



Pesticide residue accumulation in buffalo ovaries: a potential hazard to fertility

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ABSTRACT

The present study was conducted on 58 female buffaloes subjected to slaughtering at a local abattoir. The study investigated the presence of pesticide residues in their blood, ovarian tissue as well as follicular fluid samples. These samples were subjected to gas chromatography (GC) to detect the presence of residues of organochlorine pesticides, organophosphorous pesticides and synthetic pyrethroids, followed by their confirmation using gas chromatography mass spectrometry (GCMS). About 32.7% blood, 53.4% ovarian tissue and 21.4% follicular fluid samples were found positive for pesticide residue(s) and their respective alarming levels of pesticide residues were 47.7 ± 113.7 ng/ml, 124.3 ± 106.1 ng/g and 245.6 ± 477.1 ng/ml. The most detected pesticide residues in the ovarian/follicular fluid samples were DDT / endosulphan and their metabolites. In conclusion, a much higher load of pesticide residues in ovarian/follicular fluid of buffaloes as compared to their blood suggested the potential hazard of these residues to fertility status of buffaloes.

Key words: Blood, Buffalo, Follicular fluid, Ovary, Pesticide residue

Over 700 potentially toxic pesticides used worldwide have become ubiquitous in the environment (Toppari *et al.* 1996). Consequently, animals are exposed to a variety of pesticides. Although an individual component of a cocktail of pesticide residues may have no observable biological impact, but additive or synergistic impact of each residue in the mixture can exert significant damage. It is emerging that low - level, 'real - life' mixtures of pesticide residues may carry significant biological potency (Ghuman *et al.* 2012). This makes critically important to have awareness regarding the biological presence of pesticide residues (Ghuman *et al.* 2013).

The decline in fertility of dairy animals in recent decades was found related to environmental pollutants (Ghuman *et al.* 2012, Rolland *et al.* 2013). The exposure of pesticide residues leads to abortion, intra-uterine fetal demise and birth defects (Zama and Uzumcu 2010). In fact, the ability of lipophilic organochlorine pesticide residues (OCPs) to concentrate in the ovarian follicular fluid has deleterious impact on oocytes (Kamarianos *et al.* 2003). During fetal and post-natal life, the pesticides stored in the body fat of dam are rapidly mobilized, thus, young ones become susceptible due to consumption of milk with elevated amount of pesticides (Petro *et al.* 2010). The exposure during these periods causes damage to ovarian primordial

follicle and leads to reduction in the lifetime reserve of oocytes, thus producing irreversible infertility in adults (Ghuman *et al.* 2012, 2013). In Punjab state, the contamination of environment with pesticides is extremely high compared to the developed countries (Mathur *et al.* 2005). Thus, objective of the present study was to assess the presence of pesticide residues in blood and ovaries of buffaloes that are destined for slaughtering usually due to infertility problems.

MATERIALS AND METHODS

Sampling: Adult buffaloes (58) subjected to slaughtering at a local abattoir were sampled for jugular vein blood (10 ml) in non-heparinized polystyrene tube. Out of the 116 ovaries of each buffalo, 28 had large size follicles, thus, their follicular fluid was aspirated using a sterile syringe. All the samples (blood, ovaries and follicular fluid) were immediately transported to laboratory at 4°C and were stored at -20°C till analysis.

Pesticide standards: Analytical standards of organophosphorus pesticides, OPPs (chlorpyrifos, monocrotophos, dimethoate, fenitrothion, parathion-methyl, malathion, fenamiphos, profenophos, ethion, triazophos and phosalone), organochlorine pesticide residues, OCPs (α -HCH, β -HCH, γ -HCH, δ -HCH, heptachlor, aldrin, fipronil, butachlor, dieldrin, pp-DDE, op-DDD, pp-DDD, op-DDE, pp-DDT, endrin, endosulfan sulphate and β -endosulfan) and synthetic pyrethroids, SPs (cypermethrin, permethrin, cyfluthrin, deltamethrin and fenvalerate; sigma-aldrich, accu standard) had 90–99% purity. The concentration of individual pesticides was quantified from the peak area of

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sample corresponding to the external standard. The recovery of pesticides from fortified samples (50 and 100 ng/ml) was between 94–103%.

