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Impact of body burden of pesticide residues on the reproductive tract of buffalo

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ABSTRACT

The disruption of reproductive system leading to infertility can occur due to accumulation of endocrine disrupting chemicals such as pesticide residues in the reproductive tract and ovarian follicular fluid after their entry into blood of animals. Infertile adult female buffalo (100) from a slaughterhouse were utilized to assess the presence of pesticide residues in their blood and reproductive tract as well as the microscopic alterations in the tract. The pesticide residues were detected in 25% blood, 43% reproductive tract and 32% ovarian follicular fluid samples by gas chromatograph (GC) and were confirmed by gas chromatograph-mass spectroscope (GC-MS). Compared to blood, the pesticide residues were high in reproductive tract. The concomitant presence of specific pesticide residues was observed in blood, reproductive tract and follicular fluid. Histopathological alterations in pesticide-negative as well as low and highly pesticide-positive reproductive tracts of slaughtered buffaloes were 0.25±0.71, 1.87±2.23 and 3.65±1.68/sample, respectively. The major alterations were leiomyosarcoma, haemangiosarcoma, cystic ovarian degeneration, fibrotic/atrophic ovaries and cyst adenoma. In conclusion, the presence of pesticide residues in the reproductive tract of female buffalo, may be a factor contributing towards infertility.

Key words: Buffalo**,** Histopathology, Ovarian follicular fluid, Pesticide residues, Reproductive tract

The widespread usage of pesticides for agricultural production has led to an increased exposure of dairy animals to these (Directorate of Economics and Statistics 2013). Upon their entry into the blood, the residues of pesticides get accumulated in the lipid containing tissues like the reproductive tract, especially the lipoprotein membrane of ovarian follicles (Ratnakaran *et al*. 2014). Pesticide residues can disrupt the functioning of endocrine system by their action at various levels, starting from the synthesis of hormones up to their action on receptors through mechanisms disrupting enzyme functioning, expression of gene and protein (Magnarelli and Fonovich 2013). The presence of detectable amount of residues in an ovary with atretic follicles and degenerated oocytes could be related to the fact that pesticides mimic or antagonize androgens and/or estrogens resulting in infertility in experimental and farm animals (Fowler *et al*. 2008, Tiemann 2008). Moreover, the abnormal estrogenic response of pesticides can predispose to tumour development (Magnarelli and Fonovich 2013). Considering the potential adverse impact of pesticide residues on the fertility of dairy animals, this study was conducted to assess the impact of pesticide residues in reproductive tract of adult female buffalo.

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MATERIALS AND METHODS

Sampling: The study was conducted on 100 adult female buffaloes presented at a slaughterhouse. Buffaloes were sampled pre-slaughter for 10 ml blood via jugular venipuncture in heparinised vials which were stored at –20ºC for pesticide residue analysis. The reproductive tract of each buffalo was collected post-slaughter. One half of tract was stored in 10% neutral buffered formalin for histopathology and the other half was stored at –20ºC for pesticide residue analysis. Also, the ovarian follicular fluid was aspirated using 18G needle and stored at –20ºC for pesticide residue analysis.

Pesticide standards: Analytical standards of Organochlorine pesticides (OCPs; HCH and its metabolites, Methoxychlor, Heptachlor, α-Chlordane, Mirex, Aldrin, Fipronil, Butachlor, Dieldrin, DDT and its metabolites, Endrin, Endosulfan and Toxaphene), Organophosphate pesticides (OPPs; Chlorpyrifos, Monocrotophos, Dimethoate, Phorate, Fenitrothion, Parathion-methyl, Malathion, Fenamiphos, Profenphos, Ethion, Triazophos, Phosalone and Quinolphos) and Synthetic Pyrethoids (SPs; Cypermethrin, Permethrin, Cyfluthrin, Cyhalothrin, Deltamethrin and Fenvalerate) were used. The mean recoveries of different analytical methods obtained by spiking samples with multiple pesticides in concentrations ranging from 50–100 ng/g were 70–80%.

Extraction and clean up technique for pesticide residue detection in reproductive tract, blood and ovarian follicular fluid: Pesticide residues were extracted from 5.0 g reproductive tract as per Sharma (2007). Pesticide residues were extracted from blood and ovarian follicular fluid as per Gill *et al.* (1996).

