In Vitro Evaluation of the Reldan 22® Insecticide Effects on Swine Oocyte Maturation

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Abstract

Chlorpyrifos (Reldan 22®) is an widely used insecticide for the control of insect pests in agriculture and in residential areas. It is classified as moderately toxic by the United States Environmental Protection Agency and has been quantified in human biological fluids. Our aim was to test the toxicity of Chlorpyrifos (Reldan 22®) and investigate its effects in an *in vitro* model using swine oocyte maturation. Swine oocytes from ovaries harvested in a commercial slaughterhouse were cultured for 44-45h in M199 supplemented with the following Reldan 22® concentrations: 0.1, 0.5, 1 or 2 µl/ml. *Cumulus oophorous* expansion was assessed and oocytes were denuded and stained with 1 µg/ml fluorescein diacetate to estimate viability. Afterwards, oocytes were fixed in a 60% methanol in DPBS solution and stained with 50 µg/ml propidium iodide to observe the DNA stage. Our research shows that the Reldan 22® insecticide stimulated *cumulus* expansion to an extent but reduced oocyte viability which was accompanied by an increase in the number of immature oocytes and a decrease in the percentages of gametes that resumed meiosis. This leads us conclude that its presence in the oocyte environment is toxic for development at concentrations 0.5, 1 and 2 µl/ml.

Keywords: C*hlorpyrifos, maturation, oocyte, pig, toxicity*

INTRODUCTION

Chlorpyrifos (O,O-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothionate) is a broad spectrum organophosphate insecticide that has been classified moderately toxic compound by the United States Environmental Protection Agency.

It is widely employed in products like Reldan 22® to control insect pests in agricultural fields (Li *et al.*, 2015) but also in residential areas (Pollakova *et al.*, 2012). It affects the nervous system by irreversibly inhibiting the activity of cholinesterase which is necessary for the functioning of the central nervous system (Klaassen, 2001). This results in neurotoxicity in animals and humans (Zhao *et al.*, 2006) but also in birds, bees and aquatic life (http://www.beyondpesticides.org). In men, DNA damage in sperm, decreased testosterone and oestradiol, decreased sperm concentration and sperm motility and have been reported after exposure to Chlorpyrifos (Cal EPA, 2008).

Exposure to pesticides in mammals has been associated with reproductive cycle disturbances, reduced fertility, prolonged time-to-pregnancy, spontaneous abortion, stillbirths, structural abnormalities, altered growth and functional deficiencies (Bretveld *et al.*, 2006; Tiemann, 2008). Organochlorine pesticides decreased the rate of normal bovine oocyte maturation *in vitro* (Alm *et al.*, 1998) and exerted toxicity towards mouse embryos (Alm *et al.*, 1996). Their presence also altered formation of the first meiotic spindle and extrusion of the first polar body in mouse oocytes (Picard *et al.*, 2003). Although Chlorpyrifos is widely used there are fewer studies regarding its effects on oocyte and embryos.

Chlorpyrifos has been found in human biological fluids and concentrations in sera, milk and saliva range between parts per trillion to parts per million (Drevenkar *et al.*, 1994; Sanghi *et al.*, 2003; Whaytt *et al.*, 2004).

Given that the use of porcine and bovine models for testing chemicals has increased recently (Lazzari *et al.*, 2008; Santos *et al.*, 2014) we designed an experiment to test the toxicity of several Chlorpyrifos concentrations and investigate its effects on maturation of swine oocytes.

MATERIALS AND METHODS

Oocyte collection and maturation

All chemicals were purchased from Sigma-Aldrich unless otherwise specified. The medium used for harvest was M 199 supplemented with 0.1 g/l glutamine, 5.958 g/l Hepes , 100 IU/ml penicillin and 100 mg/ml streptomycin. For oocyte maturation M 199 was supplemented with 10 IU/ml Chorulon (Intervet), 10 IU/ml Folligon (Intervet), 10% foetal bovine serum, 100 IU/ml penicillin and 100 mg/ml streptomycin. Reldan® 22 (Dow-Agroscience) was dissolved in 95% ethanol to prepare a stock solution which was mixed with maturation medium to prepare the following concentrations 0.1 µl/ml, 0.5 µl/ml, 1 µl/ml and 2 µl/ml. Oocytes with a uniform ooplasm and compact *cumulus* cell mass were washed in harvest medium and then placed in 40 µl droplets of maturation medium containing Chorulon and Folligon together with the various Reldan® 22 concentrations. These were covered in paraffin oil and incubated for 24-25 h at 38°C, in an atmosphere with 5% CO_2 in air. Afterwards oocytes were moved to a medium that had not been supplemented with hormones and cultured for an additional 20 h under the same conditions.

