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EFFECT OF ORGANOPHOSPHATE PESTICIDE MALATHION ON *IN-VITRO* OOCYTE MATURATION, FERTILIZATION AND EMBRYO DEVELOPMENT OF NILI RAVI BUFFALO (*BUBALUS BUBALIS*)

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ABSTRACT

The present study evaluated *in-vitro* toxicity of organophosphate insecticide, malathion on nuclear maturation, fertilization and subsequent embryo development of the oocytes obtained from abattoir ovaries of Nili-Ravi Buffalo (*Bubalus bubalis*). The cumulus-oocyte complexes (COCs) in culture medium were exposed to four different regimens of malathion, 1.56 µmol, 3.12 µmol, 6.25 µmol and 12.5 µmol. Results demonstrated dose dependent significant difference ($P < 0.05$) in nuclear competence, oocyte maturation and degeneration. Cumulus expansion showed significant increase ($P < 0.05$) while oocyte diameter increased non significantly ($P > 0.05$). The COCs that attained nuclear maturation (MII) and were subjected to IVF and, subsequent embryonic development also showed dose dependent non-significant ($P > 0.05$) decrease in fertilization and embryonic development, however, hatched blastocyst formation was significantly compromised ($P < 0.05$). The blastocyst development with inner cell mass was again non-significantly different ($P > 0.05$). The study concludes that malathion may effect quality of preimplantation embryos developed *in-vitro* from immature oocytes pre-exposed to the pesticide.

Key words: Malathion; organophosphorous insecticides; *in-vitro* maturation; buffalo oocytes.

INTRODUCTION

Developing countries rely heavily on the use of insecticides and pesticides to control insect borne diseases and damage caused to cash crops by the insects. In Pakistan also, the use of pesticides has increased 250 times during 1980-2003 (Khooharo *et al*., 2008). Prolonged exposure of agricultural crops to organophosphate pesticides (parathion, malathion and diazanon), herbicides and insecticides is associated with reproductive and embryonic abnormalities in livestock animals (Sharara *et al*., 1998; Getenga *et al*., 2000) due to their highly toxic and environmentally persistent properties (Ecobichon, 2001). Malathion, an organophosphate insecticide (Collins and Lyne, 1985) targets mainly the nervous system of organisms by selectively inhibiting acetylcholinesterase (AChE) (Bjørling-Poulsen, 2008). It is used on a range of insect pests on a number of crops, stored grains and forests via ground and aerial sprays, aerosols, paints, pet collars, and cattle feed blocks to protect them from insects and other pests (US.EPA, 2000; Ware, 2000).

Exposure to malathion can affect both the fertility and reproductive outcomes because of its ability to interact with steroid hormone's receptors (Massaad *et al*., 2002; Meulenberg, 2002). Reduced fertility and miscarriages, genetic damage, birth defects, toxicity to fetuses, oocyte maturation, fertilization and embryo development on exposure to malathion have been reported for humans (Rutledge *et al*., 1992; Griffin, 1999; National Institute for Occupational Safety and Health, 2002). It has been proposed that differentiation of the inner cell mass is prone to environmental influence (embryo culture) with potential adverse effects on offspring (Walker *et al*., 2000). Pig morular embryos cultured *in-vitro* in the presence of different doses of malathion for 96 hrs significantly alters mitochondrial gene expression (Zayil *et al*., 2007).

The total outlay of farmers on the purchase of pesticide in Pakistan was estimated at Rs. 19.612 billion against import bill of Rs. 8.138 billion for 2003. Most pesticides used in Pakistan were insecticides (74%), followed by herbicides (14%), fungicides (9%), acaricides (2%), and fumigants (1%) (Khan, 1998). Livestock is a major financial factor in the economy of Pakistan for meat and milk. Pakistan, being the fifth largest milk producing country of the world, has an annual gross milk production of about 42,199 million tons, with buffalo contributing 71% of the total milk production (GOP, 2007-08). In this context, the most important dairy breed is Nili-Ravi buffalo. As there is a direct and indirect relation between the domestic livestock and use of pesticides in agricultural fields, production of viable and healthy embryos in cattle is being hampered due to the toxicity and bio-accumulation of pesticides residues in farm animals.

