Original Article

An Evaluation of the Plasma Levels of Frequently Used Pesticides in Dairy Cattle and Its Possible Correlation with the Occurrence of Follicular Cystic Ovarian Disease: A Case-Control Study

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Abstract

Background: Cystic ovarian disease (COD) is one of the common reproductive disorders which affecting the fertility of dairy cattle induces heavy financial burdens on herds owners. Various insecticides, fungicides and herbicides, collectively known as pesticides are frequently used in the agricultural systems of different countries. Given the fact that pesticides are known to have endocrine disrupting properties, exposure to these compounds may play a role in the development of COD.

Materials and Methods: The plasma concentrations of a complete profile of common pesticides including organophosphorus, organochlorine, and carbamate and pyrethroid compounds in the plasma of cattle with COD compared to healthy controls was examined. Moreover, plasma concentrations of inflammatory cytokines as well as oxidative stress parameters were investigated.

Results: No significant amounts of any of the pesticides investigated were detectable in the plasma of neither the healthy nor cystic cows. The plasma indices of total antioxidant capacity (TAC), thiol, lipid peroxidation (LPO), and reactive oxygen species (ROS) did not show any significant differences between the affected and the control groups. Tumor necrosis factors alpha (TNF-α), progesterone, lymphocyte, neutrophil, fibrinogen and MCHC had significantly higher amounts in the plasma of COD cows.

Conclusion: Findings of the present study do not support the notion that exposure to the studied pesticides is a contributing factor in the development of follicular cysts in dairy cattle. In addition, TNF-α might be affected as a factor in the pathogenesis of COD by an independent pathway of pesticides effect.

Keywords: Pesticide, Oxidative stress, TNF-α, Bovine, Cystic ovarian disease, Reproductive toxicity, Endocrine disruption

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Introduction

Cystic ovarian disease (COD) is one of the common causes of reduced reproductive performance in dairy cattle[1]. Although its pathogenesis is not completely understood, it has been shown to involve in the formation of cysts from follicles which fail to successfully ovulate. The persistent presence of such follicles may disrupt the normal ovarian function and give rise to several clinical outcomes[2]. The major clinical consequences include increased post-partum intervals, reduced calving rate, and decreased conception[3] among many others which ultimately end in a large benefit loss; this, in turn, may impose a heavy financial burden on the owners of industrial and non-industrial farms[4].

It is believed that the onset of the pathogenesis of COD is the result of any interruption of the hypothalamic–pituitary–gonadal axis, which finally disturbs the endocrine balance contributing to the growth of follicles lacking the required changes necessary for the rupture of the follicle and the consequent ovulation scenario[5]. Such an insult might take place at any level in the axis such as the follicular estradiol-activated release of gonadotropin releasinh hormone (GnRH) by the hypothalamus[6], the release of luteinizing hormone (LH) by the pituitary gland[7], dysfunction and irresponsiveness of cellular components of the follicle, mainly granulosa cells[8] and the inflammatory process involved in ovulation[9]. The unresponsiveness of hypothalamus to estradiol might be due to the higher concentration of progesterone, which is typically found in the cow with COD[10]. Another hypothesis is that the cystic follicles, in comparison to normally ovulating follicles, lack the adequate amounts of reactive oxygen species (ROS) required for the inflammatory reactions involved in ovulation[11]. Ruling out other potential factors that might interfere with the proper functioning of the hypothalamic–pituitary–gonadal axis can not only give us a better understanding of the pathogenesis of the disease but also it can aid in designing strategies to reduce the incidence of the disease among dairy cattle herds.

