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EFFECTS OF ACETAMIPRID, METHOMYL, AND DELTAMETHRIN ON STEROIDOGENESIS IN CULTURED BOVINE LUTEAL CELLS

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ABSTRACT

Acetamiprid, methomyl, and deltamethrinare widely used insecticides in agriculture and industry; thus, livestock may receive substantial exposure to such substances. This study was designed to evaluate the effect of these agents on bovine luteal cell steroidogenesis.Cells were incubated with acetamiprid (0.1 mM, 1 mM, or 10 mM), methomyl (0.1 mM, 1 mM, or 10 mM), or deltamethrin (0.01 mM, 0.1 mM, or 1 mM) for 5 days. Incubation with 1 mMacetamiprid resulted in significantly reducedpro-gesterone production by day 5. Incubation with 1 mM methomyl had no effect on progesterone pro-ductionby day 3 but did result in significant inhibi-tionby day 5. Incubation in 0.1 mM and 1 mMdelta-methrin resulted in significant reduction on both days 3 and 5. All three insecticides diminish luteal progesterone synthesis, with rank order methomyl<acetamiprid<deltamethrin.Excessive exposure to these agents, particularly deltamethrin, may weaken the breeding potential of bovine livestock.

KEYWORDS:
Acetamiprid; methomyl; deltamethrin; bovine luteal cells; progesterone

INTRODUCTION

Insecticides such as acetamiprid, methomyl, and deltamethrin are usedthroughout the worldto control insect pestsin agriculture and industry.However, prolonged exposure of farm animals to theseagents causes manyadverse effects, such as geno-toxicity[1], oxidative damage[2], and impaired fertility in both males[3] and females[4].

Acetamiprid is a neonicotinoidinsecticidewidelyused against soil and plant insects. A previous studyby Zhang et al. [5] reported that in vivo exposure of male mice to acetamipriddecreased body weight and the weight of testosterone-responsiveorgans, such as the testis, epididymis, seminal vesicle, and prostate. The study also reported a significant decrease in serum testosterone as well as sperm count, viability, and motility after exposure. Even short-term exposure of mouse germ cells to acetamiprid led to abnormal sperm morphology [6]. In addition, Gu et al. [7] reported that inutero exposure of mice to acetamipriddisrupted early embryonic development.

Methomyl is a carbamate insecticide usedagainst several insect classes including coleoptera,diptera, hemiptera, homoptera, and lepidoptera. It is also used as an acaricide to control ticks and spiders. However, methomyl is classified as a “restricted use” pesticide by the United StatesEnvironmental Protection Agency (EPA) because of its high toxicity to multiple non-target species, including humans[8].Chronic exposure to methomylinduced testicular damage in male rats as evidenced by significantly reduced serum testosteroneand lower testis, vesiculaseminalis, and prostate gland weight[9,3]. Shanthalatha et al. [4] reported that the administration of methomyl to female rats for 90 days decreasedovary, uterus, and fallopian tube weight;shortened the estrous cycle; and reducedthe numbers of small, preantral, antral, and preovulatory follicles.

Deltamethrin is a pyrethroid insecticide usedextensivelyin agriculture and home pest control. Previous invivo and invitro studies have demonstrated deleterious effects on the male reproductive system. Abdallah et al. [10] reported a significant decrease in rat sperm motility and viability and an increase in abnormal spermatozoa after in vitro incubation with deltamethrin at 37°C.Furthermore, the oral administration of deltamethrin to male mice for 21 days was associated with significantly decreased sperm count, motility, and viability [11].The administration of deltamethrin also decreased serum testosterone in rats [12,13].

Although many studies have reporteddeleterious effects of acetamiprid, methomyl, and deltamethrin on male and female rodent reproductive tissues,

there is currently no data regarding the effects of these insecticides on cattle, among the most valuable livestock. In this study, we examined the effects of these agents on steroid production in bovine luteal cells.

MATERIALS AND METHODS

Sample collection. Bovine ovaries were collected from a local slaughterhouse immediately after slaughter and transported to the laboratory within 45 min.Corpora lutea were judged to be at mid-cycle by the criteria of Ireland et al. [14]. Each corpus luteum was dissected from the ovary before cell isolation.