Organophosphates in common use include: malathion, chlorpyrifos, diazinon, dichlorvos, dimethoate, ethephon, methamidophos, and oxydemeton-methyl. Residential uses of chlorpyrifos and diazinon

were recently banned by the U.S. Environmental Protection Agency, Though World over, these pesticides have now been under serious consideration for ban, but are still commonly used in most developing countries, including Pakistan. A major incident of malathion poisoning occurred among field workers in 1976 (Baker *et al*., 1978) which affected 7500 workers causing the death of five workers. Extensive and unwise use of organophosphates in agricultural fields is proven by their detectable presence not only in the bodies of agricultural workers (Loewenherz *et al*., 1997) but their residues were also detected in commercial pasteurized milk of dairy animals (Salas *et al*., 2003). Research studies have shown that even non-lethal dose compromised bovine blood chemistry and physiological responses (Parido *et al*., 2007) thus, threats to buffalo health and reproduction, grazing in malathion afflicted agricultural lands need to be monitored. The present study used *in-vitro* technology and aimed at investigating the extant of morphological damage caused by malathion to early stage buffalo embryos. Parameters focused were cumulus expansion, oocyte diameter, oocyte maturation, fertilization and subsequent embryonic development.

MATERIALS AND METHODS

Procurement of ovaries and oviducts: Abattoir ovaries and oviducts obtained from slaughtered buffaloes, were transported within 2 hrs from slaughter house at Sihala Village to the Developmental Biology Laboratory, Department of Biological Sciences, Quaid-i-Azam University, in transport saline (0.9% NaCl; 100000 IU pencillin and 0.1g streptomycin) with temperature maintained between 35-37^{O}C. Immature oocytes were retrieved by aspiration method. The semen straws were provided by the Breed Improvement Department of Live-stock and Dairy Development, Government of Punjab (Pakistan) for *in vitro* fertilization of mature oocytes. The study was carried out according to the guidelines provided by the local ethics committee of the Department of Animal Sciences, Quaid-i-Azam University, Islamabad on humane use of animals for scientific research.