Pesticides are among the most widely used chemicals in the agriculture for which strong endocrine disrupting properties have been reported[12]. These agents have been shown to induce their toxic effects in body systems via interfering with hormone regulation[13]. The hypothalamic–pituitary–gonadal axis along with the immune-neuroendocrine axis serves as the target for the pesticides to induce cellular oxidative stress and the following organ injury[14]. This has been proven to affect the reproductive system as well as many other organ systems in the body[15-17]. Organophosphorus, organochlorine, and carbamate compounds such as Malathion, Dicofol, and Carbaryl are the major groups of pesticides used in the agricultural systems[18]. On such a basis, the authors of the present study hypothesized that exposure to pesticides might be a contributing factor in the development of cystic follicles in dairy cows. Thus, in the present study, researchers examined the plasma levels of 40 different pesticides of different classes in dairy cattle with or without COD to find out whether or not any relationship exists between the plasma levels of pesticides as an indicator of exposure to these poisons and the incidence of COD. Besides, the researchers studied the oxidative stress biomarkers as well as inflammatory cytokines and hematological parameters to point out the possible involved mechanisms.

Methods

Animals grouping and allocation: This case-control study was carried out on 30 cows in one of the major dairy cattle farms in Tehran, Iran. A number of 15 cows were diagnosed as cases of follicular COD by means of touché rectal and ultrasonography. This was performed twice with 10-day intervals by a veterinary obstetrician. Fifteen healthy cows of the same breed which had been previously synchronized with the animals in the COD group in terms of age, weight, milk yield, number of parity and lactating days, were considered as the control group (see Figure 1 for demographic information).

Sampling and blood analysis: Blood samples were obtained from the tail vein and were then transferred into two different tubes; one was a heparinized tube and one contained EDTA for completed blood count (CBC) analysis and fibrinogen measurement. The plasma was separated from the blood within the

heparinized tubes after 20 min of centrifugation at 3000 g and was then stored at -80°C for GC-Mass, a hormone, and cytokine analyses. CBC analysis was done to check any existing comorbidities. A number of 8 cows were found to have high lymphocyte and neutrophil counts which might be an indicator of infectious contamination. Those 8 animals were excluded from the study and the investigation was followed with a final number of 22 cows with normal CBC (11 with COD and 11 without COD-control) (Figure 2). To further confirm the existence of follicular cysts, the researchers made use of Accubind Kit, Monobind Company, USA, to measure the plasma levels of progesterone. Enzyme-Linked Immunosorbent Assay (ELISA) kits were used to measure the levels of pro-inflammatory cytokines in the plasma (TNF-Alpha Cow, Antibody Company, USA).

Assays of oxidative stress indices:

ROS measurement: To measure the ROS of plasma, DCFH-DA was used as an indicator. 50 µl of the sample was added to the mixture of 10 µl DCFH and 162 µl assay buffer. These solutions were incubated at 37°C for 15 min and subjected to a spectrophotometer[19].

LPO measurement: In lipid peroxidation (LPO) assay, TBA-reactive substances (TBARSs) were measured. The reaction between TBA and lipid peroxides in the samples leads to a measurable pink color. Briefly, the samples were diluted by buffered saline (1:5), and 800 µl of trichloroacetic acid (TCA, 28 % w/v) was added to 400 µl of this mixture and centrifuged at 3000×g for 30 min. Then, 600 µl of the supernatant was added to 150 µl of TBA (1% w/v). The mixture was incubated for 15 min in a boiling water bath, and then 4 ml n-butanol was added. Next, the solution was centrifuged and cooled, and absorption of the supernatant was read by the ELISA reader at 532 nm as was set up in our lab previously[20]. The activity was shown as micromolars.

Total Antioxidant Capacity (TAC) measurement: Anti-oxidant capacity of blood was measured based on the ability of plasma to reduce Fe3+ to Fe2+. The blue color which appears after the Fe2+ and TPTZ complex is formed, can be measured at 593 nm[21].

Total thiol measurement: To measure the thiol molecules of the plasma, the method of[22] was used. Briefly, the DNTB reagent measured total thiol molecules of plasma measured at 412 nm using a spectrophotometer.

