a]	2.88±0.09[b]
Urea (mg/ml)	20.91±1.24[a]	44.26±0.72[b]
Creatinine (mg/ml)	0.71±0.03[a]	1.06±0.04[b]
Calcium (mg/dl)	8.03±0.41[a]	6.67±0.09[b]
Inorganic phosphorous (mg/dl)	5.05±0.10[a]	4.73±0.13[a]
A.S.T(Iu/L)	33.86±0.33 [a]	76.42±3.81[b]
A.L.T(Iu/L)	29.05±0.41[a]	77.51±1.55[b]
MDA (nmol/mL)	2.73 ±0.33[a]	3.54 ±0.15[b]
TAC (mmol /L)	1.73±0.41[a]	1.19±0.31[b]

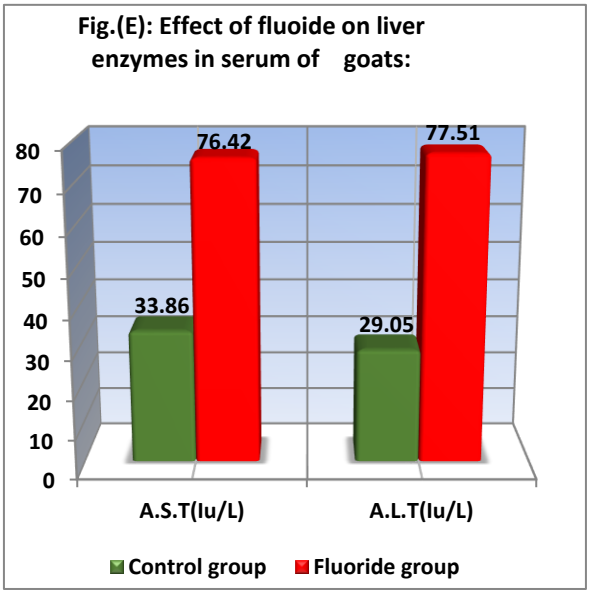
Values represent mean ± S.E.

Means with different alphabetical letters in the same row are significantly different ($p<0.05$).