Analysis: The clean up extract (1–2 µl) was injected into gas chromatography (GC) capillary column equipped with electron capture detector (ECD) for OCP and SP detection, with flame thermionic detector (FTD) for OPP detection. The analyte was identified by comparing the retention times and peak height/area with the reference standards run under similar operating conditions (Bedi *et al*. 2013). The confirmation of pesticides was done by gas chromatograph-mass spectrometer (GC-MS), in which a characteristic mass spectrum was obtained based on mass-charge ratio of a compound. Reagent and sample blank were extracted and analysed in triplicate to negate the false peaks in common.

Histopathology: The tissue samples from various parts of reproductive tract (stored in 10% NBF) of calves were processed for paraffin sectioning by Acetone-Benzene schedule. The sections of 4–5 µm thickness were used and the sections were stained with haematoxylin-eosin for morphologic studies (Luna 1968).

Statistical analysis: The concentration of pesticide residues in samples were summarized using arithmetic mean and standard deviation. One-way analysis of variance-post hoc multiple comparison test and Pearson's correlation coefficient test was applied using SPSS 15.0 software to test the difference in sample number and concentrations of pesticide residues between groups and within groups and to calculate correlation coefficient between the groups, respectively.

RESULTS AND DISCUSSION

Analysis of pesticide residues in blood, reproductive tract and ovarian follicular fluid: A higher concentration of pesticide residues (OCPs, OPPs and SPs) was detected in a significant proportion of dairy buffalo. A higher pesticide residue concentration in reproductive tract as compared to their blood and ovarian follicular fluid samples ($P<0.05$; Table 1) may be suggestive that the body burden of the residues resulting from animal's lifetime exposure, gets mobilized into blood and become available for redistribution and metabolism in the event of negative-energy balance and again reach upto lipid rich tissues including reproductive tract/ovarian follicles to act as a sink (Lerch *et al.* 2016). Also, many of the samples were positive for multiple residues which may prove deleterious in reproduction if they act synergistically. The reproductive tract and follicular fluid samples of buffalo, respectively, had higher ($P<0.05$) OCP and SP residues compared to OPPs (Table 1), suggesting a trend towards their widespread inadvertent use as well as long biological half-life of OCPs (Dobrinas *et al.* 2013).

Moreover, the pesticide residues were reported in matched samples of blood, reproductive tract and ovarian follicular fluid of buffalo. The main pesticide transferred between the three compartments was DDT and its metabolites as shown by its concomitant presence in maximum number of samples (9). Other pesticides concomitantly present in the 3 compartments were HCH, endosulfan, methylparathion, ethion and cypermethrin with correlation coefficient as high as 0.99 between them. In some studies relevant to present work, the presence of DDT and its metabolites was noticed in the ovaries/reproductive tract in varying concentrations in farm animals (Tiemann 2008, Ratnakaran *et al*. 2014) thereby hampering the reproductive cycle by altering ovarian and uterine environment. Despite its limited use in India for malaria control programmes, the abundance of DDT in samples could be related to its environmental persistence and lipophilicity (Dobrinas *et al.* 2013).

The pesticide residue concentrations detected in the present study are higher than those reported to exhibit an adverse biological impact on the reproductive system. The exposure of bovine granulosa cells *in vitro* to OCPs at 0.1–0.0001 ng/ml can decrease estrogen and progesterone secretion (Faundez *et al.* 1996). In another study, DDE @ 10 ng/ml in porcine ovarian follicular fluid affected ovarian steroidogenesis (Crellin *et al.* 1999). Additionally, *in vitro* exposure of bovine oocytes to endocrine disruptors at 0.8 ng/ml reduced blastocyst development (Krogenaes *et al.* 1998).

Although OPPs are non-persistent in nature, these can be detected in biological samples upon their acute recurrent exposure. In an *in vitro* study in buffalo, malathion caused a dose dependent decrease in oocyte and embryo development and increase in preimplantation losses (Aziz *et al.* 2012). It is also alarming that all these studies exhibited adverse impact of a single pesticide in a much lower concentration, however, the present study indicated the presence of a cocktail of pesticide residues in higher concentrations in buffalo blood and reproductive system. Since the presence of multiple pesticide residues in biological system are well known to have synergistic adverse impact (Silins and Hogberg 2011), thus, in present study, the presence of multiple residues may adversely affect the reproductive health of buffalo.

