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Purification and isolation of corticosteroidogenic cells from head kidney of rainbow trout (*Oncorhynchus mykiss*) for testing cell-specific effects of a pesticide

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Abstract

The teleost head kidneys contain corticosteroidogenic cells, chromaffin cells, lymphoid cells, and melanomacrophages. We have developed and validated a method using a Percoll density gradient and differential staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) to prepare fractions enriched with specific head kidney cell types. The proportion of steroidogenic cells to other cells in the head kidney was 1:8000 in rainbow trout, *Oncorhynchus mykiss*. To test the hypothesis that steroidogenic cells are more vulnerable to a pesticide than other cell types in the head kidney, head kidney cells were separated by a Percoll gradient and the steroidogenic cell-enriched fractions and lymphoid cell-enriched fractions were exposed to the pesticide endosulfan in vitro, and their functional integrity and viability were assessed. The effective concentration of the pesticide (EC50, concentration that inhibits 50% of the secretory response to ACTH) in the mixed head kidney cell preparation was similar to the EC50 in the fraction enriched with steroidogenic cells, but differences in viability were detected. The Percoll method for isolation of different cell types from the head kidney facilitated a study of cell-specific effects of a pesticide.

Keywords: Cortisol; Corticosteroidogenic cells; Percoll; Endosulfan; Pesticide; Fish

1. Introduction

Understanding the cell-specific mechanisms of toxicity strengthens the causal link between exposure and effects in toxicological studies, and helps us understand the biochemical, molecular and physiological processes that make specific cells vulnerable or resistant to toxicants. Cell-specific actions of toxicants have been linked to a wide range of effects, including developmental abnormalities in fish embryos (Blechinger et al., 2002), organ dysfunction (Ghosh et al., 2006) or endocrine disruption (Wojtowicz et al., 2000). Studies aimed at elucidating the cellular and molecular mechanisms of action of signaling molecules, toxicants or drugs, increasingly rely on cell cultures systems (Schirmer, 2006) which lend themselves to complex manipulations, easy replication and help us also address the ethical concerns over whole animal studies. There is an urgent need to develop and validate the use of cell cultures in lower vertebrates, including teleostean fish. Several cell systems have been already characterized, including cell lines of gill (Bols et al., 1994), liver (Bopp et al., 2006) and pituitary cells, as well as primary cultures with pituitary cells (Chang and Jobin, 1994; Bols et al., 1995), hepatocytes (Moon et al., 1999), and larval and embryo body cells (Ganassin et al., 1999). Protocols on isolation and in vitro testing of the functional integrity of teleost macrophages and lymphocytes isolated from the head kidney are also available (Pagniello et al., 2002; Zwollo et al., 2005).

The teleost head kidney is a complex tissue, composed of hematopoietic cells (lymphoid cells), immune cells (macrophages, lymphocytes), and endocrine cells (chromaffin cells and corticosteroidogenic cells, SCs) that secrete catecholamines and

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corticosteroids, respectively (Hontela, 2005). In contrast to mammalian adrenals where the distinct zonation (zona glomerularis, zona fasciculata, zona reticularis) facilitates isolation of specific cell types and investigation of cell-to-cell interactions, including cross talk between chromaffin and SCs (Schinner and Bornstein, 2005), the teleost interrenal tissue is heterogenous, with small islets of SCs scattered within the matrix of the lymphoid tissue (Fig. 1). Despite numerous studies investigating cortisol secretion in teleosts both *in vivo* and *in vitro*, there are currently no protocols for isolating and investigating SCs from the head kidney. In vitro investigations of signaling pathways leading to cortisol secretion (Rotllant et al., 2003) or toxicological studies of the mechanisms of endocrine disruption (Bisson and Hontela, 2002; Lacroix and Hontela, 2006; Gravel and Vijayan, 2006) relied on whole head kidney preparations that included all the cells, including the SCs. Therefore, in the toxicological studies with enzymatically dispersed head kidney cells, where the LC50 values (concentration of the test toxicant that kills 50% of the cells) were used as measures of cell mortality, it was assumed that the mortality of the cortisol-secreting SCs is similar to the mortality of the lymphoid cells, the chromaffin cells and the macrophages, also present within the head kidney. However, this assumption regarding the cell-specific vulnerability to toxicants has not yet been tested in the teleost head kidney tissue, mainly because protocols for isolating specific cells from this tissue have not been validated.

Percoll density gradients have been successfully used to isolate specific cell populations from teleost pituitaries (Chang and Jobin, 1994; Van Goor et al., 1994) and also from mammalian and avian adrenals (Chu and Hyatt, 1986; Carsia and Weber, 2000). Isolation and maintenance of viable SCs is a first step for establishment of cell cultures and cell lines of cortisol-secreting SCs, for investigations of cell-specific mechanisms of toxicity and of paracrine interactions (Weyts et al., 1999) between various cell types within the head kidney. Thus the objectives of the present study were to 1) validate a protocol to isolate SCs from the teleost head kidney and to maintain their secretory capacity, and 2) test the hypothesis that cell-specific differences in sensitivity to a model toxicant (pesticide endosulfan) exist in the head kidney.