Assessment of *cumulus oophorus* expansion

The expansion of *cumulus* cells was assessed by viewing the *cumulus* oocyte complexes (COCs) under an IX51 Olympus microscope (Olympus) and grading them by a subjective scoring method (Downs *et al.*, 1989). No response was scored as 0, minimum observable response as 1, expansion of outer *cumulus*-enclosed oocyte layers as 2, expansion of all *cumulus*-enclosed oocyte layers except the corona radiata as 3, and expansion of all *cumulus*-enclosed oocyte layers as 4. The number of oocytes in each expansion stage was counted and expressed as percentages.

Evaluation of oocyte viability and nuclear status

Following the assessment of *cumulus* expansion COCs were transferred to PBS supplemented with 5 mg/ml bovine serum albumin and mechanically denuded using a micropipette. Then oocytes were transferred to PBS supplemented with 1 µg/ml 3', 6' fluorescein diacetate (FDA), incubated for 7 minutes and viewed under ultraviolet illumination with an IX51 Olympus microscope. Viable oocytes were FDA positive and appeared green while nonviable ones were not coloured. Oocytes in both situations were counted and expressed as percentages.

After viability assessment oocytes were processed to investigate polar body formation. They were fixed using a combination of 60% methanol in PBS for at least 1 h at 4°C. Oocytes were then incubated for 7 minutes in PBS containing 50 µg/ml propidium iodide (PI), mounted between slide and cover glass and observed under ultraviolet light. DNA appeared bright red and oocytes were classified as immature (germinal vesicle), resumption of meiosis (germinal vesicle break down, metaphase I and anaphase I), mature (telophase I and metaphase II) or degenerated (Alm *et al.*, 1998). The number of oocytes in each development stage was counted and expressed as percentages.

Statistical analysis

For all experiments one-way ANOVA with the Tukey-Kramer post test was performed using GraphPad InStat version 3.05 for Windows 95 (GraphPad Software, San Diego California, USA). The values were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

The effects of Reldan 22® were tested after swine oocyte maturation. The first investigated aspect was *cumulus oophorus* expansion (Tab. 1).

In the presence of 0.1 µl/ml, 0.5 µl/ml or 1 µl/ml Reldan 22® *cumulus* cells expanded beyond the 0 stage. Most of those COCs probably progressed to the second expansion stage where with the same concentrations percentages were higher than the

control. The percentages of COCs that expanded to reach stage 2 were lower than the control for all Reldan 22® concentrations. However, for the last two stages the situation changed. Maturation medium supplemented with 1 µl/ml Reldan 22® induced the expansion of a significantly larger percentage of COCs than the control and 0.1 µl/ml. The overall dynamic was similar for stage 4 with the same concentration having the highest percentage. Percentages of COCs with degraded cytoplasm and *cumulus oophorus* were similar for all insecticide concentrations with the exception of 2 µl/ml.

For a better assessment of the influence of Reldan 22® on *cumulus oophorus* expansion, the five stages were divided into two categories. The first group included the lower expansion stages 0, 1 and 2 while the second was made of the higher stages 3 and 4 (Tab. 2). According to Tao *et al.* (2004) oocytes scored as 3 and 4 can be considered mature.

Grouping expansion stages showed that the presence of Reldan 22® in the maturation medium significantly increased the percentages of COCs that reached higher expansion stages while it had a reverse effect for the other category. Percentages gradually increased together with the concentration up to 1 µl/ml and then decreased slightly.

When viability was analysed (Tab. 3) it became apparent that increasing Reldan 22® concentration had a negative effect on viability which decreased below the control. Between concentrations 0.1 µl/ml and 2 µl/ml the differences were significant.

The last aspect to be investigated was DNA evolution towards the second metaphase (Tab. 4). The addition of 2 µl/ml Reldan 22® to the maturation medium significantly augmented percentages of oocytes with degraded DNA. This can be linked with the high percentage of dead oocytes to indicate that the highest Reldan concentration is toxic to the cells.

A detailed analysis of nuclear status reveals that the effect of adding Reldan 22® to the maturation medium was a higher number of oocytes that remained in an early development stage. A rise in the insecticide concentration has also caused a decrease in the percentage of gametes that progressed to the second metaphase

Tab. 1. Expansion of COCs after maturation in medium supplemented with Reldan 22®

Treatment	Number of COCs	Degraded COCs (%)	COCs at each stage of *cumulus* expansion (%)				
			0	1	2	3	4
Ctrl	78	1.79±3.59	9.79±3.59 a	21.53±4.47	35.32±4.42	23.61±1.29 a	10.42±2.12
0.1 R	66	0.00±0.00	0.00±0.00 b	29.18±1.14	32.31±1.88	21.39±0.60 a	17.12±3.17
0.5 R	70	1.28±0.70	0.00±0.00 b	24.64±0.86	29.63±2.61	25.93±0.79 ab	18.52±3.60
1 R	51	1.85±1.01	0.00±0.00 b	24.07±2.68	21.43±3.28	33.60±4.60 b	19.05±1.30
2 R	63	2.78±1.52	2.78±1.52 ab	21.11±2.13	34.44±5.34	30.56±1.52 ab	8.33±3.49

Note: Values expressed are mean ± standard error of the mean (SEM) of triplicate measurements. Different letters between means within the same column denote significant differences ($P < 0.05$).

