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Evaluation of Level of Pesticide Residue in Blood and its Effects on Hormonal Levels of Crossbred Bulls

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ABSTRACT

Background: The pesticides are potentially toxic compounds that have become omnipresent in the environment. These toxicants modulate and/or disrupt the reproductive and hormonal environment by acting on hypothalamus, pituitary and reproductive organs. Fewer studies have been carried out on pesticide residues in body fluids especially blood and semen in breeding bulls.

Methods: Study was done to assess the pesticide residues in blood in relation to the endocrine profile in crossbred breeding bulls. Nineteen blood samples were collected and were allowed to clot overnight; serum was separated and stored at -20°C until analysis. The samples were analyzed for the pesticide residues using gas chromatography (GC) for seven organochlorine pesticides (OCPs) namely Heptachlor epoxide, Chlordane, Fipronil, Lindane, Methoxychlor, op-DDT and Endrin and eleven organophosphorus pesticides (OPPs) namely Chorpyrifos, Dichlorovos, Ethion, Monocrotophos, Malathion, Parathion-methyl, Profenphos, Phorate, Triazophos, Quinalphos and Phosalone and four synthetic pyrethroids (SPs) namely Cypermethrin, Permethrin, Deltamethrin, Cyhalothrin.

Result: The blood samples of these bulls were found positive for organophosphate-phosalone and around 73.68% of the bulls were positive for phosalone at a retention time of 13.837 min. The concentration of phosalone in the blood of crossbred breeding bulls was found to be 1.89±0.98 ng/ml. In the present study, the blood hormonal profile of pesticide residues was analyzed and it showed a significant increase in the level of Estrogen there by decreasing the testosterone: estrogen ratio. The decreased testosterone: estrogen ratio may be attributed to the detection of phosalone in blood.

Key words: Blood, Crossbred bulls, Gas chromatography, Pesticide residue.

INTRODUCTION

In India, dairy farming is an effective tool for rural development, employment and sustained income and it acts as an assurance during tough times (Prasad 2011). Scientists have increasingly reported in recent years that certain pollutants are underlying causes of fertility problems in farm animals (Kamarianos *et al*., 2003b; Campagna *et al*., 2009). Over the past 60 years, a variety of pesticides have been used to increase India's agricultural productivity, the usage has increased from 2,353 MT in 1955 to 43,630 MT in 2007-2008. India ranks second in Asia and 12th largest in the world when it comes to pesticide use (Hundal *et al*., 2006).

Due to inadvertent use of potentially dangerous pesticides, dairy animals in Punjab state (especially animals in cotton belt area) have become highly vulnerable to their exposure. It is possible that the presence of endocrine disrupting compounds in the environment could be one of the reasons for the declining fertility of dairy animals (Petro *et al*., 2010).

Pesticides bind to endocrine receptors and act as hormonal ligands which affect fertility (Oliva *et al*., 2001; Figa-Talamanca *et al*., 2001). Parathion and methyl parathion are similar in structure to estrogens, hence, they may interact with hormone receptors and affect the gene transcription process (Perry *et al*., 2011). These toxicants modulate and/or disrupt the reproductive and hormonal environment by acting on hypothalamus, pituitary and reproductive organs (Zama and Uzumcu, 2010).

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Considering all the previous studies this study was designed to evaluate the pesticide level in blood of crossbred breeding bulls and its impact on hormonal level.

MATERIALS AND METHODS

Blood sampling

Blood samples were collected aseptically from the jugular vein and were allowed to clot overnight; serum was separated and stored at -20°C until analysis. Laboratory work was carried out in the Departments of Veterinary Gynaecology and Obstetrics and Pesticide Residue Testing Laboratory,

School of Veterinary Public Health, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India during the year 2020.

Pesticide residue analysis in blood serum samples

Multiple pesticide residue analysis from blood samples was carried out using gas chromatography. The steps in pesticide residue analysis were sample preparation, extraction, clean-up and analysis (identification, quantification) (Fig 1).