Isolation of cells. The dissected tissues were minced into small pieces with a single edge razor blade. Excess blood cells were removed by washing minced tissue with culture media (Dulbecco modified Eagle and Hams F-12) through a 100-µm falcon cell strainer (BD Biosciences, San Jose, CA, USA).Cells were isolated from the luteal tissue under sterile conditions as described previously [15] with minor modifications. In brief, luteal cells were dissociated by 4 successive 1-h incubations in aerated (95% O_2 and 5% CO_2, 2 min) culture medium containingcollagenase (0.03%, Type V), DNase (0.005%), bovine serum albumin (BSA, 0.5%), and antibiotic–antimycotic solution (1%) in an Erlenmeyer flaskunder rotation (90 cycles/min) at 37°C in a shaking water bath (Julabo, Labortechnik GmbH, Seelbach, Germany). The pooled supernatant from 4 incubations was filtered through a cell strainer supported by a sterile glass funnel into a sterile falcon tube (50 ml) to remove undigested tissue fragments. The filtrate was then centrifuged (400×g) for 5 min. The centrifugation was repeated twiceto wash off the chemicals (including collagenase, BSA) and cell debris.Cell viability was determined by the trypan blue exclusion test.All chemicals used for cell isolation and incubation were purchased from Sigma-Aldrich (Munich, Germany).

Identification of steroidogenic luteal cells. Luteal tissue consists of both non-steroidogeniccells (including fibroblast, macrophages, and endothelial cells) and steroidogenic cells.Steroidogenic cells constitute only 30% of the total luteal tissue cell numberin the mid-luteal phase[16]. The steroidogenic cells can be identified by staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) activity, which is essential for luteal progesterone biosynthesis[17].For this purpose, 200 µL of cell suspension was fixed in paraformaldehyde (1%) for 20 min at 37°C. After centrifugation, the cell pellet was resuspendedin 200 µl of staining solution [phosphate buffered saline (PBS), containing 0.1% BSA, 1.5mM NAD, 0.25mM nitro blue tetrazolium, and 0.2mM5β-androstene-3β-ol-17 one (prepared from 8mM stock solution in ethanol)] and incubated in the dark at 37°C for 4 h,as described previously [18].

Incubation of cells. The isolated luteal cells were cultured in 6-well cell culture plates (Corning Life Sciences, Cat no: 3516) in CO_2 incubator (BINDER GmbH, Germany). Each well included 100 µl cell suspension and 2 ml of the culture medium (Dulbecco modified Eagle and Hams F-12, 1:1), containing fetal bovine serum (10%) andantibiotic–antimycotic solution (1%). Cells were incubated without treatment for 18 h andthen with serum-free media containing 1% ITS premix (10 mg/ml insulin, 0.55 mg/ml transferrin, and 5µg/ml sodium selenite) plusthe specific insecticide or vehicle concentration for 5 days [0.1 mM, 1 mM, and 10 mM acetamiprid dissolved in ethanol; 0.1 mM, 1 mM, and 10 mM methomyl dissolved in ethanol; and 0.01 mM, 0.1 mM, and 1 mM deltamethrin dissolved in dimethyl sulfoxide (DMSO)]. As cholesterol isthe precursor forprogesteronebiosynthesis[19], all treatment groups andvehicle controls were treated with 22(R)-hydroxycholesterol (22R-HC, 10 µg/ml). During insecticide incubation, the medium was replaced every 48 h and the retrieved medium was stored at −20°C until steroid analysis by radioimmunoassay (RIA). Each insecticide dose was repeated 4 times.

Monitoring culture quality. In addition to Apart from the experimental groups, 3 separate culture dishes of untreated control cells were incubated in parallel to monitor culture quality. After 1, 3, and 5 days in culture, cells were stained for 3β-HSD activity and examined on an inverted microscope (Olympus, Tokyo, Japan) to evaluate cell growth, cell attachment, cell-to-cell contact, 3β-HSD enzyme activity, and cell viability during the culture periods (Figure 4).

Progesterone assay. Progesterone levels in the culture media were determined using a commercial RIA kit (Beckman Coulter, Inc. Czech Republic), following the manufacturer's instruction. Intra- and inter-assay coefficients of variations were below or equal to 8.15% and 8.66%, respectively. Analytical sensitivity was 0.03 ng/ml and recovery was between 80% and 106%.