2. Materials and methods

2.1. Chemicals

Porcine and human adrenocorticotropin ($ACTH_{1-39}$), minimum essential medium (MEM), Percoll, bovine serum albumin (BSA), DNase, nicotinamide, nicotinamide adenine dinucleotide (NAD), dehydroepiandrosterone (DHEA), nitroblue tetrazolium, dimethylformamide, Percoll and density beads were purchased from Sigma-Aldrich (St-Louis, MO, USA). Collagenase/dispase enzymes mixture was obtained from Boehringer Mannheim (Germany), RIA kit for cortisol determination, and 3-aminobenzoic acid ethyl ester (MS 222) for anesthesia were purchased from ICN (Costa Mesa, CA, USA). Trypan blue and culture plates (96-well) were obtained from Fisher Scientific (Nepean, Ontario, Canada) and endosulfan was purchased from Riedel de Haën (Diesenhofen, Germany).

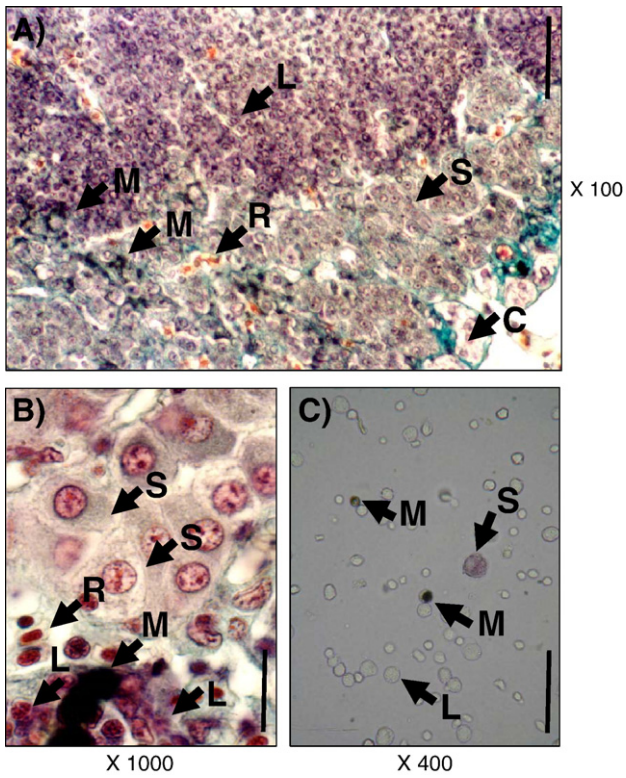

Fig. 1. Interrenal tissue of rainbow trout. A) Trichrome stain, magnified ×100, black bar measures 2 μm. B) Trichrome stain, magnified ×1000, black bar measures 20 μm. C) Cells dispersed by collagenase/dispase and stained with Tetrazolium blue, magnified ×400, black bar measures 8 μm. S, steroidogenic cell; R, red blood cell; C, chromaffin cell; M, melanomacrophage; L, lymphoid cell.

2.2. Experimental animals

Animal-use protocols have been approved by the Université du Québec à Montréal and the University of Lethbridge Animal Care Committees in accordance with national guidelines. Juvenile rainbow trout (*Oncorhynchus mykiss*) weighing 165 ± 90g were obtained from Pisciculture Laurentienne (Québec, Canada) for use in the initial Percoll validation experiments and Allison Creek Provincial trout Hatchery (Alberta, Canada) for use in the final validation experiments and exposures to the pesticide endosulfan. Animals were maintained in a 600 L freshwater tanks supplied with a constant flow rate of 3.8 L/min of filtered and air-saturated water (hardness 130 mg/L $CaCO_3$, 14–17 °C). They were fed daily with commercial trout food at the rate of 10 g/kg of fish. Experiments began after 2 weeks of acclimation following arrival of the fish.

2.3. Preparation of cell suspensions

Fish were anesthetized with MS-222 (0.1 g/L), bled from the caudal blood vessels with a heparinized syringe (Heparin sodium salt, activity 1029 U/mL, MP Biomedicals), then perfused through the caudal vein with 15 mL of NaCl 0.7% to

