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Characterization of a protein-based filtering cartridge for the removal of atrazine-induced effects on living cultured cells

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Abstract. Chronic exposure to atrazine (ATR) raises concerns about adverse effects on reproductive functions. We tested our previously validated filtering device, the OBP-based filter, onto a biological model constituted of cultured swine granulosa cells treated for 48 h with media conditioned with 0.1 or 10 μM ATR evaluating cell viability and steroidogenesis. The tested atrazine concentrations did not change granulosa cell viability and no filtering effects was observed following treatments with media prepared with differently filtered water. As for steroidogenesis, treatment of water with OBP -based filter containing 10 μM atrazine completely suppressed the stimulatory effect of 10 μM atrazine on progesterone production as well as the inhibitory effect of 0.1 μM ATR on estradiol-17β production by granulosa cells.

Our data demonstrate that the impairment of steroidogenesis induced by ATR is effectively removed after water filtration in the experimental device thus suggesting potential use in biotechnological applications on living cells and/or organisms.

Keywords: atrazine; OBP; granulosa cells; steroidogenesis

1. Introduction

Atrazine (ATR) is a pesticide widely employed in the world. Although this substance has been banned both from Italy and from the other countries of the European Union since 1992 (Ordinanza Ministeriale n° 705/91) and 2004 (Directive 2004/248/EC), several data demonstrate the persistence of ATR, as confirmed by the assessment of ^{14}C-labeled ATR in soil 22 years after the end of the treatment (Jablonowski *et al.* 2011). This strong persistence is responsible for a high risk of chronic ATR exposure for living organisms. With regard to this, it must be underlined that ATR, which is a moderately hydrophobic compound with a solubility in H_2O of 33 mg/l (Meister, R.T. (ed.). 1992. Farm Chemicals Handbook '92. Meister Publishing Company, Willoughby, Ohio), has been detected in surface and deep waters collected from areas where its use is intensive (Barbash *et al*. 2001) thus causing a significant non-occupational exposure (Curwin *et al.* 2002). It should be noted that several studies indicate that this herbicide interferes with reproduction (Hayes *et al.* 2011, Basini *et al.* 2012).

Many studies have been carried out to develop water treatment systems for the efficient removal of organic compounds (Gryta 2013, Amin and Alazba 2014, Rojas-Serrano *et al*. 2015). In particular, several studies have been devoted to purification from ATR and other triazine pesticides, but most of them have not been proved to be extremely effective (Chingombe *et al.* 2006, Jiang and Adams 2006, Bottino *et al*. 2011). As a novel contribution to those approaches that could be used for the removal of triazines form water samples, we recently validated the use of a cartridge filter where the retention element is represented by a protein carrier for small hydrophobic molecules, the Lipocalin odorant binding protein (OBP) (Tegoni *et al.* 2000), coupled to agarose particles (Bianchi *et al.* 2013).

The present research was therefore aimed to test our previously validated filtering device onto a biological model constituted by cultured endocrine cells treated with atrazine conditioned media. Swine granulosa cells were used as experimental model since atrazine was previously demonstrated to disrupt their function (Basini *et al.* 2012).

2. Materials and methods

Chemicals were purchased from Sigma (St. Louis, MO, USA) unless differently indicated.

2.1 Preparation of the OBP-based filter

The bovine OBP-based cartridge filter (bOBP), was prepared according to the procedure published by Bianchi and co-workers (Bianchi *et al.* 2013). Briefly, 8 mg of purified recombinant b-OBP, tagged with 6 histidine residues at the amino-terminal (Grolli *et al.* 2006, Bianchi *et al.* 2013), solubilized in 1.0 ml sodium phosphate (50 mM; pH 7.8), were coupled to 1.0 ml of a suspension of Ni-NTA-agarose particles (Quiagen, Milan, Italy) washed in the same buffer. After extensive washing, the resin was first

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Table 1 Water solutions employed for the preparation of the tested media

N° of the medium	ATR content of the filtered water solutions (μM)	Filtering treatment	ATR added after the filtering treatment (μM)
1	0	OBP-Ni-NTA-agarose	0
2	0	OBP-Ni-NTA-agarose	0.1
3	0	OBP-Ni-NTA-agarose	10.0
4	0.1	OBP-Ni-NTA-agarose	0
5	10	OBP-Ni-NTA-agarose	0
6	0	Ni-NTA-agarose	0
7	0.1	Ni-NTA-agarose	0
8	10.0	Ni-NTA-agarose	0
9	0	No filtration	0
10	0.1	No filtration	0
11	10	No filtration	0