Statistical analysis. Statistical analyses was conducted using the Statistical Package for the Social Sciences(SPSS version 14.0 for windows). Differences between control and treated groups were assessed by ANOVA followed by Duncan test for multiple comparisons. Differences were considered significant at $p<0.05$. All experimental data are expressed as mean ± standard error of the mean (SEM) of 4 independent experiments for each

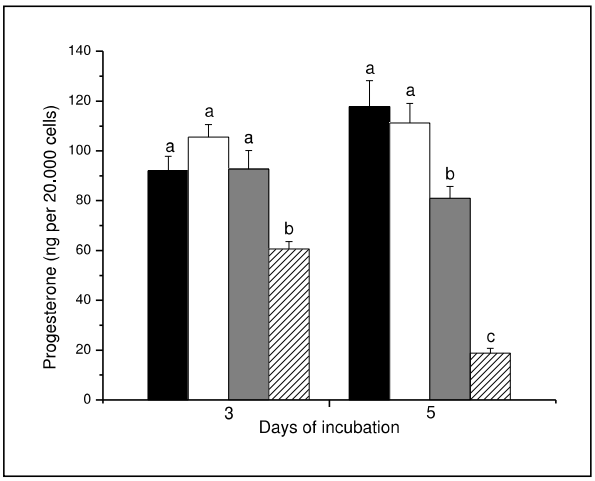

FIGURE 1
Effect of acetamiprid on bovine luteal cell steroidogenesis. Control (■); 0.1 mM acetamiprid (□); 1 mM acetamiprid (▩); 10 mM acetamiprid (■). Within each day, groups with alternate letters (a vs. b) above the error bars differ significantly (P < 0.05).

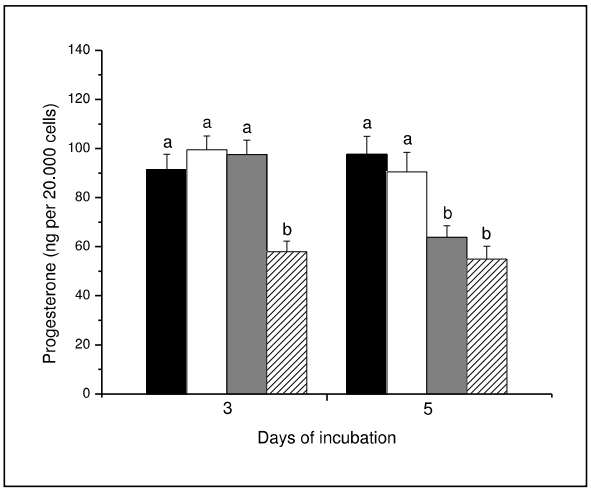

FIGURE 2
Effect of methomyl on steroidogenesis of bovine luteal cells. Control (■); 0.1 mM methomyl (□); 1 mM methomyl (▩); 10 mM methomyl (■). Within each day, groups with alternate letters (a vs. b) above the error bars differ significantly (P < 0.05).

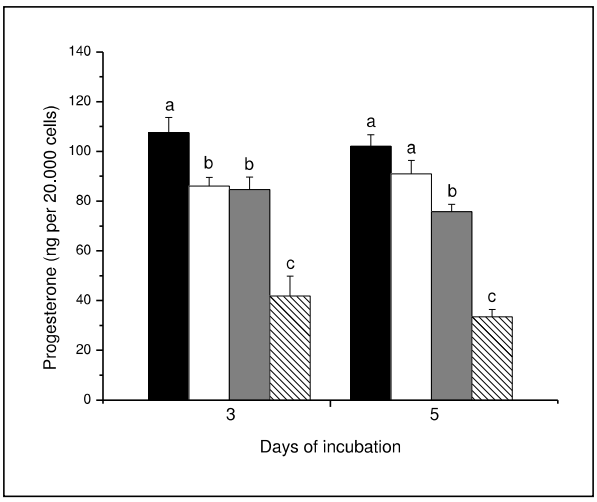

FIGURE 3
Effect of deltamethrin on bovine luteal cell steroidogenesis. Control (■); 0.01 mM deltamethrin (□); 0.1 mM deltamethrin (▩); 1 mM deltamethrin (■). Within each day, groups with alternate letters (a vs. b, a vs. c, b vs. c) above the error bars differ significantly (P < 0.05).