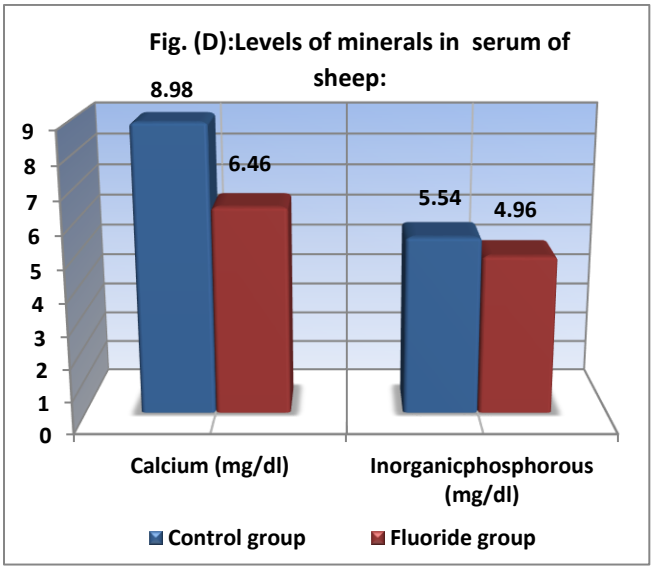


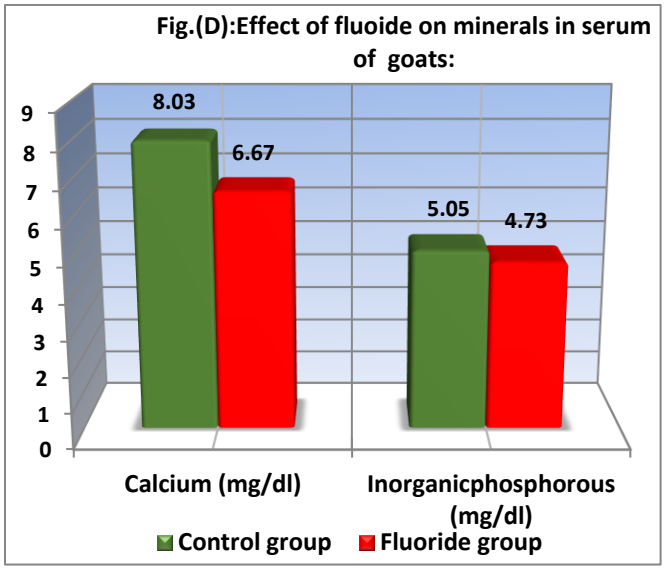


The present results demonstrated that there was a significant increase in fluoride level in GP II comparing with the control group as shown in Table (6). Serum chemistry in fluoride animals (ewes and goats) revealed a significant decrease ($p<0.05$) in values of protein profile compared with control. Values of urea, creatinine, aspartate aminotransaminase and alanine aminotransferase revealed significant increase ($P < 0.05$) in all fluoride groups (tables 6 and 7).Whereas, the values of urea, creatinine, aspartate aminotransaminase and alanine aminotransferase revealed significant increase ($P < 0.01$) in all fluoride groups .Those findings come parallel with the fluorosis induced hepatorenal histopathological changes come With agreement of previous observations by Chlubek et al., (2003) Who reported that, patients with chronic renal insufficiency or reduced glomerular filtration rates have a decreased ability to excrete fluoride in the urine and increased risk developing fluorosis even at normal recommended limit of 0.7 to 1.2 mg/l.Concerning with plasma albumin and protein levels as well as serum protein profile, we observed a

significant decrease in compared with control. Those findings came with agreement of Choubisa et al. (2011) in sheep due to fluorosis and explained as F increases the production of free radicals which ultimately react with Sulphur radicals which forming three-dimensional malfunction albumin then damaged due to the formation of disulphide bounds (Rae et al., 2004).

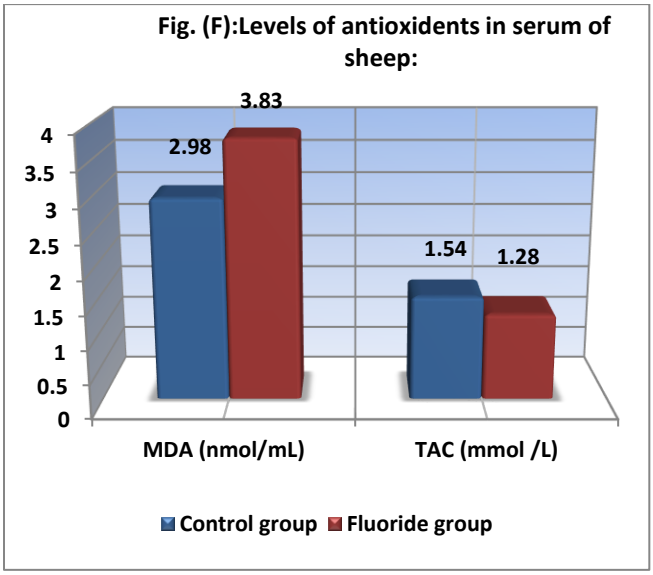
3.4.2. Macro-minerals of serum:

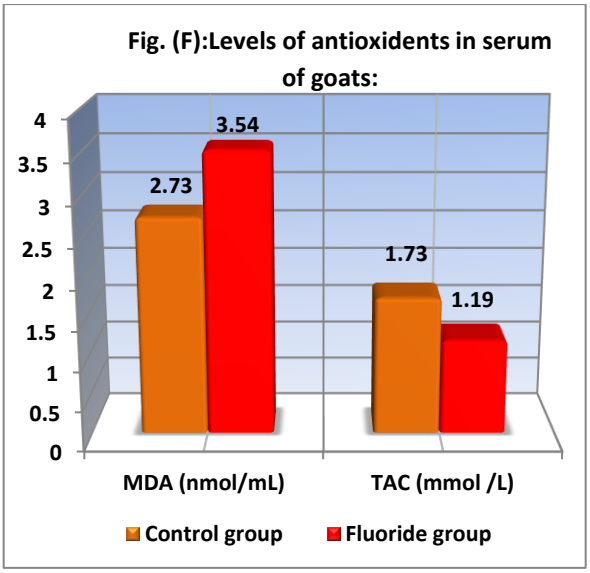




In present study, values of calcium, revealed significant decrease in all groups of animals (ewes and goats) compared with control, while inorganic phosphorus revealed non-significant changes in both ewes and goats as showed in tables 6 and 7. This might be is related to a decrease of intestinal absorption as well as enhanced excretion of calcium via urine by fluorine (Bharti *et al*., 2007). The present findings are in parallel with those reported the decline in serum calcium in cattle and buffalo due to imbalance in the serum calcium and phosphorus levels as fluoride induced Ca chelating (Madan *et al.*, 2009). Our observations on serum inorganic P was in line with earlier findings of Vashishth *et al.* (1998)