Histopathology alterations in reproductive tract: An increasing trend of mean number and co-existence of histopathological alterations was observed from pesticide-negative to highly pesticide-positive reproductive tracts (Table 2). The alterations present in pesticide-positive samples were ovarian leiomyosarcoma, ovarian haemangiosarcoma, ovarian fibrosis, cystic ovarian degeneration/polycystic ovarian syndrome, cyst adenoma and oviduct changes (Table 2).

Till date, no study on the pesticide induced carcinogenesis has been done in animals. However, the carcinogenic potential of pesticide residues can be correlated to studies in which high incidence of cancer was reported among people residing in areas with higher concentrations of OCPs in water or blood (Mathur *et al.*

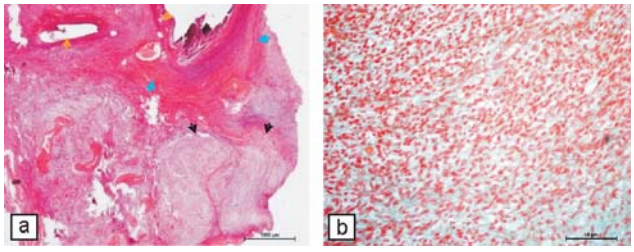
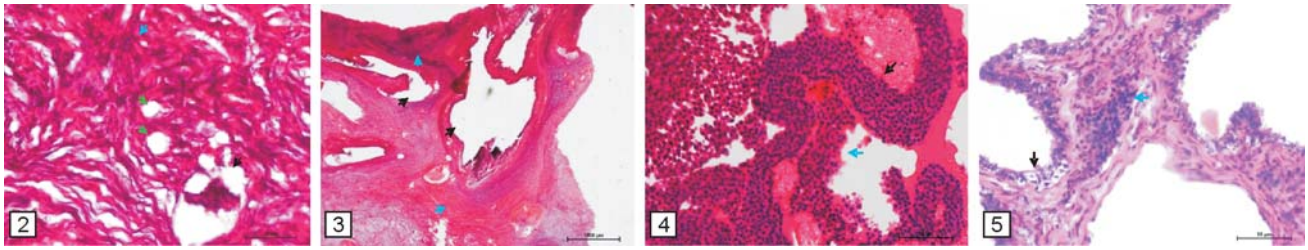


Fig. 1a-b. Ovarian leiomyosarcoma in pesticide-positive buffalo a. whorls in connective tissue (black arrows), fibrosis (blue arrows) and cysts (green arrows, 20×), b. Masson's Trichrome stained hyperplastic muscle fibres with pleomorphic nuclei (round to cigar shaped; 400×)

2008, Thakur *et al.* 2008). Pesticide residues can cause carcinogenesis by genetic and mitochondrial damage, chromosomal aberrations, transcription of cancer promoting factors and generation of reactive oxygen species which are associated with excessive mitotic potential (Magnarelli and Fonovich 2013).

Ovarian leiomyosarcoma, a rare malignant tumour of smooth muscles, was characterized by whorling of the rapidly proliferating muscle fibres (Figs 1a,b; Kaul and Sharma 2009). Haemangiosarcoma, a malignant tumour of endothelial cells of ovary, was characterized by rapidly proliferating, extensively infiltrating anaplastic cells derived from blood vessels (Fig. 2; Heo *et al.* 2011).



Figs 2–5. **2.** Ovarian haemangiosarcoma in pesticide-positive buffalo (400×) showing infiltrating cells in blood vessels (black arrows) and stroma (blue arrows) among normal vessels (green arrows). **3.** Ovarian fibrosis (blue arrows) characterised by complete loss of structural details and cysts (black arrows) in pesticide-positive buffalo (20×). **4.** Ovarian cyst adenoma in pesticide-positive buffalo (400×) showing an extensive proliferation of granulosa cells of follicle (black arrows) and invasion into stroma (blue arrows). **5.** Oviduct of pesticide-positive buffalo (400×) showing deciliation and loss of normal epithelium (black arrows) and extensive cellular infiltration (blue arrows).