Solutions and culture media: Unless otherwise indicated all chemicals used were obtained from Sigma (St. Louis Missouri USA). Stocks solutions were: 0.4% bovine serum albumin (BSA); 5 mg/ml gentamicin; 1.5% glutamine; 1% estradiol; 100000 IU/ml penicillin; 2 IU/ml human chorionic gonadotrophin/profasi; 2×10^{-4} mol sodium pyruvate; 2 mg/ml heparin; 60% sodium lactate; 10,000 U/ml hyalouronidase and 100 µl heat inactivated fetal calf serum. Oocyte collection medium (OCM) contained 1.38×10^{-1} mol NaCl; 1.5×10^{-3} mol KH_2PO_4; 3×10^{-4} mol KCl; 8×10^{-3} mol Na_2HPO_4; 2.9×10^{-3} mol glucose; 0.05% BSA (fraction V); 0.04% of glutamine; 100000 IU penicillin and 0.01% streptomycin per liter of distilled water. The pH was adjusted to 7.4. Oocyte maturation medium (OMM) contained 0.95% TCM-199; 0.002% estradiol; 0.001% hCG; 0.04% BSA; 100 µl fetal calf serum; 2×10^{-4} mol sodium pyruvate and 0.05% gentamicin in distilled water. *In-vitro* fertilization media were: Tyrode's lactate medium with HEPES (solution 1) and Tyrode's lactate medium without HEPES (solution 2). Solution 1 contained 1.14×10^{-1} mol NaCl; 3×10^{-3} mol KCl; 2×10^{-3} mol $NaHCO_3$; 3.4×10^{-4} mol hydrated $N_2H_2PO_4$; sodium lactate; HEPES; 2×10^{-3} mol $CaCl_2$; 5×10^{-4} mol $MgCl_2$ with pH of 7.3, while the solution 2 contained 1.14×10^{-1} mol NaCl;3×10^{-4} mol KCl;2.5×10^{-2} mol $NaHCO_3$; 3.4×10^{-4} mol $NaH_2PO_4.H_2O$; sodium lactate; mol HEPES; 2×10^{-3} mol $CaCl_2$;5×10^{-4} mol $MgCl_2$. For the preparation of Tyrode's albumin lactate medium (TALP) with HEPES, to 100 ml of solution 1 was added: BSA (fraction V); sodium pyruvate and gentamicin. For the preparation of Tyrode's albumin lactate medium (TALP) without HEPES, to 100 ml of solution 2 were added BSA; sodium pyruvate, gentamicin and heparin. Modified medium for sperm preparation (DPBS) contained 1.81×10^{-1} mol NaCl; 4×10^{-3} mol KCl; 2×10^{-3} mol KH_2PO_4; 12.64 mol $MgCl_2$; 1.06×10^{-2} mol Na_2HPO_4; sodium pyruvate; $4.55\times10^{+2}$ mol glucose; heparin; penicillin and streptomycin. For above media, the pH was adjusted between 7.3-7.4. Sterile culture plates were used for IVM, IVF and IVC (*in-vitro* maturation, fertilization and culture respectively) procedures. The X-plates were labeled as control and treated.

Retrieval and in-vitro maturation of oocytes: Immature oocytes were retrieved through follicular aspiration using an 18 gauge needle attached to a 10 ml disposable syringe containing small quantity of OCM. The follicular fluid along with OCM was collected in 20 ml glass beaker. Five milliliter of fluid was placed in culture dish and oocytes were searched under phase contrast microscope (Nikon). Oocyte selection criteria were: presence of germinal vesicle, homogeneous darkly pigmented cytoplasm and intact cumulus cells compactly arranged around zona pellucida.

OMM (200 µl) was pipetted into each well of X-tissue culture plates (Falcon, UK) and overlaid with liquid paraffin. Plates were acclimated for 3-4 hrs at 38.5^{O}C in a humidified incubator with 5% CO_2 in air before placement of oocytes. The number of oocytes per microdrop was kept to a minimum of 45-50.

Treatment with malathion: Cumulus oocyte complexes (germinal vesicle stage) were then exposed for 24 hrs to four increasing concentrations (1.56 µmol, 3.12 µmol, 6.25 µmol, and 12.5 µmol) of malathion under above culture conditions.

Preparation of epithelial cells for co-culture: Oviducts were placed in TCM-199 and cut surgically into 5-8 cm

pieces. Epithelial cells were then separated by pipetting. The microdrops containing bovine oviductal epithelial cells (BOEC) were placed in separate X-plates, overlaid with paraffin and acclimated for 24 hrs prior to co-culturing with 1-cell zygote.

Sperm preparation and in-vitro fertilization: For *in-vitro* fertilization, 5 ml HEPES-TALP contained in each well of 6-well culture plates was acclimated for 3 hrs in a 5% CO_2 incubator before *in-vitro* fertilization. The oocytes that attained metaphase II (MII) in both control and treated groups were placed separately in 5 ml of HEPES-TALP. Finally, matured oocytes were shifted in microdrops of IVF-TALP under liquid paraffin. Before insemination, oocytes were again acclimated for 2 hrs. For *in-vitro* capacitation of sperms, cryopreserved sperms were thawed at 37^{O}C and centrifuged. After centrifugation, dilutions were made to achieve sperm concentration of 2 x 10^6/ml in IVF-TALP. Matured oocytes were then inseminated with sperms.