Pesticide standards used

Analytical standards of organochlorines (OCPs) and organophosphorus (OPPs) pesticides were purchased from Agilent and Rankem Ltd. Purities of pesticide standards were in between 90-99%. Seven OCP standards were used namely Heptachlor epoxide, Chlordane, Fipronil, Lindane, Methoxychlor, op-DDT, Endrin. Eleven OPP standards were used namely Chorpyrifos, Dichlorovos, Ethion, Monocrotophos, Malathion, Parathion-methyl, Profenphos, Phorate, Triazophos, Quinalphos and Phosalone. Four Synthetic pyrethroids standards were used namely Cypermethrin, Permethrin, Deltamethrin, Cyalothrin.

Sample analysis

Sample analysis: Quantification

The residues in cleaned-up extracts were quantified using GC and the ECD and FTD were used for OCPs, SP's and OPPs, respectively. The cleaned-up extract measuring 2 µl was injected in GC. GC solution software on pc was used for integration and computation of signals. The compounds were identified and quantified by comparison of the retention time and peak heights/area of the sample chromatograph with those of standards run under the same operating conditions. In general, the volumes of the sample extract for injection was so chosen that it gave approximately the same area or that of the same peak height obtained with the standards.

The formula used for the quantification of residues was:

$$\text{Residues (ng/ml)} = \frac{\text{(Peak area of the sample)} \times \text{(ng of pesticide standard injected)} \times \text{(final volume of the sample extract, ml)}}{\text{(Peak area of the standard)} \times \text{(Volume of the sample (µl) injected)} \times \text{(Weight of the sample, g)}}$$

Endocrine profile in relation to pesticide residues of crossbred breeding bulls

Methodology

14 blood samples from PLDB Nabha (5 ml each) and 5 blood samples from Milkfed Khanna (5 ml each) were collected for Testosterone, Estrogen, Triiodothyronine (T3), Thyroxine (T4), Dehydro-epiandrosterone (DHEA) and Prolactin hormones analysis in blood serum using ELISA kits. The hormonal analysis was done using Biocodon ELISA kits (Biocodon technologies, Biocodon LLC 6029 Broadmoore #1006 Mission, KS 66201, USA) specific for bovines.

RESULTS AND DISCUSSION

Pesticide residue in blood of crossbred breeding bulls

The organophosphate- phosalone residue in blood sample was detected in 73.68 % bulls (14/19) (Table 2), at retention time of 13.837 min (Table 1). In this study, 80.00% bulls at the semen station Milkfed, Khanna were found positive for phosalone whereas at PLDB, Nabha semen station, around 71.42% bulls were positive. The concentration of phosalone in blood of crossbred breeding bulls was found to be 1.89±0.98 ng/ml which was significantly higher ($p<0.05$) than the previous study on monitoring of pesticide residues in human blood from Punjab, which stated the concentration of phosalone in blood of humans of age group 41-50 years and age group >51-60 years was found