Extraction and clean-up technique for blood and follicular fluid (Moreno *et al.* 2004): At room temperature, 2 ml blood or follicular fluid was mixed with 1 ml methanol followed by agitation of mixture for 1 min; to this mixture, 2.5 ml of each of n-hexane and diethyl ether was added and agitated for 2 min. The samples were centrifuged for 5 min at 2500 rpm. The upper organic phase was collected and aqueous phase was again extracted twice with 2.5 ml each of n-hexane and diethyl ether. The organic phase was evaporated and concentrated to 2 ml. The sample clean up was done as per USEPA method 3620B using florisil as adsorbent in glass column chromatography (USEPA 2003). Finally, sample was reconstituted in 2 ml mixture of n-hexane: acetone (1: 1 v/v).

Extraction and clean-up technique for ovarian tissue (Mills *et al.* 1972): Following overnight immersion of well-homogenized tissue (both the ovaries of each buffalo were pooled) in 50 ml n-hexane: acetone (1: 1 v/v), the contents were filtered, evaporated to 15 ml and partitioned thrice with 15 ml hexane saturated acetonitrile. The acetonitrile layers were dissolved in 300 ml distilled water saturated with NaCl solution and partitioned by hexane and dichloromethane. The extracted samples were cleaned by column chromatograph using silica gel (60–120 mesh) as an adsorbent in between 2 layers of anhydrous sodium sulphate. After pre-washing of the glass column with hexane, the concentrated sample extract was added. The elution rate was fixed at 2 ml/min and about 100 ml of three different eluants [eluant-1: 20% dichloromethane + 80% hexane (v/v); eluant-2: 50% dichloromethane + 0.35% acetonitrile + 49.65% hexane (v/v); Eluant-3: 50% dichloromethane + 1.5% acetonitrile + 48.5% hexane (v/v)] were used for the complete recovery of pesticide residues. The eluants were combined and concentrated to 5 ml by using rotary evaporator at 40°C. Remaining volume was evaporated completely and residues were reconstituted in n-hexane: acetone (1: 1) mixture to make final volume as 3 ml.

Analysis: The clean-up extract (2 µl) was injected into DB-5 gas chromatography (GC) capillary column (length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25µm) using auto injector. For OCP and SP analysis, an electron capture detector (ECD) was used and a flame thermionic detector (FTD) was used for OPPs. The parameters of GC for ECD and FTD were described earlier (Bedi *et al.* 2013). The confirmation of pesticide residues was done on GC-mass spectrometer equipped with a quadrupole mass

analyser and operated by auto-sampler. The column oven temperature was programmed for 80°C and held for 3 min, followed by an increase to 280°C and held for 20 min. The temperatures of injector, ion source and interface were set at 285°C, 200°C and 290°C, respectively. Helium was used as carrier gas with a column flow rate of 0.94 ml/min.

Statistical analysis: Numerical data are represented as mean $\pm$ SD, and differences were considered to be significant at $P < 0.05$. Two samples of Student's *t*-test was employed for assessing the difference between concentrations of pesticide residues in blood, ovary and follicular fluid. Statistical procedures were carried out using SPSS-16 software.

RESULTS AND DISCUSSION

In a previous study, 15.1–17.5% blood samples of normally reared dairy animals in Punjab state were found positive for pesticide residues (27.5 $\pm$ 21.0 to 65.6 $\pm$ 68.5 ng/ml; Ratnakaran *et al.* 2012). However, in the buffaloes of present study that were subjected for slaughtering, a marked percentage of blood (32.7%), ovary (53.4%) and follicular fluid (21.4%) samples were detected positive for any pesticide residue (Table 1). Moreover, out of the samples positive for pesticide residues, the proportion of blood, ovary and follicular fluid samples positive for more than one pesticide residues was between 42.1–51.6% (Table 1). In addition, the tendency of pesticide residues to accumulate in the follicular fluid (245.6 $\pm$ 477.1 ng/ml) and ovarian tissue (124.3 $\pm$ 106.1 ng/g) was alarmingly high compared to circulating blood (47.7 $\pm$ 113.7 ng/ml; Table 1). Overall, these findings suggested that the presence of pesticide residues in high concentrations in the buffaloes destined to be slaughtered might be one of the causative factors underlying their ill-health and/or infertility scenario. The extent of damage to fertility by the observed level of pesticide residues in the present study can be extrapolated from the fact that a mixture of pesticides even at 1.0 ng/ml exerted adverse impact on bovine oocyte maturation and embryonic development (Pocar *et al.* 2001). The maturation stages of oocyte and early embryo have susceptibility even towards background doses of pesticide residues (Pocar *et al.* 2001). Moreover, quality and viability of cumulus cells decreased, developmental competence of exposed oocytes got affected and the blastocyst rate and quality reduced in a dose-dependent manner (Campagna *et al.* 2001).