Gas Chromatography Mass (GC-Mass) analysis: To perform the analysis, 5 ml of each sample was initially transferred to a centrifuge tube and two steps were followed. Fifteen milliliters of 0.1% acetonitrile and acetic acid solution was first added to the tube and agitated for 2 min. Next, the extraction powder containing 1.5 g sodium acetate and 4 g magnesium sulfate was added. The solution was agitated once more for 2 min. The sample was then centrifuged at 4000 g for 5 min and 8 ml of the supernatant was taken and added to the cleanup kit to remove the debris. After vortex, the sample was centrifuged at 5000 g for 5 minutes and 5 ml of the supernatant was added to a vial, then it was concentrated using nitrogen gas and finally 2 µl injected to the mass spectrometer to perform the analysis. A Thermoquest trace Mass Spectrometer with a 30 m long DB-5 column and an internal diameter of 250 µm was used to perform the GC-Mass analysis of 40 pesticides including Carbaryl, Quintozene, Diazinon, Pirimicarb, Chlorpyrifos-methyl, Pirimiphos-methyl, Metalaxyl, Chlorpyrifos, Dicofol, Fenthion, Malathion, Fenithrothion, Propanil, Endosulfan I, Penconazol, Methidathion, Profenofos, Pretilachlor, Oxadiazon, Endosulfan II, Hexythiazox, Bioresmethrin, Fipronil, Edifenphos, Propiconazol I, Propiconazol II, Propargite, Biphenthrin, Bromopropylate, Iprodion, Phosalone, Permethrin I, Permethrin II, Cypermethrin I, Cypermethrin II, Cypermethrin III, Cypermethrin IV, Fenvalerate I, Fenvalerate II, Indoxacarb, Deltamethrin I, and Deltamethrin II. Helium gas was used as the career at a speed of 1 ml/min. The temperatures of injection port and interface were 270 and 280°C, respectively. Initial oven temperature of 60°C was held for 1 min, followed by an increase to 130°C (hold for 0 min) at a rate of 6°C/ min, 230°C (0 min hold) at a rate of 5°C/ min, and 280°C (hold for 20 min) at a rate of 10°C/ min. Three major ions of each pesticide were selected to be used which are shown in Table 1. Dichlorobenzene (an internal standard) was used as a scale to measure the levels of other pesticides. The detection limit of the spectrometer was 30 ppb and the detection was done in the plasma[23].

Statistical analyses: All data were expressed as

Mean±SEM. The One-Sample Kolmogorov-Smirnov test was used to determine the normality of the data and Independent t-tests were used to determine the differences between the levels of plasma parameters in the control and the COD groups. The Mann-Whitney test was used as the alternative in case the data did not follow a normal pattern of distribution.

Results

The plasma levels of all the pesticides investigated were below the detectable limit (30 bbp) in both the control group and the group with COD, which was shown in the GC-Mass analysis. Results of plasma cytokine assay revealed a dramatic difference in the plasma levels of TNF-α between the control and COD groups ($p<0.001$). The levels of progesterone in the plasma were also significantly different between the two groups ($p<0.001$). However, the TAC, Thiol, LPO, and ROS indices did not show any significant difference between the healthy cattle and those with COD ($p>0.05$).