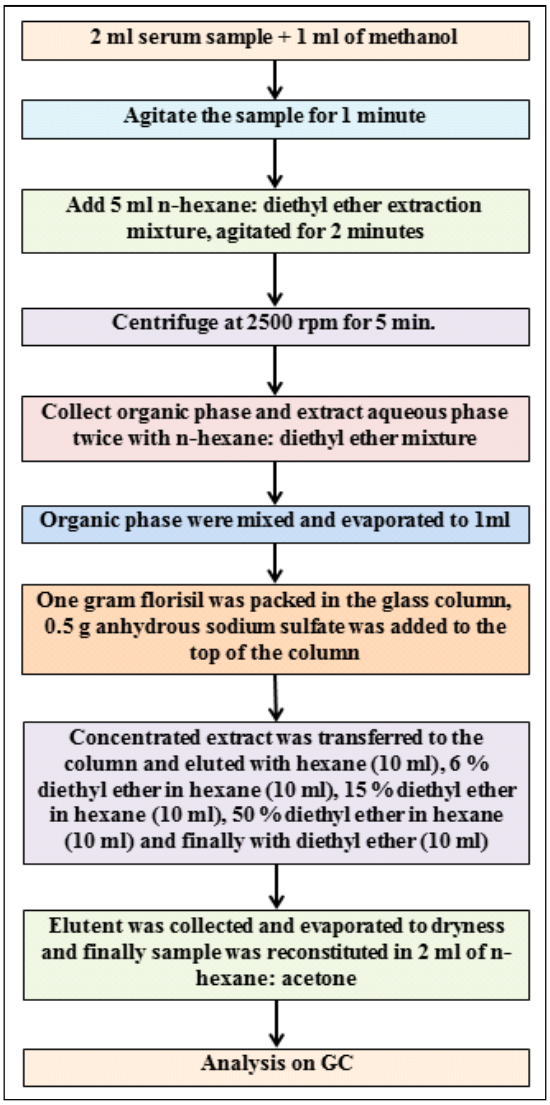

Fig 1: Extraction and clean up followed in serum/blood for estimation of pesticide residues (modified method of Moreno *et al.*, 2004).

to be 1.67±7.49 ng/ml and 0.49±2.16 ng/ml respectively (Sharma *et al*., 2015).

It has been reported that the oral consumptions of one fourth of LD_{50} phosalone over 48 days decreased body weight, testicle, and epididymis weight in the treated wistar rats compared to the control counterparts. Numerous studies have reported that many pesticides affect the pituitary, hypothalamus or both (Wissem *et al*., 2011).

In a another study 35 per cent of the total human blood samples, taken from various districts of Punjab state, were found positive for pesticide residues of a-HCH, b-HCH, p,p-DDD, p,p-DDE, p,p-DDT, endosulfan, monocrotophos, phosalone and profenophos (Sharma *et al*., 2015). Presence of OPs residues in the blood samples indicates a shift in consumption pattern of pesticides from organochlorines to organophosphate pesticides. Few studies have been conducted on analysis of pesticide residues in blood of animals (Sharma *et al*., 2015). All organophosphorus pesticides (OP) are lipophilic, and these environmental xenobiotics are recognised to have a considerable affinity for membrane phospholipid component of the bio-membrane is believed to be the site of action of OP insecticides (Datta *et al*., 1994).

Studies in dairy animals on blood serum concentrations of pesticide residues are not available, hence in the present study; the comparisons have been made with human blood samples. Among various OPPs, the blood samples of humans in Punjab state were positive for monocrotophos (94.8 ng/ml), chlorpyriphos (66.2 ng/ml), malathion (30.1 ng/ml) and phosphamidon (36.6 ng/ml; (Mathur *et al*., 2005). In previous study, dairy animals of low pesticide usage and high pesticide usage area were positive for chlorpyriphos (19.92±23.7 ng/ml), methyl parathion (10.5±2.8 ng/ml), whereas dimethoate OPP was detected (25.2 ng/ml) in slaughter house buffaloes (Ratnakaran *et al*., 2014).

Table 1: Retention time of various pesticide standards in gas chromatography (GC) procedures.

Pesticides					
Organochlorine	Retention time (min)	Organophosphorus	Retention time (min)	Synthetic pyrethroids	Retention time (min)
Heptachlor epoxide	18.881	Chorpyrifos	7.048	Cyhalothrin	38.104
Chlordane	21.949	Dichlorovos	2.478	Permethrin	40.233
Fipronil	21.17	Ethion,	10.829	Cypermethrin	43.555
Lindane	8.168	Monocrotophos	5.047	Deltamethrin	48.666
Methoxychlor	33.763	Malathion	7.759		
op-DDT	27.14	Parathion-methyl	8.092		
Endrin	25.102	Profenphos	9.875		
		Phorate	5.241		
		Triazophos	11.129		
		Quinalphos	8.957		
		Phosalone	13.837		

Table 2: Percentage (%) and serum concentration (ng/ml); (Mean±SEM) of phosalone in blood of crossbred breeding bulls.

Semen stations	Number of samples	Animals positive for phosalone (%)
Milkfed Khanna	5	4/5 (80.00%)
PLDB Nabha	14	10/14 (71.42%)
Overall	19	14/19 (73.68%)
Serum concentration (ng/ml); (Mean±SEM)		
Animals positive for phosalone (%) (n=14)		1.89±0.98

Table 3: Mean±SEM values of various hormones in blood samples collected from crossbred breeding bulls (n=19).