Different treatments of the pure water samples and of the ATR water solutions employed for the preparation of the cell culture media. CM=culture medium, that is the same for all the preparations, is DMEM/Ham's F12 supplemented with sodium bicarbonate (2.2 mg/ml), bovine serum albumin (BSA 0.1% W/V), penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml), selenium (5 ng/ml) and transferrin (5 μg/ml).

equilibrated with distilled H_2O and then used for the filtration of the water employed for the preparation of the media used for the cell culture experiments. The functionality and the ligand binding capacity of the filter were evaluated by loading supra-saturating amounts of both the OBP ligand 1-aminoanthracene (AMA) (50 μM), and ATR (25 μM) dissolved in sodium phosphate (50 mM; pH 7.8), to 1.0 mL aliquots of bOBP-his-tag-agarose suspension trapped inside 2.0 ml disposable plastic syringes. Comparison of the absorption spectra (between 210 and 340 nm) of the eluates, to those of the same loaded AMA and ATR solutions, allowed to evaluate the amounts of ligands retained by the bOBP coupled to the resin. The quantities of both AMA and ATR unspecifically retained were determined with the same procedure, but using the uncoupled NiNTA agarose resin. The capability of the OBP based filter in the removal of atrazine from the water used for the preparation of the culture media (Table 1) was then evaluated by a solid-phase microextraction (SPME)-GC-SIM-MS analysis method (Bianchi *et al.* 2013), which is technique allowing to detect trace amounts of ATRA (at the submicromolar level) that cannot be determined spectrophotometrically.

2.2 Granulosa cell collection and culture with atrazine conditioned media

Swine ovaries and granulosa cells were isolated as previously described (Basini *et al.* 2008). Briefly, large follicles (> 5 mm) were aseptically harvested by aspiration with a 26-gauge needle and and granulosa cells were centrifuged for pelleting. After vital staining with trypan blue (0.4% w/v) cells were seeded in DMEM/Ham's F12 (Gibco, Grand Island, NY, USA) plus sodium bicarbonate (2.2 mg/ml), bovine serum albumin (BSA 0.1% w/v), penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml), selenium (5 ng/ml) and transferrin (5 μg/ml), hereafter referred as culture medium (CM).

In a previous study (Basini *et al.* 2012), we demonstrate a biological effect of ATR (0.1 or 10 μM) on swine granulosa cell steroidogenesis. The concentrations chosen are in accordance with the ovarian concentrations detected after atrazine exposure (Quignot *et al.* 2012). Dimethyl sulfoxide (DMSO) was used as the carrier solvent and its final concentration was less than 0.1% v/v, which is a value that has no effect on the examined parameters.

Once seeded, granulosa cells were incubated with culture media prepared by dissolving the different components either into pure water, or into atrazine water solutions that had been prepared according to the scheme shown in Table 1 (no filtration, filtration onto Ni-NTA-agarose and filtration onto OBP- Ni-NTA-agarose), thus assessing the potential effect of the OBP-based filter.

The cells were cultured for 48 h at 37°C under humidified atmosphere (5% CO_2), and the effects of atrazine as well as that of its removal by the OBP-based filtering device were evaluated on cell viability and steroidogenesis.

2.2.1 Granulosa cell viability

Granulosa cells ($2 \cdot 10^5$ cells/ 100 μl culture media/well) were grown in 96-well microplates and incubated for 48h in the presence of culture media prepared as described in Table 1. Intracellular ATP content, as a marker for cell viability, was assayed using luminescent ATPlite (PerkinElmer Inc., Waltham, MA, USA) according to the manufacturer's instruction. The luminescence was measured using a microplate reader (Multilabel Counter Victor, Perkin Elmer, Boston, USA).

2.2.2 Granulosa cell steroidogenesis

Granulosa cells were seeded as previously detailed (Basini and Tamanini 2000) and treated for 48 h with culture media prepared as reported in Table 1. At the end of incubation, media were assayed for progesterone (P4) and 17β estradiol (E2) by RIAs (Grasselli *et al.* 1993).

P4 assay sensitivity and ED50 were 0.24 and 1 nmol/l, respectively; E2 assay sensitivity and ED50 were 0.05 and 0.2 nmol/l. The intra- and inter-assay coefficients of variation were less than 12% for both assays.