3.4.3. Effect of fluoride on antioxidants activity (Malondialdehyde (MDA) and Total Antioxidants Capacity (TAC) :





The free radicals generation constituted one of the mechanisms of fluoride intoxication as Reactive Oxygen Species (ROS) play key roles in many physiologic and pathogenic processes (Finkel and Halbrook, 2000). In the present study, MDA level were increased significantly ($p<0.05$) while TAC was significantly decreased ($p<0.05$) which indicated that chronic fluorosis induced oxidative stress and in response to increase fluoride in water compared with normal control group tables (6 and 7).These results were consistent with the results reported by Shivarajashankara and Shivashankara 2002) in pigs. Chinoy et al. (2004) had reported that, fluoride can pass through the blood brain barrier and accumulates in brain tissue and causes impaired antioxidant defense system. While significantly decreased serum TAC were noticed in our work which was similar to the results published by Li et al. (2003), who reported that high fluorine intake significantly decreased serum T-AOC concentrations in the serum, liver, kidney, spleen and brain of goats. These enzymes activities showed a dose-effect correlation and have significantly negative correlations with different levels of fluorine.

3.5. Pathological results:

The incidence of histopathological alterations of different reproductive and hepato-renal disorders in the collected animal tissues from the abattoir in the same assigned endemic area for fluorosis was illustrated in table (8 &9). which, are corelated with the significant increase of serum fluoride in fluoride group both in does and ewes (15.01 and 13.03) respectively compared to the control group (3.30 and 2.90) as well as oxidative stress resulted in impaired reproductive function and structure in ovine.

Table (8): The incidence of reproductive disorders and hepato-renal histopathological changes under the effect of fluorosis:

Animals	Ewes	Does	No.	%
1-Animals having reproductive and thyroid disorders	5	2	7	70
2-Animals free of reproductive or thyroid disorders (having variable hepato-renal disorders	3	0	3	30
Total Number of animal	8	2	10	100

% were calculated according to total number of abattoir collected animal tissues (n=10)

Table (9): Histopathological alterations in tissues of ewes and does from the abattoir in the same assigned endemic area for fluorosis:

Tissue	Lesion	Ewes	Does	NO	%
Ovaries & their uteri	1-Smooth inactive ovaries with atrophied uteri and uterine fibroleiomyoma with papillary hyperplastic proliferations of the uterine gland epithelium	1	1	2	29
	2- Follicular cystic ovary associated with chronic cystic endometritis	4	1	5	71
Thyroid	Hypertrophied with hypothyroidism	5	2	7	100
Liver	1-Enlargement, congestion, dilated congested blood vessels	3	1	4	57
	2-Pale, scattered necrotic foci with fibrosis.	2	1	3	42
	3-Ballooning degeneration, hepatic necrotic foci	3	1	4	57
	4-Hyperplastic proliferative changes as well as portal and periportal fibrosis with moderate leukocytes infiltration	2	1	3	42
Kidneys	Degenerative and necrotic changes in tubular epithelial cells with interstitial nephritis	3	1	4	57
	Atrophied glomeruli, peri-glomerular fibrosis, Interstitial edema and congestion were observed	2	1	3	42

% were calculated according to total number of examined animal tissue sample (n=7)

In the present study, 70% of the examined tissue samples revealed reproductive and thyroid disorders and 100% showed variable hepato-renal histopathological changes that are explained with the significant increase of serum fluoride in fluoride group both in does and ewes (15.01 and 13.03) respectively compared to the control group (3.30 and 2.90) as well as oxidative stress resulted in impaired reproductive function and structure in ovine as showed in table (8&9).

3.5.1. Effect of fluoride on female reproductive organs:

As showed in table (9), out of the ten examined animals, two cases showed smooth inactive ovaries with atrophied uteri associated with fibroleiomyoma that appeared as a single large reddish white fleshy mass with multiple foci of necrosis and trabeculated. Whereas, the other five animals showed follicular cystic ovaries with chronic cystic endometritis were appeared grossly. Microscopically, ovaries showed excessive fibrous connective tissue proliferation with complete absence of follicular or luteal developments with interstitial vascular congestion in cases of smooth inactive ovaries. In addition to, the associated uteri revealed that chronic metritis with focal and/or diffuse subepithelial mononuclear inflammatory cell infiltrations mainly lymphocytes and plasma cells as well as fibrous connective proliferation (Fig. 1). Also, a noticed fibroleiomyoma in which endometrial hyperplastic proliferation of smooth muscle and perivascular and periglandular fibrous connective tissue proliferation with vasculitis were observed. The uterine glandular epithelial lining showed papillary hyperplastic proliferations (Fig. 2). On the other hand, the macroscopic examination of ovaries of five animals (four ewes and one doe) showed follicular cystic ovaries with edematous, congested and focal hemorrhagic uteri. Microscopically, follicular cysts that have thick vascular connective tissue wall lined by many layers of atrophied, degenerated granulosa cells containing homogenous eosinophilic secretion with noticed interstitial edema, congestion, hemorrhages and monocyte infiltrations (Fig. 3). Also, the decreased and degenerated ovarian follicles with an increase in atretic follicles were appeared.