Hormone	(Mean±SEM) n=19	Mean±SEM (+ve for phosalone) (n=14 bulls)	Mean±SEM (-ve for phosalone) n=5 bulls
Testosterone (ng/ml)	4.43±0.12	4.12±0.16	4.45±0.20
Estrogen (ng/L)	75.45±6.95	86.27±2.32[a]	54.09±1.21[b]
DHEA (ng/L)	80.27±5.65	79.97±3.14	80.12±2.33
T3 (ng/ml)	1.51±0.16	1.52±0.18	1.50±0.12
T4 (ng/ml)	4.82±0.54	4.76±0.87	4.54±0.65
Prolactin (ng/ml)	86.69±2.73	85.94±1.12	85.32±2.3

[a,b] Values with different superscripts show significant difference ($p<0.05$).

Earlier, with regard to OCPs, it was observed that majority of the blood samples were found positive for DDT and its metabolites both in the animals of low pesticide usage and high pesticide usage area (49.6±74.1 ng/ml) and slaughter house (54.3±58.2 ng/ml) (Ratnakaran *et al.*, 2014). The concentrations of DDT (19.7-204.7 ng/ml) observed in the animal blood samples of study were comparable to human blood samples reported in Punjab (65.2 ng/ml; Mathur *et al.*, 2005), higher in comparison to Madurai (8.0-26.0 ng/ml) (Subramaniam and Solomon 2006) and in Lucknow (2.0-33.0 ng/ml) (Kaphalia and Seth, 1983) and lower than reports from Delhi (710.0 ng/ml) (Ramachandra *et al.*, 1984, Saxena *et al.*, 1987). It has been reported that DDT and its metabolites, despite the decrease of concentrations in environment, still have a high biological activity and are able to impair the cell function (Wojtowicz *et al.*, 2007). Thus, their adverse effect on reproductive processes of animals can be prolonged by their metabolites formed during biodegradation. In present study, 73.68% crossbred breeding bulls were found positive for pesticide residue Phosalone detected at retention time of 13.837 min. (Table 2). Previous studies using serum samples have also detected higher prevalence in the pesticide polluted area (Deepa *et al.*, 2008). They found higher DDT levels in dairy animals reared in polluted area in comparison to those reared in non-polluted area.

In brief, in this study none of the sample was found positive to organochlorines due to the major factor that the animals are stall fed with chopped fodder cultivated at the semen station premises and there is very little pesticides being sprayed on the fodder. Also 73.68% samples were positive for organophosphate-phosalone which may also be attributed to shift to the use of organophosphates since most of the organochlorines are strictly prohibited to use.

Few chromatograms of standard and blood sample for the pesticides mostly used in Punjab have been shown (Fig 2-5) which depicts the peaks at a particular retention time specific to a particular pesticide residue.

Assessment of endocrine profile in relation to pesticide residues of crossbred breeding bulls

Serum testosterone level

Studies regarding analysis of hormones in relation to the pesticide residues in bullsare not available and the testosterone concentration recorded in serum of crossbred breeding bulls is presented in Table 3.

The present study revealed no significant change in the values of testosterone and the value of testosterone was found (4.12±0.16 ng/ml) in the bulls that were tested positive for phosalone, therefore showing a very little variation, attributing to the blood testis-epididymis barrier (Kamarianos *et al.*, 2003a) and shorter half-life of