Peripheral blood CBC analysis showed that the cattle

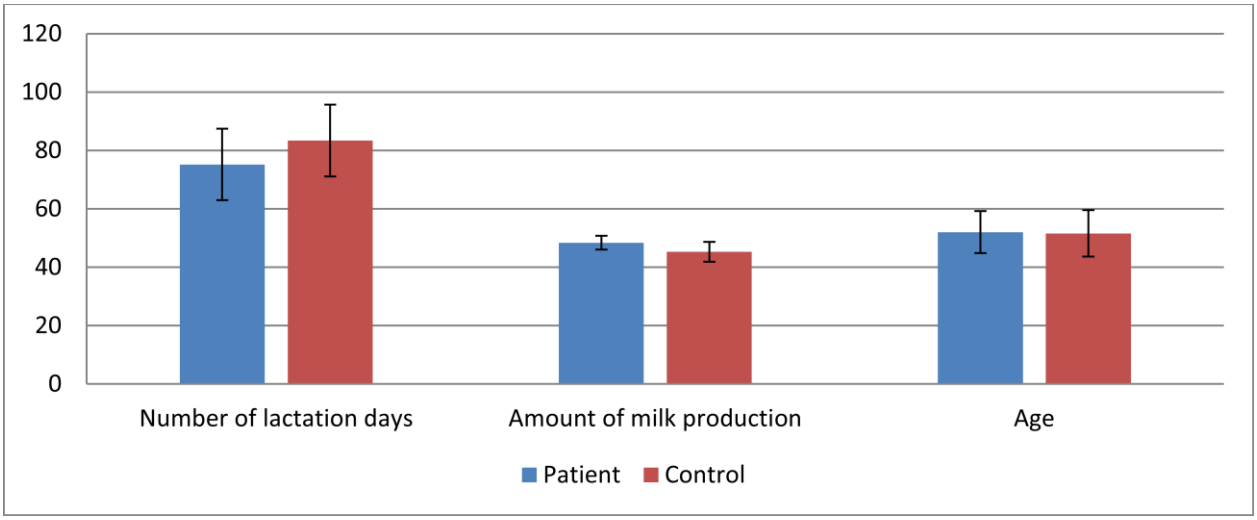

Figure 1. Demographic information of the studied cattle. The cows in the control and COD groups were the same regarding the number of lactating days, milk yield, and age. The measurement scale for number of lactation days, amount of milk production and age are days, litters and months, respectively.

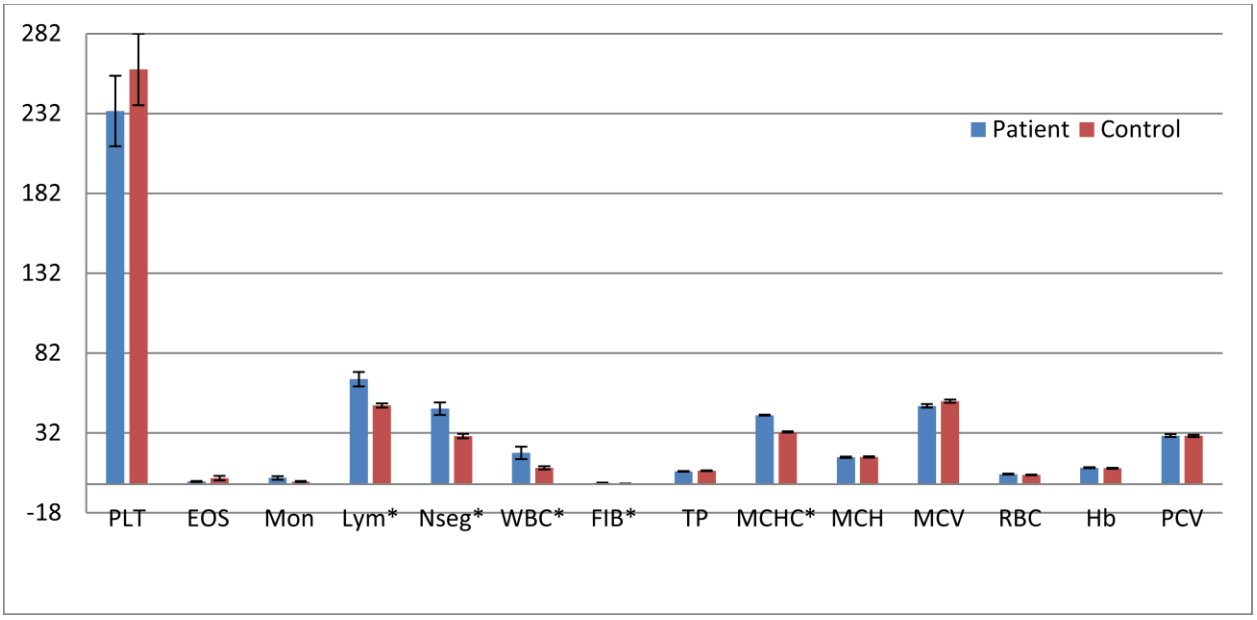

Figure 2. Complete blood count and fibrinogen analyses for the animals in the control and COD groups. The neutrophil and lymphocyte counts were higher in the COD group compared to the control group. * Marker indicates statistically significant.