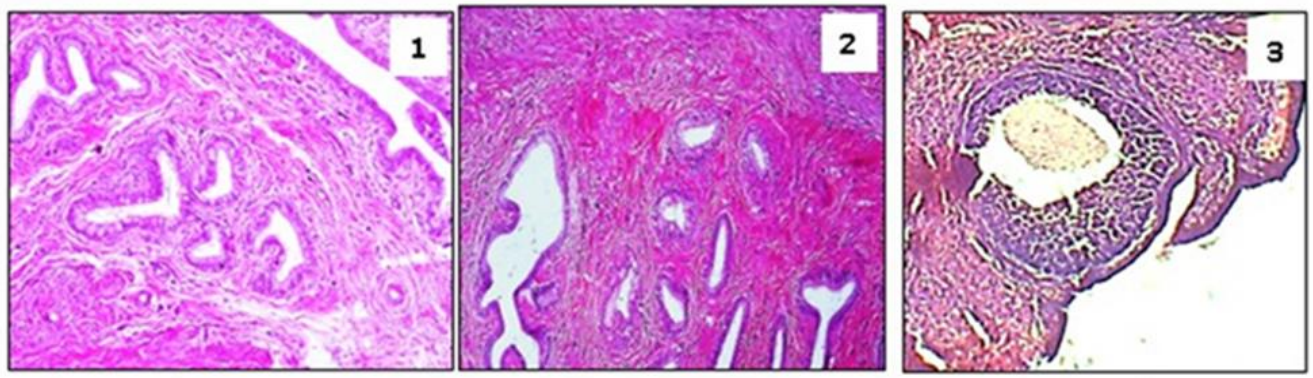


Fig.1: Uterus of doe showing chronic metritis with focal and diffuse subepithelial and periglandular mononuclear inflammatory cell infiltrations as well as fibrous connective proliferation (H&E, X100).

Fig. 2: Uterus of doe showing fibroleiomyoma with endometrial hyperplastic proliferation of smooth muscle and fibrous connective tissue as well as papillary hyperplastic proliferations of the glandular epithelium (H&E, X100).

Fig .3: Ovary of ewe showing follicular cyst that forming thick vascular connective tissue wall lined by many layers of atrophied, degenerated granulosa cells, contained homogenous eosinophilic secretion with interstitial congestion and leukocytes infiltrations (H&E, X40).

The uterus revealed chronic cystic metritis and myometrial hyperplasia with noticed congestion and edema as well as glandular cystic dilatation and peri glandular mononuclear cellular infiltrations were observed (Fig. 4).

Our findings were nearly similar with the previous studies of Sharma *et al.* (2007) and EL-Hallawany and El-Metwally (2011) in rabbits and Dhurvey et al. (2017) in rats. We suggesting that the observed smooth inactive ovaries in this work attributed to the folliculogenesis failure as consequence to suboptimal