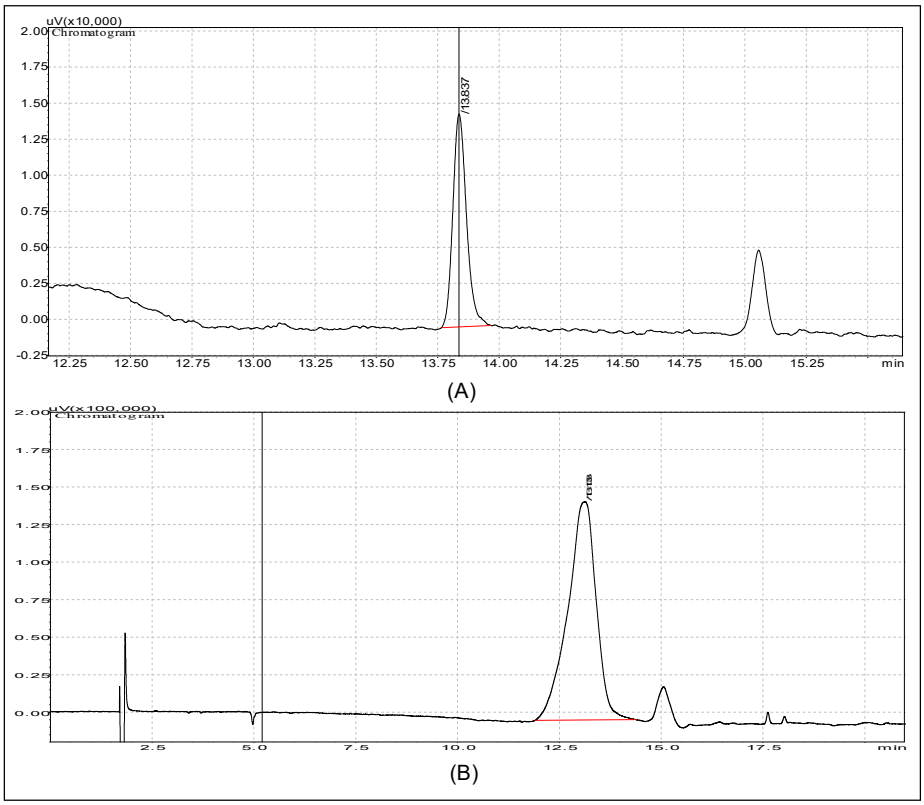

Fig 2: Chromatogram of A) Standard (at retention time of 13.83 min.) B) Blood (peak showing sample positive for phosalone) for phosalone.

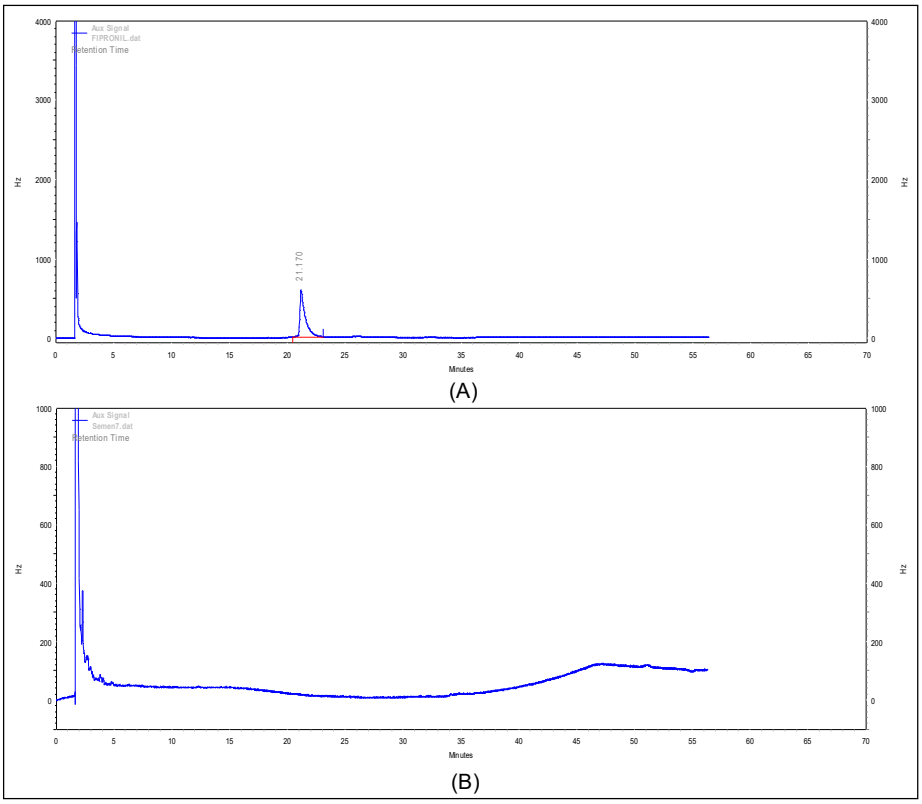

Fig 3: Chromatogram of A) Standard (at retention time of 21.17 min.) B) Blood for fipronil, (showing no peak in blood sample).