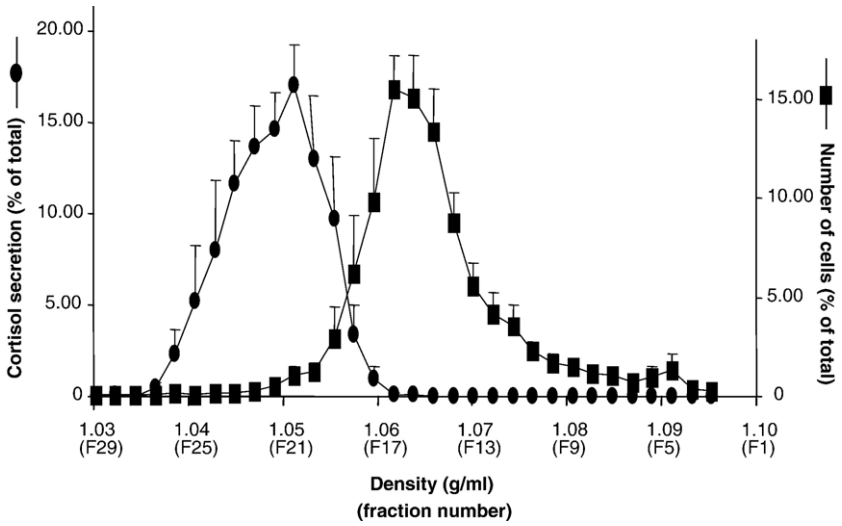

Fig. 2. Separation of head kidney cells by continuous Percoll density gradient. Squares (■) represent the percentage of total number of cells eluted at specific densities (1.10–1.03) and collected in specific fractions (F1–F29), and circles (●) represent the percentage of total cortisol produced in response to 2.4×10^{-6} M porcine ACTH. ($n=7$ as cell preparations from individual fish, mean±SEM).

remove red blood cells, according to a protocol described previously (Leblond and Hontela, 1999). Head kidneys were dissected out and washed in MEM supplemented with 5 g/L BSA and 2.2 g/L $NaHCO_3$, pH 7.4 (complete MEM), cut into pieces and incubated in 2.5 mL of complete MEM containing 2 mg/mL collagenase/dispase and 1.2 mg/mL DNase for 60 min at 20 °C with slow agitation. Cell suspension was filtered through nylon mesh (30 μm) (B&SH Thompson, Montréal, Canada) and centrifuged at 200 g for 5 min. Viability of cells prepared by this method, as determined by Trypan Blue exclusion test, was >95%. Following dispersion, cells were resuspended in 10 mL of MEM for the Percoll experiments or the cell concentration was adjusted to 75×10^6 cells/mL for experiments with the primary cell suspension. In all experiments, cells were plated in 96-wells microplate in a volume of 150 μl for a 120 min preincubation in complete MEM and then they were stimulated with porcine ACTH at the optimal effective dose (2.4×10^{-6} M; 1 IU/mL; Leblond et al., 2001) for 60 min at 15 °C. Cortisol content in the supernatant was assayed by RIA using a commercial kit obtained from Medicorp (Catalogue # 07-221102).

2.4. Isolation of corticosteroidogenic cells in a Percoll gradient

Following the enzymatic digestion with collagenase/dispase and DNAse, cells were resuspended in 10 mL of complete MEM and loaded onto a continuous density gradient column of 9% Percoll at the top to 72% at the bottom generated with a gradient maker and a peristaltic pump. The 72% Percoll was made of 14.4 mL of Percoll, 1.6 mL buffer A (BSA 5% w/v, KCl 13 mM, KH_2PO_4 7.3 mM, NaCl 694 mM and $Na_2HPO_4\cdot7H_2O$ 40.3 mM) and 4.0 mL buffer B (BSA 0.5% w/v, KCl 1.3 mM, KH_2PO_4 0.73 mM, NaCl 69.4 mM and $Na_2HPO_4\cdot7H_2O$ 4.0 mM). The 9% Percoll was constituted of 1.8 mL Percoll, 0.2 mL buffer A and 18 mL buffer B. The column was centrifuged for 25 min × 1200 g at 15 °C. In parallel, a Percoll column was loaded with density beads of 1.036, 1.052, 1.064, 1.075 and 1.087 g/mL to validate the Percoll gradient and to determine the density of the SCs. Fractions were then collected in 1 mL volume, 0.4 mL of MEM was added to each fraction to allow cells to pellet and fractions were centrifuged for 10 min × 200 g at 15 °C. Fractions were preincubated for 120 min in 150 μl of complete MEM and either stained with nitroblue tetrazolium to identify the SCs, stimulated for 60 min with 2.4×10^{-6} M porcine ACTH to assess the steroidogenic capacity of the cells, or exposed to the test toxicant (endosulfan) for 60 min, then stimulated with ACTH.

2.5. Staining of steroidogenic cells

To identify the islets of SCs within the head kidneys, the tissue was processed for histology and stained with Trichrome de Masson. To stain the SCs in cell suspensions, a modified method of Shepherd and Holzwarth (1998) was used. Cells resuspended in microplate wells were fixed for 5 min in 0.067 M phosphate buffer (PBS), pH 7.4 containing 2.6% paraformaldehyde. Cells were then washed with PBS 0.01 M pH 7.4 and centrifuged for 3 min at 200 g. To reveal the enzymatic activity of 3β-hydroxysteroid dehydrogenase responsible for the conversion of 17α-

Table 1
Enrichment factor for cortisol production by steroidogenic and lymphoid cell-enriched fractions eluted from the continuous Percoll gradient (mean±SD, $n=4$)

Cell preparation	Cortisol secretion (ng/mL)	Cell count (number of cells/mL)	[a]Enrichment factor $\frac{C_f/N_f}{C_p/N_p}$
Primary suspension	9.6±5.3	75×10^6	1
Fraction 11 (density 1.064 g/mL)	0	32×10^6	0.000461±0.00011
Fraction 21 (density 1.050 g/mL)	48.6±16.6	1.7×10^6	223.7±146.2

[a] Where C_f = cortisol secretion, N_f = number of cells in fraction, C_p = cortisol secretion in primary suspension, N_p = number of cells in primary suspension.

hydroxypregnenolone to 17α-hydroxyprogesterone in steroidogenic tissues, fixed cells were incubated for 90 min at 20 °C in 0.01M PBS pH 7.4 containing nicotinamide adenine dinucleotide (NAD) 1.5 mM, nicotinamide 4.1 mM, dehydroepiandrosterone (DHEA) 0.35 mM, dissolved in dimethylformamide, and nitroblue tetrazolium 0.61 mM. Following a wash with 0.01 M PBS, cells were mounted on slides and the blue-stained steroidogenic cells were counted.