In the present study, the reason underlying the predominant presence of OCP residues as compared to OPPs and SPs (Table 2) could be the long biological half-life of lipophilic OCPs, which leads to their accumulation in the animal body during their life - time (Ghuman *et al.* 2012).

Table 1. Pesticide residues in dairy buffaloes slaughtered at a local abattoir

	Blood (n=58)	Ovarian tissue (n=58)	Follicular fluid (n=28)
No of samples positive (%)	19 (32.7%)	31 (53.4%)	6 (21.4%)
Mean conc. of pesticide residue (mean $\pm$ SD)	47.7 $\pm$ 113.7 ng/ml	124.3 $\pm$ 106.1 ng/g	245.6 $\pm$ 477.1 ng/ml
No of samples positive for >1 residue	8 (42.1%, 8/19)	16 (51.6%, 16/34)	3 (50.0%, 3/6)

Table 2. The presence of different organophosphorus (OPP), organochlorine (OCP) pesticide residues and synthetic pyrethroids (SP; mean±SD) in blood, ovary and follicular fluid of dairy buffaloes slaughtered at a local abattoir

Pesticides		Blood		Ovarian tissue#		Follicular fluid	
		Sample (n=19)^	Pesticides (ng/ml)	Sample (n=31)^	Pesticides (ng/g)	Sample (n=6)^	Pesticides (ng/ml)
OPP	Profenophos	0	ND*	0	ND	1	688.2
OCP	DDT + metabolites	17	54.3±58.2 ^a	11	107.8±110.1 ^a	4	282.6±471.7 ^b
	Endrin	2	9.4±1.6	2	16.2±2.8	2	774.8±1059.0
	Endosulphan + metabolites	3	13.9±9.6	16	65.8±97.4	1	9.7
	HCH + metabolites	1	696.0	3	28.7±22.0	0	ND
	Fipronil	3	9.4±1.8	0	ND	1	21.2
	Dieldrin	0	ND	0	ND	2	8.3±2.1
SP	Fenvalerate	0	ND	5	51.3±35.0	0	ND
	Cypermethrin	0	ND	4	222.8±311.1	0	ND

*ND: Non-detectable; #Tissue of 2 ovaries of a buffalo was pooled; ^some samples were positive for >1 pesticide residue; ^a vs ^b P<0.05, within a row.

Studies have not been conducted till date to record the concentrations of pesticide residues in blood of animals, however presence of various pesticide residues was reported in human blood (Mathur *et al.* 2005).

Among all the OCP residues detected in blood, ovary and follicular fluid, DDT and its metabolites were observed as the main pesticide residue followed by endosulphan and its metabolites (Table 2). It is pertinent to mention here that although the use of DDT is banned in India, but due to lack of suitable alternative for malaria control, it is still permitted to use up to 10,000 tonnes for vector control programs (UNIDO 2009). The spraying of DDT for mosquito control in cattle shed was reported to contribute towards contamination of dairy milk (Battu *et al.* 1989). Moreover, the illegal diversion of DDT to agriculture could be one of the sources of detectable residues in agricultural commodities (Curtis and Lines 2000). In fact, in present study, the detection of pp-DDE, a metabolite of DDT, reflected past usage of DDT.

The lipophilic OCPs have the ability to concentrate in follicular fluid thus having deleterious effect on animal fertility by causing toxicity to oocyte plasma membrane (Kamarianos *et al.* 2003). Previously, various pesticide residues ranging from 10–30 pg/ml were detected in the ovarian follicular fluid of Belgian dairy cattle with the most frequent presence of DDT and its metabolites (Petro *et al.* 2010). In the present study, the follicular fluid concentrations of DDT were higher (P<0.05) compared to their blood and ovarian tissue counterparts (Table 2). Among OPPs, only profenofos residues were observed in present study (Table 1) that could be due to short biological half-life of OPPs (Ghuman *et al.* 2013). The presence of SPs in ovarian tissue (Table 2) suggested their lipophilic nature and the increasing use as replacement to OCPs.

Alarming high concentrations of pesticide residues in buffaloes destined to be slaughtered suggested the impact of pesticide residues, as generally ill or infertile buffaloes are sent for slaughtering. High concentrations of pesticide residues (DDT and endosulphan) in ovarian tissue in

comparison to their blood concentrations were conclusive regarding the tendency of pesticide residues to accumulate in the ovaries of dairy buffalo that could be a potential hazard to their fertility.

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