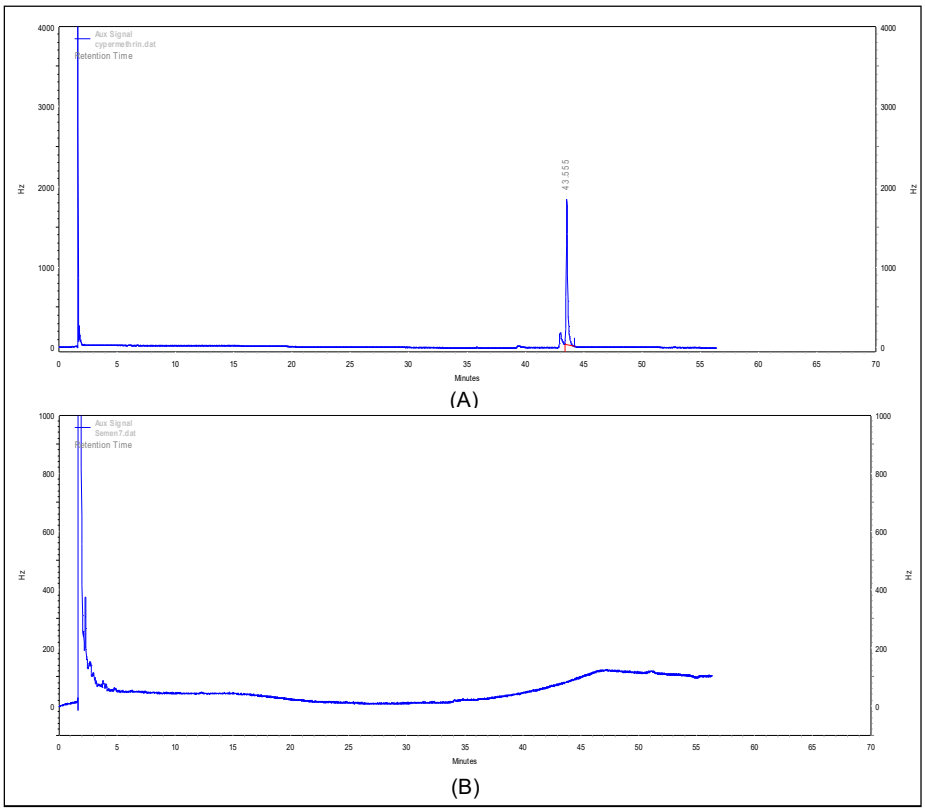

Fig 4: Chromatogram of A) Standard (at retention time of 43.555 min.) B) Blood for cypermethrin (showing no peak in blood sample).

organophosphates (Smith 1995). The differences in the permeability of the blood-testis and epididymis barriers, in the metabolism and excretion by the reproductive tract and the ability of the reproductive system to retain pollutants are the major factors contributing to retention of pesticide residue in tissue (Kamarianos *et al*., 2003a).

Serum estradiol level

In this study, estradiol concentration was significantly higher ($p<0.05$) (86.27±2.32 ng/L) in bulls found that were positive for phosalone. The level of estradiol was found near normal in bulls that were found negative for phosalone. Increased estrogen in comparison to testosterone is associated with poor libido in breeding buffalo bulls (Muller *et al*., 2012). Certain amount of estradiol is required for the function of postpubertal bull testes and to regulate sperm motility (Devkota *et al*., 2008). Increased concentration of estradiol can affect libido as testosterone and estradiol have been found to be negatively correlated to each other (Javed *et al*., 2000).

In our study, testosterone level was near to normal values in all the bulls and significant difference was observed in estradiol levels. So, the difference in testosterone to estradiol ratios bulls was due to the differences in estradiol level rather than testosterone. The ratios of testosterone to estrogen are more important than their individual values in regulating libido (Singh *et al*., 2009). Leydig cells produce testosterone, which are converted to estradiol by aromatization in sertoli cells, adipose tissues and hypothalamic pre-optic area (Michael *et al*., 1987). Increased aromatization of testosterone to estradiol causes decreased testosterone to estrogen ratio. Hence, higher estradiol level might be associated with poor libido in breeding buffalo bulls.