2.6. Exposure to the pesticide

Following the preincubation, the supernatants were removed and the cells were resuspended in 150 μL of Ringer solution with the pesticide (endosulfan at 20, 40, 100, 300 and 360 μM) or without the pesticide (control wells), and incubated for 60 min at 15 °C. Endosulfan was first dissolved in dimethyl sulfoxide (DMSO) at a final DMSO concentration of 5% v/v. Blanks with DMSO were used to demonstrate that 5% DMSO has no effect on cell viability or cortisol secretion. Following exposure to endosulfan, the plates were centrifuged (300 g × 3 min) and cells were washed with 200 μL of Ringer solution, then stimulated with ACTH. Cortisol content in the supernatant was assayed by RIA after 60 min of stimulation. Cell viability was estimated with trypan blue, as described previously (Leblond and Hontela, 1999).

2.7. Statistical analysis

All data analyses were performed with JMPin 3.2.1 software 1988–1997 from SAS Institute. Regression analysis on probit transformed data were tested for significance using the Fisher test ($p < 0.05$), otherwise ANOVA and Student's *t*-test were used.

3. Results

3.1. Morphology of the head kidney

A histological preparation (trichrome stained) of the head kidney is shown in Fig. 1A and B. Islets of large, round SCs, surrounded by lymphoid tissue made up of small dark staining lymphoid cells with packets of black melanomacrophages, were

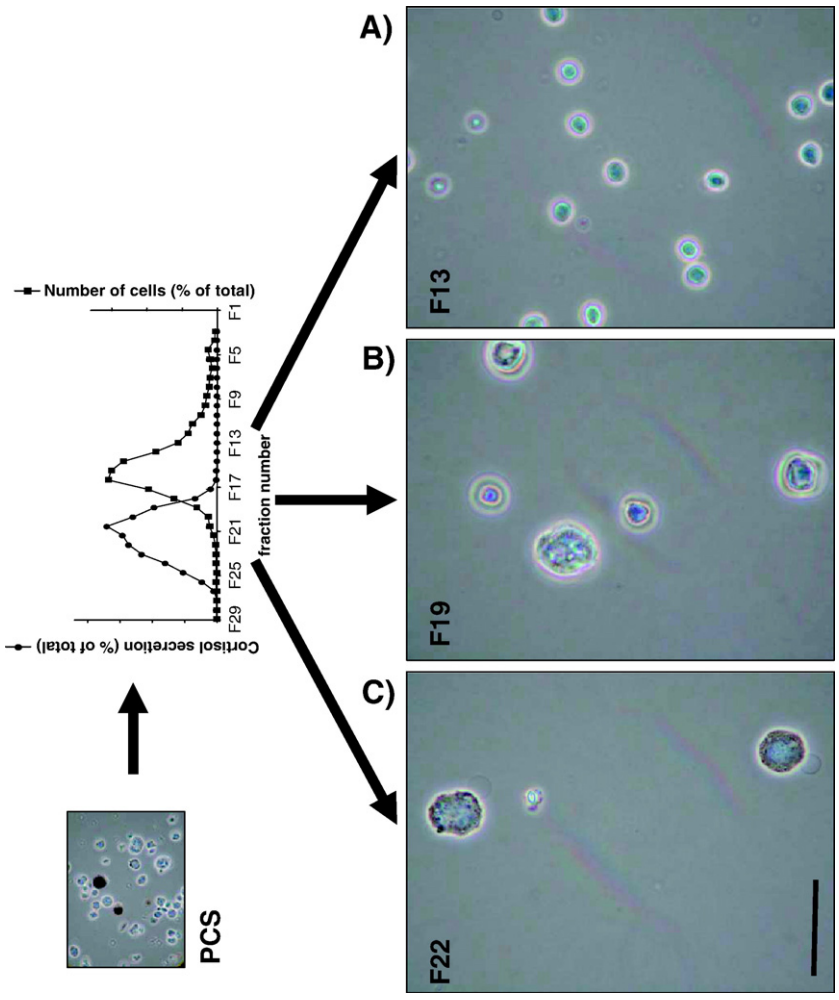

Fig. 3. Morphological characteristics of rainbow trout head kidney cells observed in the primary cell suspension (PCS) and in fractions eluted from the Percoll gradient. A) Lymphoid cells in fraction 13 (F13), B) lymphoid and steroidogenic cells in fraction 19 (F19), and C) steroidogenic cells in fraction 22 (F22) Magnified ×1000. Black bar measures 20 μm.

located mostly in the proximity of the cardinal vein sinuses. Pale staining cells, presumably chromaffin cells (Grassi Milano et al., 1997) were observed in the lining of the blood vessels. It is important to note that the steroidogenic islets are scarce — Fig. 1A and B focus on one islet while most of the head kidney (not shown) is made up by lymphoid tissue. Head kidney tissue cells enzymatically dispersed by collagenase/dispase and stained with Tetrazolium nitroblue stain are shown in Fig. 1C. All the cells of the head kidney, including large blue staining SCs, can be identified, except the chromaffin cells. The SCs represented approximately 1:8000 of the total cells in the primary suspensions of the head kidney.