release of gonadotrophins that resulted in reduced the production of ovarian steroids as well as lipid and cholesterol deficiencies which resulting in hypothyroidism. Also, it may be due to lack of available proteins necessary for division and differentiation of germ cells during oogenesis. This suggestion comes parallel with the explanation of Tanaka *et al.* (2007). Whereas, the cystic ovarian disease noticed in this study is explained by Bartolome et al. (2005) and Tanaka *et al*. (2007) as consequence to insufficient release of the luteinizing hormone. The later induced hypothyroidism and hormonal imbalance that alter the normal sex hormones metabolism and leading to no ovulation. Those ovarian alterations were leading to the endocrine imbalance and the reduction in circulating estrogen and progesterone which supported by histopathological results under the effect of fluorosis. These findings were in accordance with the results of Zhou *et al.* (2013) and Dhurvey *et al*. (2017) in rats. Moreover, the reduction of estrogen and progesterone is related to a decreased number of healthy follicles (Madan, *et al* 2009). In addition, fluorosis induced ovarian toxicity through increasing the ROS production which could affect multiple physiological processes from oocyte maturation to fertilization, embryo development and pregnancy (Vashishth *et al.*, 1998). Uterine fibroleiomyomas noticed in this work are similar to those observed previously by Jhala et al. (2004) who recorded that there was a significant positive correlation between fluoride concentration in drinking water and uterine cancer. Also, EL-Hallawany and El-Metwally (2011) were recorded endometrial clear cell carcinoma in fluorinated rabbits. The same uterine histopathological changes described by Guney et al. (2007) and Kamble and Velhal (2010). In addition, Zeiger et al. (1993) reported that fluoride ions is genotoxic and capable of inducing neoplastic transformation. Whereas chronic cystic endometritis and impaired reproduction that observed in this work, were reported previously among hypothyroidism in goats by Choubisa (2015). Our mentioned ovarian and uterine fluoride induced histopathological alterations were parallel the significant decrease in T3, progesterone and estrogen hormone.

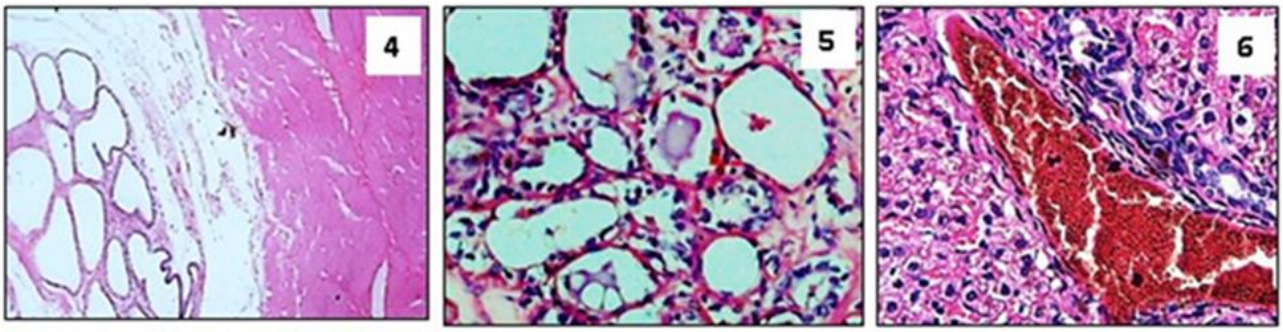


Fig. 4: Uterus of ewe showing chronic cystic metritis with congestion, edema as well as peri glandular mononuclear inflammatory cell infiltrations and glandular cystic dilatation (H&E, X100).

Fig .5: Thyroid gland of ewe showing vacuolated or exfoliated follicular epithelial cell lining cells as well as reduction or vacuolation of follicular colloid (H&E, X400).

Fig. 6: Liver of ewe showing distorted hepatic cords, with dilated congested central vein and hepatic sinusoids as well as vacuolar denegation of hepatocytes and hyperplastic proliferative changes in biliary epithelium as well as periportal fibrosis with moderate leukocytes (H&E, X100).

3.5. 2. Effect of fluoride on thyroid glands:

In the present work, variable thyroid gland histopathological alterations were noticed in 5 ewes and 2 does. Grossly, they appeared hypertrophied and congested of variable degrees. Microscopically, hypertrophied thyroid follicles appeared with various degenerative and necrotic changes of follicular epithelial cells as well as reduction or vacuolation of follicular colloid with congestion of the interseptal blood vessels (Fig. 5). Our noticed histopathological changes in the thyroid glands came parallel with a significant decrease in T3 hormones concentrations

which was in agreement with findings those described previously by Wang et al. (2009) and Dhurvey et al. (2017). This result explained as, the gland has a strong capacity for absorbing and accumulating fluoride instead of iodine which damage the gland and disturb its function. Moreover, enlargement of the thyroid gland mostly due to increase the level of TSH, which is responsible for the proliferative activity of follicular cells that alters thyroid hormones levels in the serum (Barry , 2005).