3. Statistical analysis

The experiments were repeated at least 5 times (6 replicates/treatment). Experimental data are presented as mean ± SEM; statistical differences between treatments were calculated with ANOVA using Statgraphics package (STSC Inc., Rockville, MD, USA). When significant differences were found, means were compared by Scheffè's

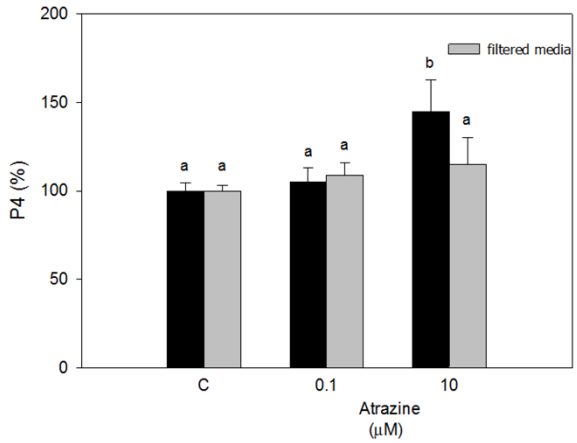

Fig. 1 Effect of the treatment with ATR (0.1 or 10 μM) for 48 on P4 production by granulosa cells. Black bars are for unfiltered media. Different letters indicate a significant difference ($p<0.05$) among treatments as calculated by ANOVA and Scheffè' F test

F test; p values < 0.05 were considered to be statistically significant.

4. Results and discussion

4.1 Treatment of the water used for the preparation of the cell culture media

Atrazine has been demonstrated to affect reproduction (Kniewald *et al.* 2000, Stoker *et al.* 2000, Friedman 2000, Trentacoste *et al*. 2001, Swan 2006, Cooper *et al*. 2007, Solomon *et al.* 2008, Dehkargani *et al.* 2012) In a previous study (Basini *et al.* 2012), we demonstrate that the herbicide interferes with granulosa cells steroidogenesis. These results raise concern and confirm that this herbicide can impair reproductive efficiency.

Therefore, we proceeded to set up a novel protein based filtering device (Bianchi *et al.* 2013) to remove ATR from water, as an alternative to previously developed filtering matrixes, such as conventional and surface modified activated carbons (Croll *et al*. 1991, Duguet 1994, Chingombe *et al*. 2006, Jiang *et al*. 2006), polymeric phases (Yoon *et al.* 2008) and carbon nanotubes (Yan *et al.* 2008).

The filtering retention element is the bovine form of the lipocalin odorant binding protein, which is a polypeptide that binds a large spectrum of structurally unrelated hydrophobic molecules (Tegoni *et al.* 2000) including ATR and other compounds of the same tirazine category. (Bianchi *et al.* 2013). For this application the filter has been set up as a cartridge, where an engineered form of bovine OBP tagged at the amino-terminal with six histidine residues, has been coupled to Ni-NTA-agarose particles.

The binding capacity of the filter, determined with the OBP ligand AMA, was about 360 nmoles of AMA/ml of agarose suspension. This value is in agreement with previous evaluations and indicates that about 85% of the OBP binding sites of the protein coupled to the resin are potentially available for the removal of atrazine from the water samples used for the preparation of the cell culture media.

To verify the effectiveness of the filter in terms of removal of the endocrine disruptor activity exerted by atrazine on swine granulosa cells, both the water and the atrazine water solutions used for the preparation of experimental media were first filtered either on the OBP-based filter and on the uncoupled Ni-NTA agarose resin alone according to the scheme reported in Table 1 (media 1-8). The evaluation of the atrazine content in these samples, determined by SPME-GC-SIM-MS analysis, that is a technique allowing to detect submicromolar amounts of ATR, showed that the removal of the herbicide was always greater than 97%, thus confirming previous determinations on water solutions of three different triazine compounds (Bianchi *et al.* 2013). As control of the effect of the filtration by the uncoupled resin on the cell cultures, three additional media were prepared with pure water, directly taken from the dispenser of the nanopure distiller (media 9-11).

4.2 Granulosa cell viability and steroidogenesis

It should be noted that previous studies performed in pigs reported multiple cysts and persistence of corpora lutea in the ovaries, while lowered plasma E2 concentrations were measured thus suggesting that ATR negatively impacts on reproduction (Gojmera *et al.* 1996, 1999).

As already demonstrated in our previous study (Basini *et al.* 2012), the examined atrazine concentrations did not change granulosa cell viability (media 9-11).

As for filtering effects, in our culture model granulosa cell viability was unaffected following treatments with media prepared with differently filtered water. Furthermore, we did not find any effect on cell viability due to the resin alone or to the presence of bOBP coupled to the agarose particles (media 1-8).