3.2. Separation of cells on Percoll

To enrich the cell preparation with SCs, the primary cell suspension was placed onto a continuous Percoll density gradient ranging from density of 1.10, collected as fraction 1 (F1) to density of 1.03 (F29), as shown in Fig. 2. Head kidney cells collected in individual fractions were counted and the cells were stimulated with ACTH to assess the capacity of the cells to secrete cortisol. As shown in Fig. 2, the cortisol-secreting SCs are located in fractions with low density (1.04 to 1.06 g/mL) with a peak around 1.05 g/mL (F21). However, the majority of cells in the head kidney are denser, with a peak situated at 1.065 g/mL. The enrichment factor for the fraction corresponding to density of 1.050 g/mL was 223.69 (Table 1), representing one cell in 44, while no cortisol secretion was observed in fractions denser than 1.060 g/mL. The efficiency of cell recovery after the Percoll enrichment step was 80%.

3.3. Cell morphology

The most abundant cells in the head kidney, collected at density 1.06–1.07 (F13–F17), were round small cells with distinct nuclei (Fig. 3A). These cells, identified as lymphoid cells, did not secrete cortisol in response to ACTH stimulation. All of the cortisol-secreting capacity was detected in fractions F21–F25 where large, granular, dark staining cells were eluted (Fig. 3C). Fractions F19 contained a mixture of cells, mostly lymphoid cells (Fig. 3B), and cortisol secretion was undetectable.

3.4. Effect of endosulfan on head kidney primary cell suspension (PCS)

The PCS was exposed in vitro to endosulfan for 60 min and the capacity to secrete cortisol in response to ACTH stimulation and cell viability were assessed. Cortisol secretion by the primary cell suspensions (at 75×10^6 cells/mL) not exposed to endosulfan (controls) in response to 1 IU/mL of ACTH was 13.4±1.2 ng/mL ($n=33$). Endosulfan inhibited ACTH-stimulated cortisol secretion and cell viability in a concentration-dependent pattern; the concentration that inhibited 50% of the secretion (EC50) and the concentration that killed 50% of the cells (LC50) were determined as 43 µM (Fig. 4A, $R^2=0.949$) and 70 µM (Fig. 4B, $R^2=0.866$), respectively. The concentration-dependent pattern of the effects of endosulfan on cortisol secretion and cell viability is also clearly evident in Fig. 4C, with the loss of cortisol secretion occurring at a lower concentration of endosulfan than the loss of cell viability.

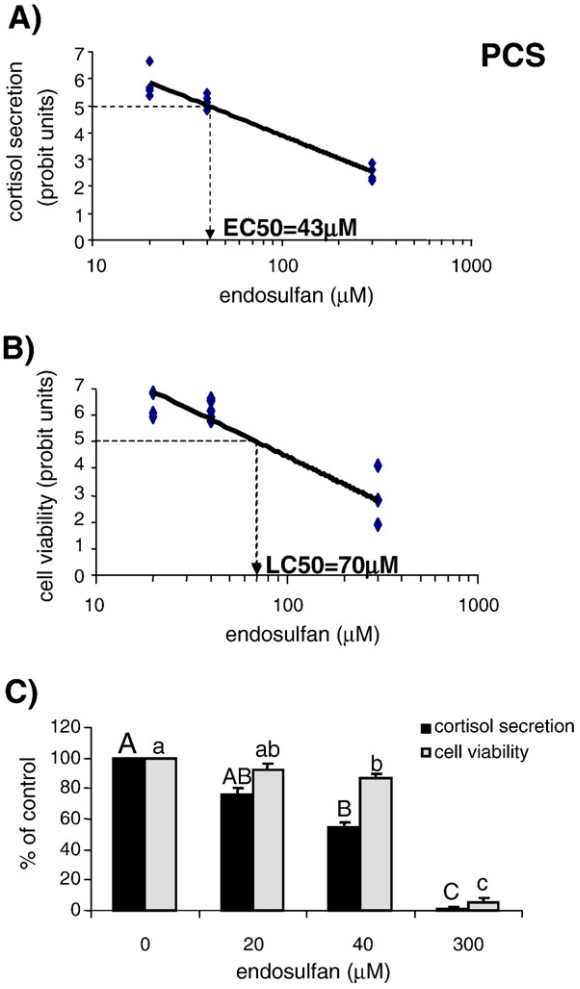

Fig. 4. Effects of endosulfan on head kidney primary cell suspension (PCS). A) Cortisol secretion in response to ACTH and the EC50 (effective concentration of endosulfan that inhibits 50% of cortisol secretion); the curve described as: $y=-1.2263\ln(x)+9.5392$, $R^2=0.949$; B) cell viability and the LC50 (lethal concentration that kills 50% of the cells); the curve described as: $y=-1.5019\ln(x)+11.372$, $R^2=0.866$, with both cortisol secretion and cell viability expressed in probit units, probit 5 corresponding to 50%, and C) cortisol secretion and viability of PCS, expressed as % of control (100%, cells not exposed to endosulfan). Regression analysis on probit transformed data were tested for significance using the Fisher test ($p<0.05$); means indicated by different letters (capital letters for cortisol secretion, small letters for viability) are significantly different at $p<0.05$ (ANOVA).