In vitro culture and subsequent embryonic development: After 24 hrs of co-incubation of sperms and oocytes, the presumptive zygotes were shifted to 5 ml pre-warmed washing medium, excessive cumulus cells and sperms were removed from surface of zygotes mechanically, through pipetting. Hyaluronidase, was used in washing medium (HEPES-TALP). The rate of fertilization was assessed by the presence of second polar body in perivitelline space and formation of male and female pronuclei. The microdrops of 200 µl of IVCM (*in-vitro* culture medium) with BOEC were formed in X-plates under paraffin oil, re-acclimated in incubator at 37^{o}C and at 98% relative humidity. The presumptive zygotes were washed and shifted into IVCM microdrops. The numbers of zygotes were kept 25 in each microdrop. The embryonic development was observed at subsequent days using the phase contrast inverted microscope (Nikon).

Observations: Immature oocytes; oocytes at meiosis-II; insemination or IVF; 1-cell zygote; 2-4 cell embryo; 8-16-cell embryo; morula; hatched blastocyst; blastocyst with inner cell mass were examined morphologically. Five replicates were run for each set of observations throughout the study.

Statistical analysis: Data were analyzed using pad prism (Version Inc., USA). The control and treated groups were compared using student's t-test while time-related survival was analyzed through linear regression. $P < 0.05$ was considered significant difference.

RESULTS

Oocyte retrieval: Of total 741 ovaries, n=2983 follicles were aspirated (average 4.03 follicles per ovary), of which 1563 immature eggs or oocytes were retrieved giving 52.39% retrieval success. On average 2.11 oocytes per ovary were obtained.

Cumulus expansion: For control COCs, mean increase in the expansion was 222.85±3.57 µm to 325.11±5.08 µm within 24 hrs of culturing (P = 0.0082). Where COCs were treated with 1.56, 3.12, 6.25 and 12.5 µmol malathion, the mean expansion of cumulus cells increased significantly (P=0.018; P=0.0025; P=0.048; P=0.1069 respectively, Table 1). Regression analysis showed a significant (P=0.004) decrease in cumulus expansion in dose dependent manner (b = -11.24 ± 1.41; $F_{(1, 3)}$ =63.89).

Oocyte diameter: Mean diameter for control oocyte was 144.58±0.95 µm at '0' hours that increased to 152.26±0.90 µm after 24 hrs of culturing (P=0.989). For the treatment groups mean oocyte diameter showed non-significant difference from control (P=0.57; P=0.97; P=0.65; P=0.95 respectively for 1.56, 3.12, 6.25 and 12.5 µmol malathion doses) (Table 1).

Nuclear competence: The nuclear status was evaluated at '0' hrs germinal vesicle stage (GV) and at 24 hrs (MII stage) of culture. For control COCs, 65.39% (170/260) attained MII stage after '24' hrs of culture. The COCs exposed to different doses of malathion at zero hrs demonstrated decreased nuclear competence 62.26 % (160/257), 60.56% (152/251), 57.48% (146/254) and 58.11% (147/253) at low and high doses. The regression analysis showed significant dose dependent decrease (b = -1.93 ± 0.36; $F_{(1,3)}$ = 27.34, P = 0.0136) in the maturation rate of oocytes.

Embryonic development: Total fertilized zygotes, and those that successfully achieved cleavage and developed to later stages are given in Table 2. In the treated groups, the post fertilization and embryonic development of pre-exposed COCs with malathion showed dose dependent drop in 1-cell stage zygote, 2-4 and 8-16 cell stage embryos, morulae, hatched blastocysts and blastocysts with inner cell mass. When the control group was compared with group treated with highest tested dose of malathion (12.5 µmol), the rate of fertilization, 2-4 cell and 8-16 cell stage embryos, morulae and blastocysts with inner cell mass showed non-significant differences. Only the hatched blastocysts however showed significant (P=0.0114) decrease than the control (Table 2).