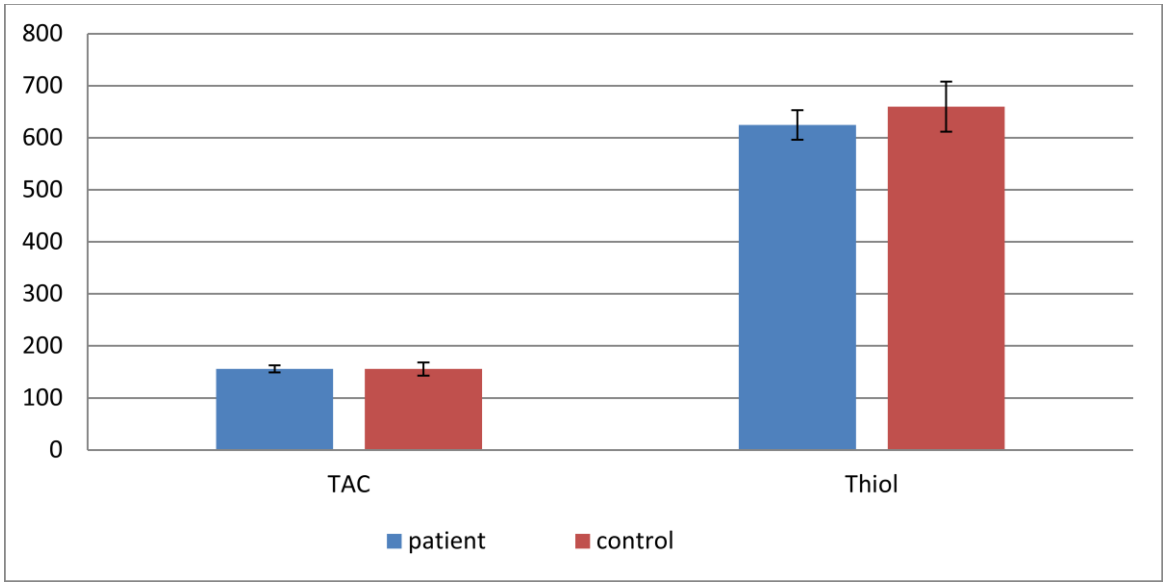

Figure 3. The plasma concentrations of TAC and Thiol indices in the control and COD cows. There was no significant difference between the two groups (p>0.05). The measurement scale for TAC and Thiol are µM and mM/L, respectively.