Serum DHEA

The serum DHEA levels were also not affected in the bulls found positive for phosalone (80.27±5.65 ng/L) and no such previous research works have been done to evaluate the effect of levels of DHEA in crossbred breeding bulls.

Serum thyroid hormones

Thyroid hormones concentrations in serum of crossbred breeding bulls are presented were found to be normal *i.e*., the pesticides didn't affect the levels of thyroid hormones. Lack of relationship between sexual behaviour and circulating levels thyroid hormones was found by Boyd and Corah (1988). Normal circulating levels of T3 and T4 have not been found to be correlated with libido. However, hypothyroidism reduces the concentration of serum sex hormone binding globulin (SHBG) (Olivo *et al*., 1970), which might alter the plasma testosterone concentration (Ford *et al*., 1992) thereby affecting libido.

Serum prolactin level

In our study, serum prolactin levels were found similar ($p>0.05$) in (85.32±2.3 ng/ml to 85.94±1.12 ng/ml) in all the crossbred breeding bulls. It has been observed that serum prolactin level at ejaculation increases six times (59.0 ng/ml)

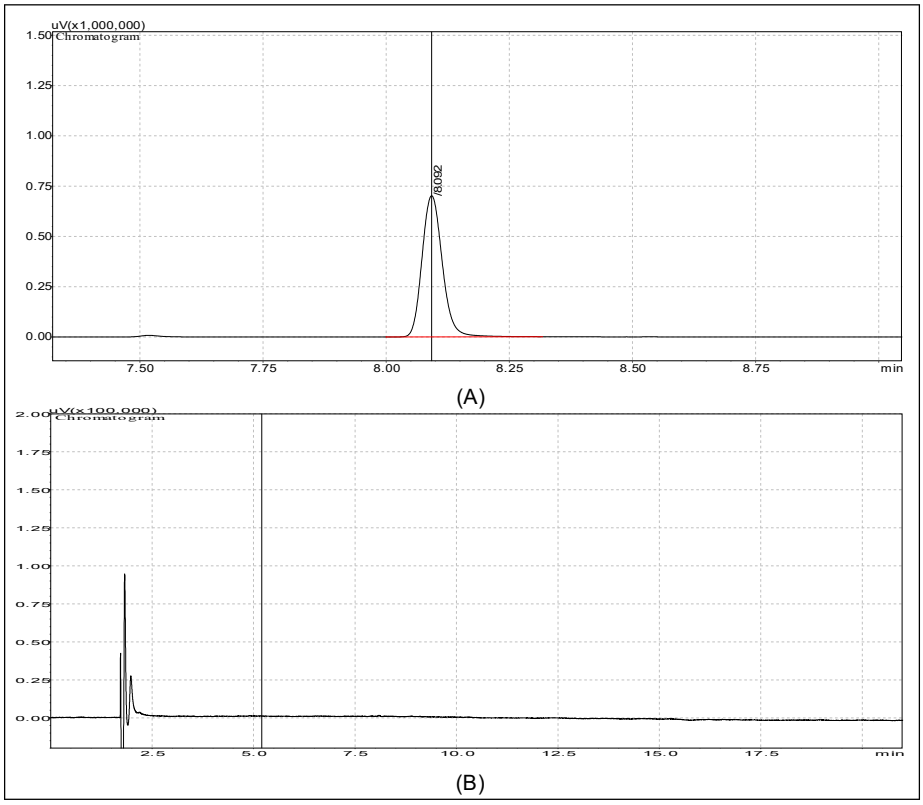

Fig 5: Chromatogram of A) Standard (at retention time of 8.092 min.) B) Blood for parathion (showing no peak in blood sample).

the normal basal level (10.8 ng/ml) (Convey *et al*., 1971). Prolactin is required for maintaining good libido and manifestation of sexual activity. Prolactin secretion is heritable and its level in sire could be predictors of lactational ability in daughters which could be useful in identification of superior dairy animals (Klindt 1988). However, we could not find any difference in serum prolactin level in crossbred breeding bull.

CONCLUSION

In well managed bull semen stations, organophospahate (phosalone) was detected in serum of 73% bulls. Testosterone: Estradiol ratio was decreased in bulls which may be attributed to phosalone.

Data availability statements

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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