3.5. Effect of endosulfan on head kidney cells in fractions F20–25 and F14–19

The effects of endosulfan on cortisol secretion and cell viability of F14–19 (fractions enriched with lymphoid cells) and F20–25 (fractions enriched with SCs) are shown in Fig. 5A and B. Reflecting the cell enrichment, cortisol secretion by control cells (not exposed to endosulfan) in fractions 20–25 (at 1.7×10^6 cells/mL) in response to 1 IU/mL of ACTH was 17.0±

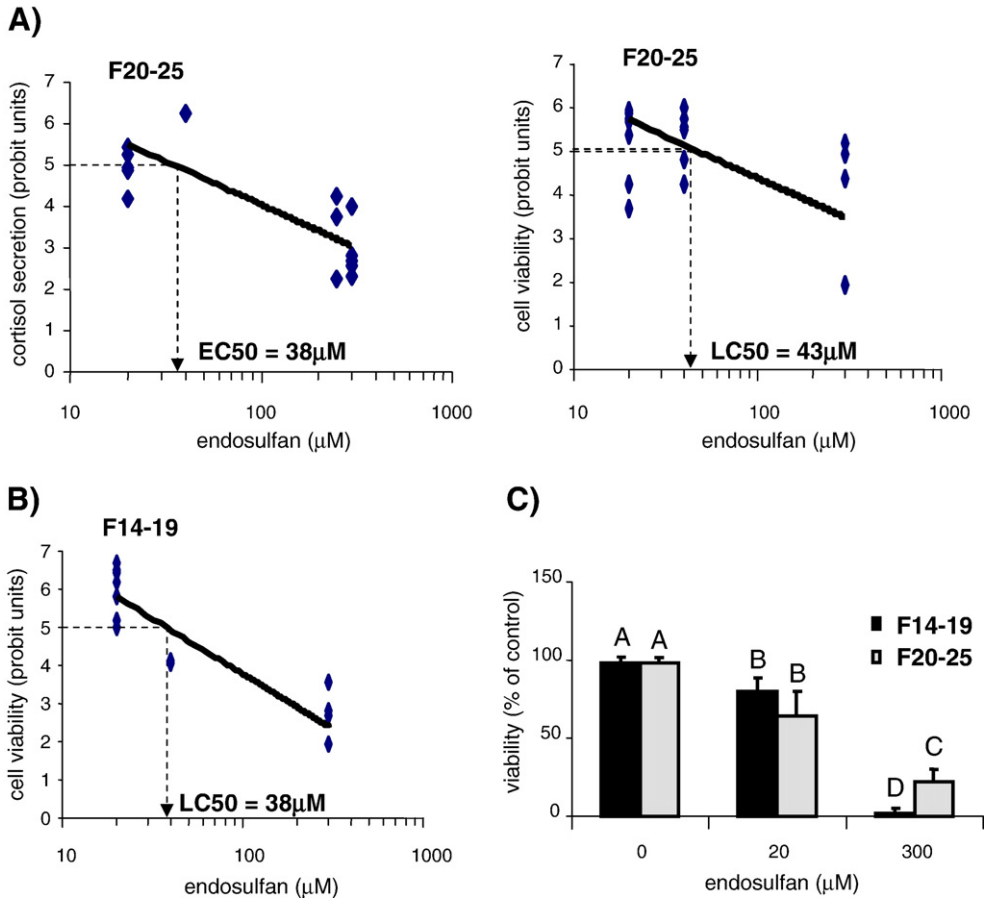

Fig. 5. Effects of endosulfan in cells eluted from the Percoll density gradient. A) Cortisol secretion and cell viability in fractions 20–25 (F20–25) enriched with steroidogenic cells, the curve described as $y=-0.9108\ln(x)+8.2408$, $R^2=0.634$ for cortisol secretion, and $y=-0.8249\ln(x)+8.1914$, $R^2=0.417$ for cell viability; B) cell viability in fractions 14–19 (F14–19) enriched with lymphoid cells; the curve described as $y=-1.2554\ln(x)+9.558$, $R^2=0.852$. Cortisol secretion was undetectable in F14–19. The EC50 and the LC50 are indicated at probit unit 5. C) Cell viability in cells from F14–19 and in F20–25, expressed as % of control (cell not exposed to endosulfan). Means indicated by different letters are significantly different at $p<0.05$ (ANOVA).