3.5. 3. Effect of fluoride on liver tissues:

In this investigation, the collected liver tissue samples of fluoride group as showed in table (8) revealed 100% histopathological alterations. Grossly, enlargement, congestion in 4 cases where as 3 cases showed paleness with necrotic foci and fibrosis. Microscopically, 4 cases showed distorted normal lobular pattern of hepatic cords with dilated congested central vein and hepatic sinusoids as well as vacuolar denegation of hepatocytes and hyperplastic proliferative changes in biliary epithelium as well as periportal fibrosis with moderate leukocytes (Fig. 6). Also, degenerative, necrotic and hyperplastic proliferative changes which appeared as binucleated hepatocytes associated with mononuclear cells (Fig. 7). Those findings supported with the biochemical results and came in accordance of Ersan et al. (2010) in mice and EL-Hallawany and El-Metwally (2011) in rabbits, as well as Ulemale *et al*. (2010) in cow. Sahu *et al.* (2015) attributed those histopathological changes to the oxidaive stress induced by chronicfluorosis and accumulation of ROS.

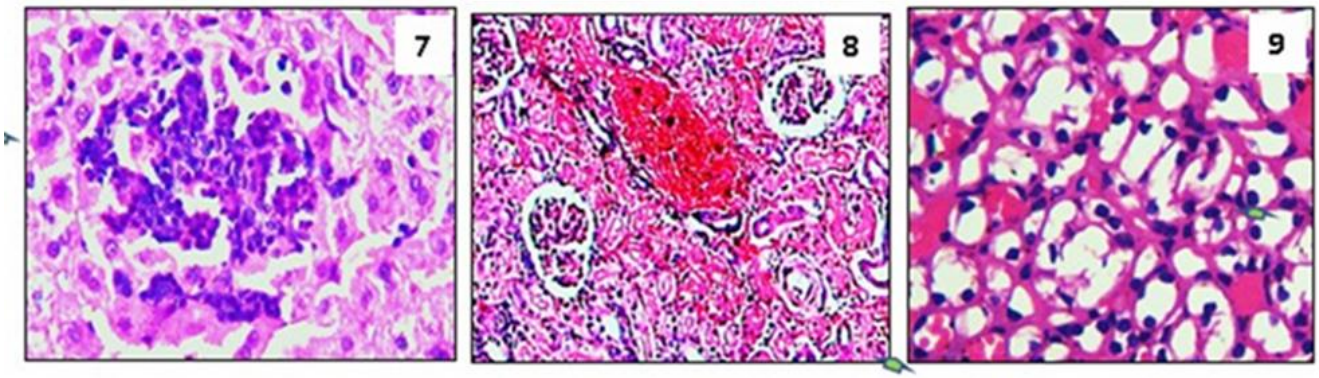


Fig .7: Liver of ewe showing degenerative, necrotic and hyperplastic proliferative changes in hepatocytes as binucleated hepatocytes and mononuclear cellular infiltrations (H&E, X400).

Fig .8: Kidney of doe showing peritubular vascular congestion and hemorrhages with as well as glomerular atrophy and interstitial nephritis (H&E, X100).

Fig .9: Kidney of doe showing sever peritubular vascular congestion and tubular epithelium vacuolar degeneration with lymphocytic infiltrations (H&E, X400).

3.5.4. Effect of fluoride on kidney tissues:

In this study, In this investigation, collected kidney tissue samples of the 3rd group as showed in table (8&9) revealed 100% histopathological alterations.

Grossly, they appeared enlarged with congestion and necrotic foci on one or the two kidneys. Microscopically, they revealed sever peritubular vascular congestion and hemorrhages with lymphocytic infiltrations as well as glomerular atrophy and interstitial nephritis (Fig. 8). Most renal tubular epithelial cell lining revealed degenerative and necrotic changes with variable degrees (Fig. 9). Our histopathological and histochemical findings are nearly similar to those observed by Ulemale *et al*. (2010) in cattle which referred to the oxidative stress and accumulation of ROS induced by chronic fluorosis that significally increased serum levels of urea and creatinine.

3.5.5. Immunohistochemical evaluation:

a- Caspase-3 immunoreactivity: Immunoreactivity for Caspase-3 for determining the apoptotic cells in the uterus and thyroid gland which revealed that, marked increase of caspase 3 reaction was detected as brown colorations in the uterine epithelial lining, peri glandular and perivascular (Fig. 10 &11) comparing to

normal (Fig. 12). Also, marked increase of caspase 3 reaction in thyroid gland at the follicular epithelial lining was noticed (Fig. 13).