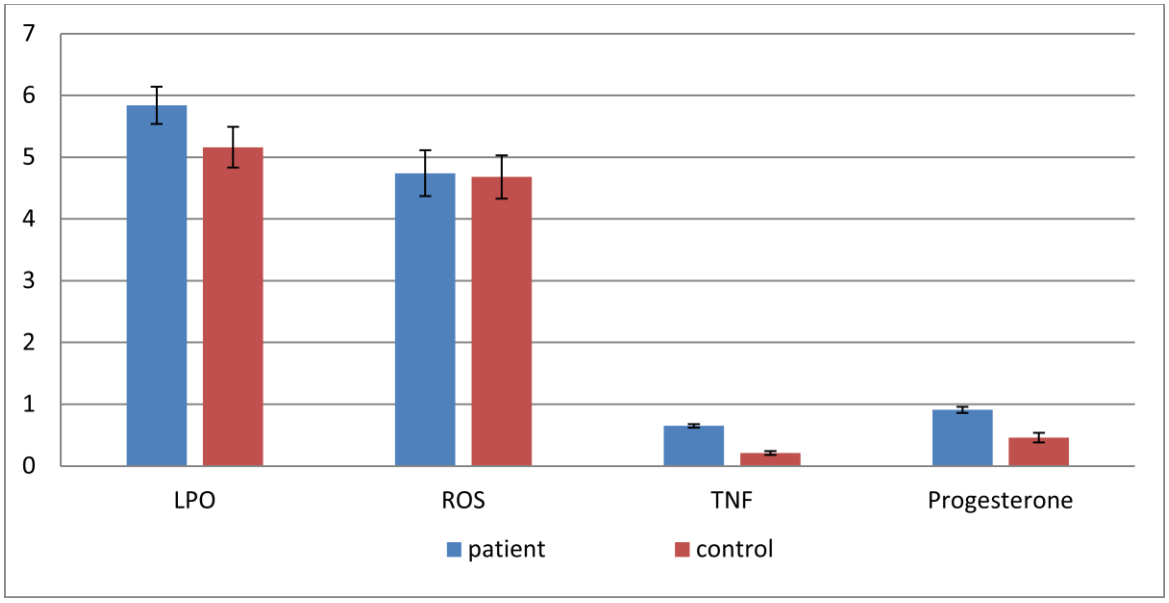

Figure 4. The plasma concentrations of LPO, ROS, TNF, and progestrone in the control and COD groups. There was no significant difference between the two groups regarding LPO and ROS (p>0.05). Progesterone and TNF-α had higher levels in the COD group compared to the control group (p≤0.05). Measurement scale for LPO, ROS, TNF and Progesterone are µM/ml, unit/mg, pg/ml and ng/ml, respectively.

with COD have significantly higher WBC counts in comparison to the animals in the control group ($p<0.05$) which might be due to the lymphocyte fraction, since the animals with COD had higher lymphocyte counts ($p<0.05$). The segmented neutrophil counts (N-seg) were also higher in the COD group ($p<0.05$). Moreover, red blood cell analysis showed lower MCHC levels in the COD cattle when compared to the animals without COD ($p<0.05$). Neither of the remaining WBC nor RBC parameters were significantly different between the COD and the control groups ($p>0.05$). Plasma concentration of fibrinogen protein was more in COD group than controls significantly ($p<0.05$) (see Tables 3 and 4).

Table 1: Three selected ions of each pesticide for GC-MS analysis.

Name	Application	M/Z ion ratio		
Carbaryl	insecticide	144	116	115
Quintozene	Fungicide	237	214	295
Pirimicarb	insecticide	166	238	72
Chlorpyrifos-methyl	insecticide	286	125	288
Pirimiphos-methyl	insecticide	290	276	305
Metalaxyl	Fungicide	206	279	249
Chlorpyrifos	insecticide	197	258	314
Dicofol	acaricide	139	111	75
Fenthion	acaricide/insecticide	278	109	125
Fenithrothion	insecticide	277	125	109
Propanil	herbicide	161	163	217
Endosulfan I	insecticide	195	170	241
Penconazol	Fungicide	159	161	248
Methidathion	insecticide	145	125	85
Profenofos	insecticide	208	206	139
Pretilachlor	herbicide	238	162	311
Oxadiazon	insecticide	175	177	258
Endosulfan II	insecticide	195	170	241
Hexythiazox	acaricide	184	158	156
Bioresmethrin	Herbicide	123	128	171
Fipronil	insecticide	367	213	369
Edifenphos	Fungicide	310	173	109
Propiconazol I	fungicide	259	191	175
Propiconazol II		259	191	175
Propargite	Acricide	135	173	
Biphenthrin	insecticide	181	166	165
Bromopropylate	Acricide	183	185	341
Iprodion	Fungicide	187	189	244
Phosalone	acricide/insecticide	182	121	184
Permethrin I	insecticide	183	165	163
Permethrin II		183	165	163
Cypermethrin I	insecticide	181	163	91
Cypermethrin II		181	163	91
Cypermethrin III		181	163	91
Cypermethrin IV		181	163	91
Fenvalerate I	insecticide	125	167	181
Fenvalerate II		125	167	181
Indoxacarb	insecticide	59	150	203
Deltamethrin I	insecticide	181	208	253
Deltamethrin II		181	208	253

Discussion

Organophosphoruses, organochlorines and carbamates are among the most widely used pesticides in all indoor and agricultural systems[18]. The fact that these agents as well as other pesticides possess endocrine-disrupting properties made the researchers of the present study hypothesize that they might contribute to the development of cystic follicles and may thus have a relationship with the incidence of COD in dairy cattle. In this sense, the researchers of the present study attempted to measure the plasma levels of a complete profile of pesticides in dairy cattle diagnosed with COD compared to healthy cattle.