Table 1. Pesticide residue (OCP: organochlorine pesticides, OPP: organophosphorus pesticides, SP: synthetic pyrethroids) concentrations (mean±SD) in blood, reproductive tract and ovarian follicular fluid samples (100 each) of buffalo

	Blood (ng/ml)		Reproductive tract (ng/g)		Follicular fluid (ng/ml)	
Overall pesticide residue concentration	42.0±47.4[a]		107.3±115.6[b, c]		63.6±55.5[d]	
Samples positive for a residue	25%		43%		32%	
Samples positive for >1 residue	32%		21.9%		31.25%	
Concentrations of a specific pesticide residue category (n, samples positive)						
	n	ng/ml	N	ng/ml	N	ng/g
OCPs	13	33.8±52.9[a]	24[*]	124.0±133.2[b,c]	25[*]	55.3±34.4[d,e]
OPPs	11	29.3±20.5	7[*]	60.2±75.3	9[*]	48.7±45.2[g]
SPs	5	70.8±54.6[a]	8[*]	150.7±99.5	5[*]	130.8±47.9[b,f,h]
Concentrations of a specific pesticide residue (n, samples positive)						
	n	ng/ml	N	ng/ml	N	ng/g
OCPs						
DDT metabolites	9	27.8±16.8	12	133.1±75.9	19	58.8±57.1
γ-HCH	4	20.3±16.2	4	74.1±27.5	3	41.7±19.7
β-HCH	0	ND	3	30.4±16.2	2	16.2±2.5
Endosulfan	3	20.4±16.3	4	132.9±136.6	5	31.3±12.6
Aldrin	2	9.6± 4.7	2	48.3±16.7	2	90.1±19.1
OPPs						
Ethion	3	50.4±38.9	5	41.9±31.6	2	37.3±2.5
Chlorpyriphos	4	19.0±7.2	6	28.8±18.7	4	27.6±12.1
Methylparathion	4	29.6±9.8	4	43.2±22.8	3	98.4±31.6
SPs						
Cypermethrin	5	70.8±54.6	2	101±82.1	3	123.2±57.5
Permethrin	0	ND	6	167.4±105.8	3	138.5±47.5

ND, Non-detectable; [*]P<0.05 between row; [a] *vs* [b, c] *vs* [d] P<0.05-within a row, [e] *vs* [f, g] *vs* [h] P<0.05-within a column.

Table 2. Histopathological alterations in the reproductive tract of buffalo in relation to pesticide presence

	Pesticide negative tract (n=10)	Low pesticide positive tract (n=15)	High pesticide positive tract (n=15)
Pesticide concentration (ng/g, mean±SD)	0	53.3±22.8	194.0±123.2
Histopathological alteration per sample (n, mean±SE)	0.25±0.71[a]	1.87±2.23[b]	3.65±1.68[c]
Specific histopathological alteration			
Leiomyosarcoma	0	0	4
Leiomyosarcoma + cystic ovary + atrophy	0	0	3
Haemangiosarcoma	0	0	3
Haemangiosarcoma + cystic ovary + atrophy	0	0	2
Cyst adenoma	0	3	2
Cystic ovary	0	5	5
Fibrotic + atrophic ovary	2	5	7
Oviductal changes	0	2	3

a *vs* c, b *vs* c $P<0.05$.

The pesticide-mediated endocrine disruption may be a contributor to ovarian fibrosis due to loss of follicular activity (Fig. 3; Fowler *et al.* 2008). Furthermore, the mechanism underlying cystic ovarian degeneration / polycystic ovarian syndrome could be pesticide-induced prevention of the conversion of androgen secreted from the granulosa cells to estrogen. This retards growth of follicles to the successive stage, leads to their persistence and cyst formation (Fig. 3; Steckler *et al.* 2005, Forsdike *et al.* 2007). Cyst adenoma, a benign tumour arising from the surface epithelium of ovary, was characterized by hyperplastic proliferation of granulosa cells with no demarcation between granulosa and theca cell layers of a follicle (Fig. 4; Kamel 2010).

Oviductal changes in pesticide-positive samples could be related to the role of pesticide residues as endocrine disruptors as the differentiation, proliferation and motility of ciliated epithelial cells is regulated by the interaction of estrogen and progesterone hormones (Fig. 5; Hernandez-Ochoa *et al.* 2009). Various histopathological alterations in the low pesticide-positive reproductive tracts indicated that reproductive system is also sensitive to low exposure of pesticide residues (Table 2). This could be correlated to a histopathological study where oral exposure of malathion at a dose of 200 ng (sublethal dose) for 30 days in fish increased the number of atretic oocytes showing changes like cytoplasmic degeneration, vacuolation and clumping along with degeneration of vitelline membrane (Dutta *et al.* 1994).

Presence of pesticide residues in blood and reproductive tract of adult female buffaloes as well as the presence of histopathological alterations in the pesticide residue-positive reproductive tracts is suggestive of the adverse impact of body burden of pesticide residues on the reproductive health of adults.

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