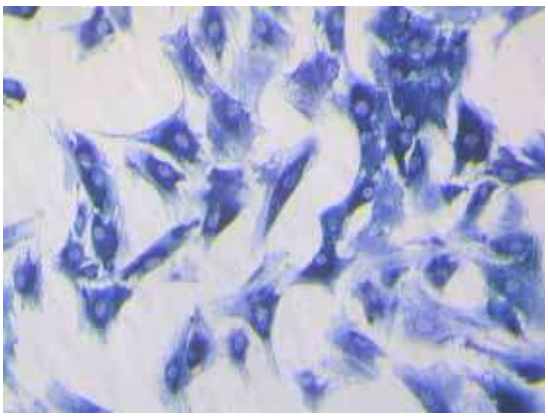

FIGURE 4
Bovine luteal cells stained for 3β-hydroxysteroid dehydrogenase activity after day 5 in culture.

treatment protocol. Progesterone synthesis is expressed as ng/20000 cells.

RESULTS

Effect of acetamiprid on progesterone synthesis. Luteal cells were incubated with 3 concentrations of acetamipridor equal volumes of vehiclefor 5 days.There was no significant difference in progesterone productionbetween control and 0.1 mM acetamiprid treatment groups on days 3 and 5, whereas1 mM resulted in significant reduction ($p<0.05$) on day 5. In contrast, cells treated with 10 mMacetamipridexhibited a34% decrease in progesterone production on day 3 and 84% decrease onday 5 compared with controls (Figure 1).

Effect of methomyl on progesterone secretion. Treatment with 1mM methomyl had no effect

onprogesterone production by day 3 but resulted in significant suppression by day 5 ($p<0.05$). The addition of 10 mMmethomyl resulted in a significantsuppression ($p<0.01$) on both days 3 and 5, whereas0.1 mM methomyl did not reduce progesterone production compared with control at either time (Figure 2).

Effect of deltamethrin on progesterone secretion. The addition of 0.01 mM deltamethrin did not alter progesterone production by day5.On the contrary,both 0.1 mM and 1 mM deltamethrin resulted in significant reductions ($p<0.05$)in progesterone accumulation on both days 3 and 5 of incubation.Luteal cells treated with 1 mM deltamethrin exhibited a61% decrease by day 3 and 67% decrease by day 5 (Figure 3).

Monitoring culture quality. To assess cell growth and cell–cell attachment, parallel untreated cultures where stained for 3β-HSD enzyme activity and examined by light microscopy. Cells became elliptical after attachment to the culture plate and extended membrane protrusions to contact neighboring cells.The cell nuclei become visible because oflower staining intensitythanthat of the cytoplasm (Figure 4). When poor cell growth and attachment were observed in any stage of incubation in untreated control cultures, incubation was stoppedand excluded from the study. These incubations were repeated later by preparing fresh luteal cell culture.

DISCUSSION

In mammals, acetamiprid, methomyl, and deltamethrin have a wide range of deleteriouseffects on theimmune system[20], nervous system [21,22], female reproductive system [4], and male reproductive system[9].Although several studies have reported effects of acetamiprid, methomyl, and deltamethrin onsteroidogenesis in the testis, there is currently no data on the effects of these insecticides on luteal cell steroidogenesis. The present study demonstratingreduced steroidogenesis by cultured bovine luteal cellsin vitro suggests that the exposure of bovine livestock in the field may reduce breeding by impairing female fertility.

Although all three insecticides used in the present study reduced luteal cell steroidogenesis, deltamethrin was the most damaging. Methomyl at 1 mm or 10 mM was required to significantly reduce progesterone synthesis within 3−5 days and the concentration-dependence was modest at the latter incubation time. Nonetheless, methomyl does appear to have broad effects on mammalian male reproductive tissue, as oral administration of 1.0 and 0.5 mg/kg body weight for 65 consecutive days [3] and chronic exposure [9] significantly decreased serum testosterone in male rats. Acetamiprid at 1 mM resulted in significant reduction of progesterone only on day 5, whereas 10 mM was required for a substantial reduction after 3 days' exposure. These findings do, however, provide indirect support for a previous study showing that acetamiprid administration (30 mg/kg body weight for 35 days) to male mice decrease serum testosterone by 40% [23].In contrast to methomyl and acetamiprid, incubation with 0.1 mM deltamethrin inhibited steroidogenesis, even in the presence of the progesterone precursor22R-hidroxycholesterol(10 µg/ml) to maintain constant synthesis [24].Deltamethrin also reduced serum testosterone in rats [12,13].

In conclusion,acetamiprid, methomyl, and deltamethrin diminishedbovine luteal cell steroidogenesis, with deltamethrin showing the highest potency. In general, the suppressive effects increased with dose and exposure time. Therefore,field applicationshould be conducted with caution to limit the exposure offarm animals.

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