Pathologic ovarian cysts are classified into follicular and luteal cysts. The luteal cysts possess thicker walls and consequently contain more luteal tissue giving rise to plasma progesterone levels of more than 1 ng/ml. They occur sporadically and are mostly seen in the left ovary, whereas follicular cysts are more prevalent and are mostly seen in the right ovary[24,25]. In the present study, the measured progesterone levels of less than 1 ng/ml in the COD group were supported by the results

of clinical examinations which had shown the cyst types to be luteal.

It has been shown that the inhibition of the activity of acetylcholinesterase at the neuromuscular junctions and the consequent nervous system toxicity are not the sole mechanisms by which organophosphorus compounds exert their toxic effects. Induction of oxidative stress is another mechanism of the toxic effects of these pesticides, which interferes with the neuro-hormonal system, especially hormones of the reproductive system[26]. A steady balance between the ROS and antioxidants is required for normal ovulation process and any factor that increases ROS production may cause COD[27].

In a study, pesticides including phthalates, tobacco, and bisphenol A were shown to be the most common environmental pollutants, which have negative impacts on ovarian function[28]. Pesticides have also been shown to be the cause of polycystic ovarian syndrome (PCOS) via dysregulating the activity of aromatase, a cytochrome P450 enzyme critical for many endocrine pathways[29].

In the present study, the plasma concentration of oxidative stress indices including TAC, Thiol, LPO, and ROS in the plasma did not show any significant differences between the healthy and cystic cows, indicating that the disbalance of oxidative metabolites is not a contributing factor for COD, at least at this stage of the disease. Although many of the pesticides investigated in the current study are well known to induce oxidative stress, the data collected in this investigation showed that the plasma concentrations of these pesticides were extremely low and that there is no difference between their concentration in the animals with and without COD. The obtained results do not support the notion that exposure to pesticides is a contributing factor in the development of follicular cysts in dairy cattle at least in the studied population. However, previous studies have shown various pesticides to be responsible for some ovarian pathologies. Among them is a study on a Chinese population, which linked the exposure to dichlorodiphenyltrichloroethane (DDT) with the pathogenesis of PCOS because of affecting the hormone levels[30]. In line with the mentioned study, another study showed that the retention of some organic pollutants and pesticides such as p, p'-dichlorodiphenyldichloroethylene (p,p'-DDE) is associated with the occurrence of PCOS in humans[31]. Despite the results of the mentioned studies and many others which have introduced the exposure to pesticides as a risk factor for PCOS, as far as the authors of the present study are concerned, studies on the possible association of these environmental pollutants with COD in cattle are rare.

It has been agreed that ovulation resembles the process of an acute inflammation, which involves a variety of pro-inflammatory cytokine[9]. The results obtained from this study showed that the concentration of TNF-α as a major inflammatory cytokine was significantly higher in the healthy group when compared with the COD group, as demonstrated in some other previous studies[32-35]. This seems controversial with the basic notion that a decrease in the secretion of inflammatory cytokines may be due to an impaired inflammatory process, which is necessary for ovulation that play a role in the pathogenesis of COD.

Conclusion

Taken together, the findings do not show any relationship between the plasma concentrations of the studied pesticides with the presence of COD in the dairy cattle included in the present study. Neither the oxidative stress parameters nor the antioxidant factors, with the exception of TNF-α, indicated different plasma concentrations between the healthy cows and those with COD. Although this does not completely undermine the possibility of any link between pesticide exposure and the development of COD, it rejects the association between certain blood concentrations of the studied pesticides and the incidence of COD. Indeed, conducting further studies with a greater sample size is recommended to remove ambiguities in this regard.

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