Tab. 2. COCs in the two expansion groups after maturation in medium supplemented with Reldan 22®

Treatment	Expansion groups (%)	
	0+1+2	3+4
Ctrl	64.19±3.03 a	34.03±1.87 a
0.1 R	61.49±2.98 a	38.51±2.98 ab
0.5 R	54.27±3.45 ab	44.44±3.28 ab
1 R	45.50±3.56 b	52.65±4.39 b
2 R	58.33±3.49 ab	38.89±4.24 ab

Note: Values expressed are mean ± standard error of the mean (SEM) of triplicate measurements. Different letters between means within the same column denote significant differences ($P < 0.05$).

Cumulus cells surround and communicate with the oocyte via paracrine factors and through gap junctions (Albertini *et al.*, 2001). Through these pass pyruvate (Paradis, 2009), ATP and glutathione (Cui *et al.*, 2009) and *cumulus* cells can arrest oocyte meiosis via generating oocyte maturation inhibitors (Chaning *et al.*, 1980). *Cumulus* cells undergo expansion induced by the pre-ovulatory luteinizing hormone (LH) surge (Assidi *et al.*, 2010). Therefore, *cumulus oophorus* cells play an essential role during *in vitro* maturation of oocytes (Tanghe *et al.*, 2002). The pesticide used in this experiment triggered expansion in a larger number of COCs probably by stimulating *cumulus* activity under the influence of LH or the exchanges between oocyte and *cumulus* cells.

In the bovine follicle communication between oocyte and *cumulus* cells is completely interrupted around the metaphase I stage (Hyttel, 1987). However, Brevini *et al.* (2004) have found that Aroclor-1254, a commercial polychlorinated biphenyl mixture can delay the closure of the gap junctions in porcine COCs and this may be the conduit used to transport pesticide into the oocyte during maturation. Oocyte response has also been found to be more profound in the presence of hormones such as FSH (Pocar *et al.*, 2006). The same could happen in the swine oocytes used in this study.

In somatic cells Chlorpyrifos can induce membrane and DNA damage and inhibit cell viability (Li *et al.*, 2015; Pollakova *et al.*, 2012). Nandi *et al* (2011) have found that Chlorpyrifos inhibited buffalo oocyte growth and development *in vitro* directly and through *cumulus* cells and also that the adverse effects of exposure extended to fertilization and embryonic development even after the removal of the pesticide. These results are similar to ours. It is interesting to point out that Chlorpyrifos doses used in the study of Nandi *et al* (2011) were similar to those observed in the biological fluids of humans (Casey, 2005). However, any extrapolation to *in vivo* situations should be done with caution and after further study.

CONCLUSION

Our research shows that the Reldan 22® insecticide with Chlorpyrifos as an active ingredient stimulated *cumulus* expansion to an extent but reduced oocyte viability which was accompanied by an increase in the number of immature oocytes and a decrease in the percentages of gametes that

Tab. 3. Oocyte viability after maturation in medium supplemented with Reldan 22®

Treatment	Number of oocytes	Live oocytes (%)	Dead oocytes (%)
Ctrl	74	88.79±3.38 ab	11.21±3.38 ab
0.1 R	76	93.68±1.98a	6.32±1.98 a
0.5 R	78	84.76±2.68 ab	15.24±2.68 ab
1 R	61	84.98±5.34 ab	15.02±4.14 ab
2 R	74	76.57±3.81 b	23.43±3.81 b

Note: Values expressed are mean ± standard error of the mean (SEM) of triplicate measurements. Different letters between means within the same column denote significant differences ($P < 0.05$).

Tab. 4. Nuclear status of oocytes after culture in medium supplemented with Reldan 22®

Treatment	Number of oocytes	Degenerated DNA (%)	Meiotic stage (%)		
			Immature (GV)	Resumption of meiosis (GVBD-AI)	Mature (TI-MII)
Ctrl	65	20.63±3.48a	11.75±4.73	33.49±4.54a	34.13±0.43
0.1 R	63	19.75±2.05a	12.86±4.13	30.86±4.13a	38.62±4.83
0.5 R	70	31.24±4.72ab	17.48±4.08	16.97±0.67b	34.32±0.28
1 R	57	25.08±1.36ab	20.47±1.25	25.08±3.50a	34.32±3.57
2 R	70	40.48±6.16b	7.14±3.91	27.38±3.08a	32.94±6.70

Note: GV, germinal vesicle; GVBD, germinal vesicle break down; AI, anaphase I; MII, metaphase II. Values expressed are mean ± standard error of the mean (SEM) of triplicate measurements. Different letters between means within the same column denote significant differences ($P < 0.05$).

resumed meiosis. This leads us to conclude that its presence in the oocyte environment is toxic for development at concentrations 0.5, 1 and 2 µl/ml.

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