3.2 ng/mL ($n=21$), and was not significantly different from the secretion by the primary cell suspensions, even though fewer cells were used for the preparation. The EC50 of endosulfan for F20–25 was 38 µM ($R^2=0.634$), similar to the EC50 determined in the PCS. The EC50 could not be determined in F14–19 (fraction enriched with lymphoid cells) since there was no detectable cortisol secretion in this fraction. The LC50 of endosulfan in the F20–25 was 43 µM ($R^2=0.417$), similar to the LC50 in F14–19 (38 µM, $R^2=0.852$). Thus the LC50s of the cells eluted from the Percoll was lower than the LC50 of the PCS (70 µM). Again, the concentration-dependent pattern of the effects of endosulfan on cortisol secretion and cell viability was clearly evident and highly significant (Fig. 5C).

4. Discussion

The teleost head kidney, a functionally and anatomically complex tissue, has an important role in both immune and endocrine function, including phagocytosis by macrophages, hematopoiesis, cortisol secretion by SCs and catecholamine secretion by chromaffin cells (Weyts et al., 1999; Hontela, 2005). Previously published in vivo and in vitro investigations of the head kidneys provided knowledge important for understanding how fish react to environmental challenges, including chemical stressors, and how they defend themselves against pathogens and foreign particles. The approach traditionally used by researchers was to focus on one type of responses (e.g. immune or endocrine endpoints), using the whole head kidney, with all its cellular constituents, both in vivo or in vitro (Rotllant et al., 2003; Gravel and Vijayan, 2006). Our study was designed to develop and validate a method for isolation of specific cells from the head kidney and for testing in vitro the cell-specific responses to the pesticide endosulfan, a model environmentally relevant toxicant.

A Percoll density gradient was used in the present study to separate the SCs from other cell populations in the head kidney, to estimate their total number, and to compare their functional integrity and responses to a model toxicant with those of the other head kidney cell types. Except for chromaffin cells, all other cell types that were observed in the trichrome stained histological preparation of the head kidney were present in the enzymatically dispersed PCS, including lymphoid cells, darkly pigmented melanomacrophages, red blood cells and SCs. Chromaffin cells, localized mainly in the walls of the posterior cardinal veins (Reid et al., 1998) may have been damaged by the enzymatic digestion. Consistent with the absence of chromaffin cells in the PCS,

epinephrine and norepinephrine secretion activity (without any substrate or enzyme additions) were also not detected in Percoll fractions tested (S. Perry, personal communication). The number of SCs in the primary cell suspension identified with the Tetrazolium stain was small (1:8000), as expected from observations of the low number of small islets of SCs within the lymphoid tissue matrix, in trichrome stained histological sections of head kidney tissue (Fig. 1A). The small number of the SCs within the head kidney represents a significant challenge for any studies requiring pure cell preparations or preparations enriched with these particular cells.

The Percoll gradients have been used with success to isolate specific cell types from various tissues, including adrenals of mammals and birds (Chu and Hyatt, 1986; Carsia and Weber, 2000). Using the continuous Percoll density gradient (density gradient of 1.03 to 1.10) in the present study, we were able to isolate the specific cell types and estimate their numbers. The performance and results of the Percoll gradient separation were reliable and remarkably consistent. The largest number of cells eluted at densities ranging from 1.07 to 1.06; these cells were relatively small, round and did not have the capacity to secrete cortisol when stimulated with ACTH. Based on these morphological (size, appearance) and functional (cortisol secretion) characteristics, we identified these cells as lymphoid cells. Cells having the capacity to respond to ACTH by secreting cortisol were identified in fractions eluting at lower densities, between 1.045 and 1.055. A large cortisol secretion response was associated with these cells, which were large, granulated, and very scarce (<1% of the total cell population of the head kidney). It is noteworthy that these cells, although large, were eluting at relatively lower densities than some of the smaller cells, e.g. the lymphoid cells, possible because they are filled with lipid-rich materials, linked to their steroidogenic capacity. Melanomacrophages, also relatively scarce in the head kidney tissue, as observed in histology sections, were not eluting in the Percoll gradient because they were collecting on the Nitex filter during one of the first steps following the enzymatic digestion. Red blood cells, also present in the tissue, were scarce since the fish were bled and perfused with saline before the dissection of the head kidney.

Following the isolation of the SCs (Fractions 20–25), we were able to characterize and compare the responses of SC-enriched cell preparations to a model toxicant, the organochlorine pesticide endosulfan, with responses of cells in PCS and cell preparations enriched with lymphoid cells (Fractions 14–19). The effects of endosulfan on the viability and cortisol secretion of the primary cell suspension were estimated by the LC50 (concentration of the toxicant that kills 50% of the cell population) and the EC50 (concentration of the toxicant that inhibits the cortisol secretory capacity by 50%), respectively. Dose-dependent effects of endosulfan on cortisol secretion and viability were observed, as reported previously. The EC50 value (43 μM endosulfan) obtained with the PCS in the present study was similar to values reported previously (38 μM, Bisson and Hontela, 2002), while the LC50 in the present study (70 μM) was lower, possibly because trypan blue exclusion method was used while LDH release was measured previously. In the present study, we expressed the cortisol and the viability data as Probit units, a transformation often used with toxicological data to determine the LC50 (Finney, 1971). This transformation is appropriate for data expressed as %; moreover it facilitates the interpretation of the data since the results are plotted onto a straight line, rather than a sigmoid curve.