The regression analysis showed non-significant (b = -2.35 ± 1.26; $F_{(1, 3)}$ = 3.53, P = 0.1568) difference for 1-cell zygotes in control and treated groups. A non-significant (b = -0.94 ± 0.493; $F_{(1, 3)}$ = 3.644, P=0.1523) difference was observed for 2-4 cell stage embryos while non-significant (b = -1.115 ± 0.434; $F_{(1, 3)}$ = 6.588, P = 0.0827) deviation was shown by 8-16 cell stage embryos. The non-significant (b = -1.321 ± 0.47; $F_{(1, 3)}$ = 7.891, P = 0.0673) difference was observed for the development of

morulae. There was statistically significant (b = -1.633 ± 0.241; $F_{(1, 3)}$ = 45.95, P=0.0066) deviation expressed by the hatched blastocysts in dose dependent manner. While the formation of blastocysts with inner cell mass showed non-significant (b = -0.805 ± 0.2887; $F_{(1, 3)}$ = 7.774, P= 0.0685) difference in dose dependent fashion.

Morphological observations: Treatment with malathion doses led to abnormal release of polar bodies of variable size in the maturational stage of oocyte (Fig. 1 B-D) as compared to normal formation the polar body and oocyte maturation (Fig. 1A). Prominent degenerative changes representing massive vacuolation, fragmentation, disrupted zona pellucid and disintegrated ooplasm were readily noticeable at 1-cell zygote (Fig. 1F-H) as compared to the normal embryo formation from the control non-treated oocyte (Fig. 1E).

DISCUSSION

Extensive use of pesticides in agricultural practices has adversely affected the environment, human and animal health. Many of the pesticides are notorious for being persistently present in the soil, water and air to finally bioaccumulate in plants, animals and (Amer *et al*., 2001) human bodies. Use of organophosphate pesticides is still on the rise raising major concerns about the health status of populations living in such agricultural areas.

Although little has been studied as regards the embryo toxicity on exposure to pesticides, studies carried out in the past have indicated alarming situation in the context of infertility, poor fertilization and birth defects (Smith *et al*., 1997; Fuortes *et al*., 1997; Greenlee *et al*., 2003). In contrast to humans, livestock embryos are perhaps directly exposed to pesticides through contaminated fodder and drinking water. Once in the animal bodies these pesticides can lead to production of abnormal gametes and defective embryos (Amer *et al*., 2001; Yamauchi, et, al., 2003). Nili Ravi Buffalo (*Bubalus bubalis*) is an economically important animal in Asia owing to its milk and meat production (Madan *et al*., 1996). Heavy use of pesticide in Pakistan's agricultural fields and horticultural farms (Khan, 1998) has become a major threat to buffalo health and survival (Pardio *et al*., 2000). The utilization of immature oocytes from slaughtered animals is an effective non-invasive *in-vitro* toxicological model to study early stage embryo toxicity on a wide range of insecticides and pesticides.

Presently, we tested *in-vitro* embryotoxicity of malathion on Nili Ravi buffalo. Our results demonstrated that malathion affected the cumulus expansion at the highest dose, oocyte diameter remained unaffected, while nuclear competence was compromised in a dose-dependent manner. Those oocytes that survived or attained maturation after 24 hrs exposure to the toxicant were followed from fertilization through to the blastocyt stage. Embryo viability at all successive developmental stages was compromised whereby a dose-dependent decrease was observed in each developmental stage. Morphologically, degeneration, vacuolation, ooplasm disintegration and abnormal polar body release were the outcome.