Caspases are a family of cysteine proteases as inactive proenzymes and become activated when apoptosis (programmed cell death) is initiated (Llopis *et al.*, 2003). This Oxidative stress resulting from the production of ROS more than the body's natural antioxidant defense mechanisms capacity, so leading to DNA, proteins and lipids damage. Disruption of the outer mitochondrial membrane by apoptotic stimuli results in the release of cytochrome c into the cytoplasm where it initiates a cascade of caspase activation and results in apoptosis (Zhang, 1992 and Casellato *et al.*, 2014). In our study marked positive immunoreactivity to caspase 3 as apoptosis markers in the uterine cells and thyroid gland follicles. This results confirmed our histopathological and biochemical results which agreed with that of Elgawish and Abdelrazekb (2014) who explore caspase-3 in mice testes to lead acetate.

b- Desmin and Actin immunohistochemistry: Fibroleiomyomas cases showed positive immunoreactivity for desmin (Fig. 15) and SMA (Fig. 16).

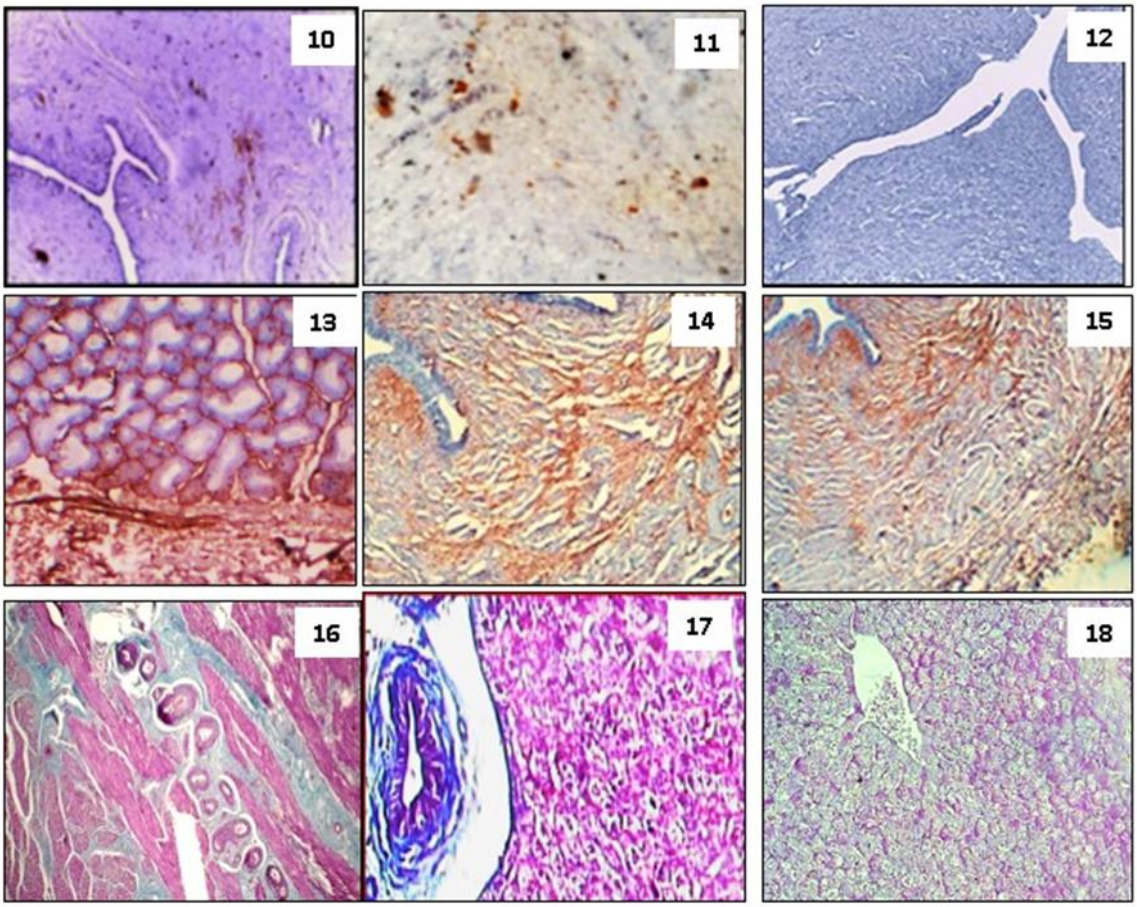


Fig .10: uterus of ewe showing marked positive caspase 3 immunoreactivity as golden-brown colorations as of epithelial, and perimuscular(Caspase-3 immunostain, X 40).

Fig .11: uterus of ewe showing marked positive caspase 3 immunoreactivity as golden-brown colorations of epithelial, peri glandular, perivascular and perimuscular (Caspase-3 immunostain, X 400).

Fig .12: uterus of ewe showing negative expression of caspase 3 (Caspase-3 immunostain, X 100).