In the present study, the effects of endosulfan were investigated, for the first time, in cell-specific fractions isolated from the head kidney of rainbow trout. The LC50s for endosulfan in SC-enriched preparations was 38 μM, a value very similar to the EC50 identified in the PCS. These results suggest that the endocrine toxicity of endosulfan to SCs does not change significantly whether the cells are exposed together with other cells of the head kidney, as during the exposure of the PCS, or whether the SCs are exposed by themselves. Use of the Percoll gradient thus seems to be an effective method for preparing cell-specific fractions for cell-specific toxicity testing; moreover, the secretory capacity of the SCs is not significantly altered by the Percoll gradient and all the steps associated with the method. On the other hand, the LC50s for both the steroidogenic fraction (F20–25) and the lymphoid cell fraction (F14–19) decreased (43 μM for F20–25 and 38 μM for F14–19) compared to the PCS (LC50 70 μM). Thus the Percoll procedure does make the cells more vulnerable to endosulfan (lower LC50) but the mortality is not cell-specific since the LC50 for both the SC fraction and the lymphoid cell fraction were similar.

The similarity of the EC50 and LC50 of endosulfan for the SC fraction suggests that the mechanism of the endosulfan-induced cortisol secretion impairment may be mediated by a loss of viability of the SCs. It has been proposed earlier that a large ratio of LC50/EC50 indicates that the loss of a secretory capacity is not caused by cell mortality but rather by a disruption of the cellular processes leading to hormone synthesis. The LC50/EC50 value for e.g. Cd was 65 (Leblond and Hontela 1999), indicating that the loss of cortisol secretion occurred at concentration of Cd 64 times lower than the loss of viability. The LC50/EC50 ratio reported previously for endosulfan in enzymatically dispersed trout head kidney cells was 10.6 (Bisson and Hontela, 2002), suggesting that some of the loss of cortisol secretion may be associated with endosulfan-induced mortality of steroidogenic cells. Our study with the Percoll gradient enriched fractions strongly suggests that the loss of cortisol secretion was due to cell mortality since the EC50 and LC50 were similar. Our data also suggest that the SCs may be slightly more resistant to the cytotoxicity of endosulfan since the viability of the SC fraction at 300 uM of endosulfan was higher in these cells than in cells from F14–19. The greater tolerance (higher LC50) to endosulfan of the PCS containing all the head kidney cells, compared to the SC-enriched fraction (F20–25) or lymphoid cell fraction (F14–19) suggests the presence of certain cell populations in the head kidney that are more resistant to endosulfan than the two cells types tested in our study. Alternatively, the interactions of different cell types may be important in modulating cell vulnerability to endosulfan. It is also possible that the Percoll gradient separation and all the steps associated with it made cells more susceptible to the toxic effects of endosulfan. Our study did not test these three

alternative hypotheses, but the protocol presented here could provide new data on these relative vulnerabilities in the future.

Although cortisol secretions in response to ACTH of the PCS and the F20–25, adjusted for cell numbers, of the control treatment (no endosulfan) were not different (13.4 ng/mL and 16.9 ng/mL, respectively), the procedure used to prepare PCS does slightly lower the response to ACTH, when compared to head kidney tissue used directly. The secretory response (expressed as ng cortisol produced by 1 mg of tissue) of head kidney tissue, simply cut into fragments and exposed to a control 1 IU/mL ACTH, was 1.5 ng/mg of head kidney (data not shown), about 7.5 times higher than the response of PCS (estimated at 0.2 ng/mg of PCS pellet). Such a lowering of cortisol response to ACTH in PCS is to be expected since the PCS has been exposed to enzyme treatment and additional physical manipulations relative to the fragments. However, responses to ACTH using head kidney fragments were very variable (CV > 23%, standard error/mean) possibly due to the heterogeneity in the distribution of SCs among the tissue pieces. Such variability is one of the disadvantages of using fragments rather than cell suspensions (CV < 9%) in mechanistic studies. Interestingly, adjusting for the amount of tissue present, the pooled F20–25 fractions still produced more cortisol per mg (11.5 ng/mL) than both the head kidney fragments and the PCS, indicating that the Percoll density separation protocol resulted in enrichment of responsive SCs relative to both primary preparations.

In conclusion, the Percoll gradient can be used for preparation of cell-specific fractions from a complex tissue such as the teleost head kidney. Using this method, quantitative data on number of specific cells in the tissue can be obtained. We report that the number of SCs relative to the total cell population in rainbow trout head kidney is very small, approximately 1:8000. The effects of endosulfan, a model pesticide, were assessed in the primary cell suspension, and in fractions enriched with either SCs or lymphoid cells. The elution from the Percoll gradient and the enrichment steps did not have an effect of the endosulfan-induced loss of cortisol secretion but did make the cells more vulnerable to the toxicant. The availability of cell preparations enriched with specific head kidney cell types should facilitate development of specific cell cultures and eventually cell lines, making possible studies of functional interactions between different cell types (e.g. endocrine and immune) in the head kidney.

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