Our results demonstrated that cumulus expansion was arrested at the highest tested dose. Similarly, oocyte maturation was decreased with each increasing dose of the pesticide. It appears that malathion might have caused disruption of meiosis. Yin *et al*. (1998) have shown mitotic spindle disruption on exposure of oocytes 3 hrs to 50 µg/ml Trichlorfon. Madrigal-Bujadiar *et al*. (1993) and Doherty *et al*. (1996) also reported that the exposure of somatic cells to organophosphate leads to mitotic delay. As we also observed that cumulus expansion was compromised, hence maturation of oocyte could not have supported. Ducolomb *et al*. (2004 and 2008) used extraordinary high doses of malathion and demonstrated that exposure of porcine oocytes to malathion led to decreased *in-vitro* fertilization, further development and morulae formation, the cleavage decreased to 31% while morula development decreased to 9% at 500 µmol dose. It should be noted that we observed these changes at very low doses implicating that malathion could be highly toxic even at low doses. In the present study, malathion application did not alter oocyte diameter. As nuclear competence was significantly decreased dose-dependently, malathion application could therefore be devastating in the sense that it decreased the availability of viable female gametes for subsequent embryonic development. Bonilla *et al*. (2008) used 250 µmol malathion and found significant decrease of fetal mouse oocytes survival after 24-hrs.

Currently, of 170 oocytes in the control, only 7% reached the blastocysts with inner cell mass stage, oocyte retrieval rates have been comparatively more successful (2.11/ovary) as compared to the previous reports where oocytes obtained per ovary ranged from 0.4 to 1.82 (Totey *et al*.,1992; Yousaf and Chauhan, 2003). In the present study the cleavage was 75.78% and blastocyst formation was 14.06% comparable with the findings of Hakan *et al.,* (2004) and blastocyst formation was 16.1% in cattle oocyte *in-vitro*. However, it has already been reported earlier that following *in-vitro* maturation and fertilization, only about 30-40% bovine oocytes ever reach the blastocysts stage (Lonergan *et al*., 1994). Treatment with malathion caused further decrease in this number in a dose-dependent manner to 3% in the highest tested dose. In order to ensure uniformity we used TCM-199 with nutritive additives throughout the cultures. Moreover, the technique adapted here was co-culturing of oocytes with BOEC to enhance the nutritional requirements of embryo and detoxification of

the external medium (Rexroad, 1989; Abe and Hoshi, 1997).

Evaluation of subsequent zygote formation, 2-4 cell 8-16 cell stage, morula formation and blastocysts with inner cell mass, oocytes pre-exposed to malathion doses showed non-significant differences from control oocytes. However number of hatched blastocysts decreased significantly and this decrease was 7%. This could be due to that extremely low doses of malathion were used. It should be noted that doses similar to our have been used for atrazine on fish (*Oreochromis niloticus*) and significant nuclear abnormalities were found indicating the mutagenicity and genotoxicity of this herbicide (Ventura *et al*., 2007). Endocrine disrupting potential of malathion (Kime, 1995) behind the normal growth of oocytes and embryos as observed presently cannot be ruled out, since supplementation of hormones in culture media supports the IVM of immature oocytes.

In-vitro cultured non-invasive morphological studies of preimplantation buffalo embryos for evaluation of risk assessment of embryo toxicity caused by the insecticides is an invaluable technique. To the best of authors' knowledge, the present is the first report on early embryo toxicity of malathion using the buffalo oocytes. Although we did not find any significant difference in the development of 2-4 cell 8-16 cell stage, morula formation and blastocysts with inner cell mass except the hatched balstocyts, the comparison is difficult to be made since no data exist the effect of malathion on immature oocytes and subsequent developmental stages. However, the present findings do indicate that malathion can be highly toxic to embryos developing *in-vitro* even when applied at low dose concentrations. We suggest that buffalo embryos can prove to be a reliable and cost effective *in-vitro* model system to evaluate the toxicity of agrochemicals. Moreover, our study also indicates that similar kind of abnormalities may be encountered for human subjects in the occupational set up, especially workers in the pesticide industry and farmers who apply these pesticides to their fields.

In future, detailed information can be gathered as regards damage to the DNA content and cellular physiology of sperms, eggs and preimplantation buffalo embryos using the invasive research means along with non-invasive methodologies. .

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