Granulosa cells basal progesterone output was 73.5±8.4 ng/ml (mean±SEM). The cultures carried out with the differently prepared media showed that the pattern of secretion of progesterone by porcine granulosa cells is not significantly modified by ions and/or other molecules potentially released from the resin or by bOBP (media 6-8).

Treatment of water containing 10 μM atrazine with the OBP-based filter, completely removed the stimulatory effect of the endocrine disruptor on P4 production by granulosa cells (Fig. 1).

Granulosa cells, in basal condition produce 3.5±0.8 ng/ml of estradiol-17β. In agreement with the results obtained for the progesterone production, also estradiol-17β secretion was not significantly affected by contaminants from the filtration technique (media 6-8). Interestingly, through the filtration of the water used for the production of the culture medium, the radioimmunoassay showed that the OBP coupled to the resin is able to remove the inhibitory effect of 0.1 μM ATR on estradiol-17β production (Fig. 2).

Therefore, taken together the impairment of steroidogenesis induced by atrazine (Basini *et al.* 2012) is effectively removed after water filtration in the experimental device thus suggesting its potential in biotechnological applications on living cells and/or organisms, where ultrapure water, free of atrazine and/or

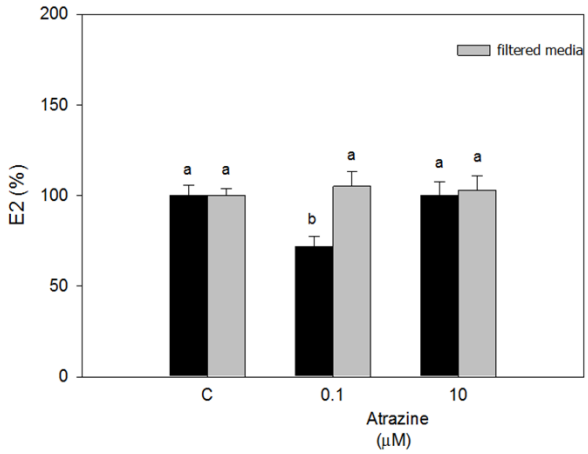

Fig. 2 Effect of the treatment with ATR (0.1 or 10 μM) for 48 on E2 production by granulosa cells. Black bars are for unfiltered media. Different letters indicate a significant difference ($p<0.05$) among treatments as calculated by ANOVA and Scheffè' F test

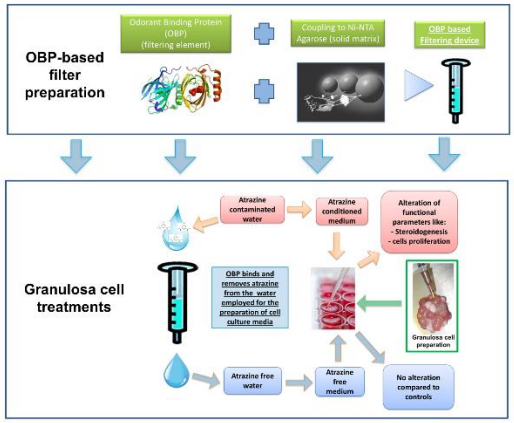

Fig. 3

other endocrine disrupters substances that the OBP can bind, are required. Examples of applications could be the production of solutions of pharmaceuticals, or the preparation of reconstituted milk and/or food preparations for the early childhood. Moreover, due to the characteristics of the filtration device, the treatment could be useful as alternative method for COD removal (Madaeni and Samieirad 2010). Since the his-tagged OBP can be eluted from the Ni-NTA-agarose particles, and the bound ligands removed by organic solvent extraction, the reuse of both solid matrix and protein for following filtration cycles represents a hypothesis that is worth to be considered and verified, following the protocol on cell living cells that has been set up in the present investigation.

5. Conclusions

Atrazine was introduced as pesticide in the 1950s. In the European Union has been banned and its employment has been restricted in other countries, but it is still used worldwide.

Atrazine is moderately hydrophilic with significant aqueous solubility, indicating that it may have a high leaching potential. Significant concentrations of this herbicide or its metabolites have been reported at great depths in ground water and in surface runoff, thus suggesting a significant risk for exposure for animals and humans. Recently, a significant amount of data indicate environmental chemical contaminants to cause alterations in the animal reproduction. The experimental results collected in our present study (Fig. 3) let us to suggest the novel OBP-based filtration device as an effective method in water purification from ATR. This is of particular interest since the impairment of steroidogenesis induced by ATR determine serious reproductive concerns.

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