Fig .13: thyroid gland of doe showing marked increase of caspase 3 reaction as brown colorations in the follicular epithelial lining (Caspase-3 immunostain, X400).

Fig.14: uterus of doe showing fibroleiomyomas with positive immunoreactivity to desmin (Desmin immunostain, X 100).

Fig .15: uterus of ewe showing fibroleiomyoma with positive immunoreactivity to SMA (SMA immunostain, X 40).
Fig .16: uterus of ewe showing marked fibroblastic proliferation as greenish blue colorations peri muscular and peri glandular (Masson-trichrome stain, x100).
Fig .17: liver of ewe showing portal fibroblastic proliferation as greenish blue colorations (Masson-trichrome stain, x100).
Fig .18: liver of ewe showing decrease in the polysaccharides content of the hepatocytes (PAS stain, x100).

The present study revealed diffuse and uniform immunoreactivity to Desmin and SMA uterine leiomyomas and similar observation recorded by Cooper and Valentine (2002). Desmin is a class-III intermediate filament protein expressed on cells and is useful in diagnosis of tumor with myoid origin. Also, the antibody recognizes actin isotypes alpha of smooth muscle and those cells with myofibroblast differentiation. It labels smooth muscular cells, myofibroblasts, and myoepithelial cells that is a useful marker for the identification (D'angeloe and Prat, 2009).

3.5.6. Histochemical Evaluation:

In the present work, the endometrial peri muscular, peri-glandular, perivascular fibrosis in as well as periportal as well as portal fibrosis which demonstrated with Masson-trichrome stain in the uterine (Fig.17) and hepatic (Fig. 18) tissues. Also, hepatocytes appeared markedly with less PAS-positive matter of glycogen particles (Fig. 19).

Hepatic histochemical and histopathological alterations noticed in this work mimic to that observed by El-Khayat et al. (2010) and came parallel with the significant decrease ($p<0.05$) of calcium, protein, albumin, globulin and significant increase ($p<0.05$) of AST, ALT, which referred to liver dysfunction and disturbance in the biosynthesis of those enzymes and change in the permeability of hepatocytes (Khalil and El-Sheikh, 2010). Moreover, chronic fluorosis was leading to glycogen depletion in the hepatic tissues and the reduction in carbohydrate components which could be due to the release of hydrolytic enzymes from the ruptured lysosomes (Rae et al., 2004).

4. CONCLUSION:

1-This study suggested that the excessive fluoride ingestion had a significant adverse effect on the growth performances and serum indexes of ovaries. In the same time, high fluorine intake can markedly altered MDA and T-AOC levels and some enzymes activities associated with free radical metabolism in ovine.

2-The consumption of excess fluoride in adult ovine leads to adverse degenerative effects on the ovary, uterus, thyroid, liver and kidneys. Hypothyroidism might lead to certain ovarian malfunctions due to pathological lesion accompanied with increasing in the rate of apoptosis and turn in the ovine infertility because of thebthyroid hormones play an important role in the regulation of folliculogenesis.

3-The carcinogenic effect of chronic fluorosis was observed in uterus as endometrial hyperplasia and hyperplastic hepatic changes.

4- Our data might provide some evidence for further studying the mechanism of excess fluorine accumulation on the impairment of soft tissues.

5. RECOMMENDATIONS

Protection of well from pollution should be considered and there is a need for more stringent standardization & supervision. Groundwater in this area cannot be considered safe for drinking unless properly treated. To achieve this safety, we recommend the following:

• Recognition of the existence of the problem and don't blackout either by the decision makers or the multimedia.

• A monitoring program should be initiated for the St. Katherine area, aiming to study the effect of over fluorosis on the human health.

• Defluoridation using the Nalgonda Technique by establishment of modified purification units by the government on all water wells.

• Activation of environment law 4/1994 against hotels, tourist camps which watering plants by black water.

• Apply a suitable technique for wastewater recycling through construction of wetland system of the Gravel Bed Hydroponic (GBH) type is highly recommended.

• Enhancement of natural aquifer recharge through establishing simple traditional retardation dams for floodwater in Wadi Sedod, El-Arbaein and/or Wadi ElTalaa (upstream parts away from the contaminated areas).

• Further study is recommended to identify risk factors in order to reduce the prevalence of enamel fluorosis and employ methods to manage such risk factors.

• An extensive survey by a team from Authority of Nuclear Materials is highly recommended to measure the level of radiation in the ground water wells in Saint Katherine

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