The Toxic Effect of Toluene on Ovarian Cells Can Be Prevented by the MicroRNA miR-152

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Summary

The potential of microRNAs to protect the female reproductive system from the toxic influence of oil-related environmental contaminants has not yet been examined. The aim of the present study was to examine the ability of the microRNA miR-152 to prevent the toxic effects of toluene on ovarian cells. Porcine ovarian granulosa cells transfected or not transfected with miR-152 mimics were cultured with or without toluene (0, 10 and 100 ng/ml). The expression of miR-152; cell viability; proliferation (accumulation of PCNA, cyclin B1 and BrdU); cytoplasmic/mitochondrial apoptosis (accumulation of bax and caspase 3); and release of progesterone, testosterone and estradiol were quantified *via* RT-qPCR, the Trypan blue exclusion test, quantitative immunocytochemistry, the BrdU assay and ELISA. The addition of toluene reduced cell viability, decreased the levels of all the measured markers of proliferation and the release of all the measured steroid hormones, and promoted the expression of apoptosis markers. Transfection of cells with miR-152 mimics increased the expression of miR-152, cell proliferation, and progesterone release but reduced apoptosis and the release of testosterone and estradiol. Moreover, miR-152 prevented or inhibited all the toluene effects in addition to its inhibitory effect on testosterone and estradiol release. The present results demonstrate that miR-152 can protect ovarian cells from the harmful influence of toluene.

Key words

miR-152 • Toluene • Ovary • Hormones • Apoptosis • Proliferation

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Introduction

The toxic effects of oil-related contaminants and the drug toluene on female reproductive processes are well known. Toluene and its analogs can suppress ovarian folliculogenesis and fecundity [1-3]. *In vitro* studies on porcine ovarian granulosa cells demonstrated that the adverse effects of toluene on female reproduction can be explained by its ability to directly inhibit ovarian cell proliferation and release hormones and to promote ovarian cell apoptosis. Taken together, these changes result in a decrease in ovarian cell viability [4-6].

The search for natural protectors of ovarian cells against the toxic action of toluene is important from both biological and practical viewpoints. The protective action of some medicinal plants [6,7] and plant flavonoids [4] has been reported, but none of these protectors prevented all the adverse effects of toluene on ovarian cells.

The alternative physiological protectors could be noncoding microRNAs (miRs). The expression of many miRs in blood [8,9], urine [9], lung [10], central nervous system [11] and cultured HL-60 cells can be altered by toluene. The response of miRs to toluene exposure might indicate that these miRs could be responsible for the resistance of target cells to the adverse influence of this

environmental contaminant. The involvement of miRs in the control of the ovarian cell response to toluene has not yet been studied. This study aimed to determine whether some miRs could physiologically protect ovarian cells against the adverse influence of toluene and whether manipulations of these miRs could be applied for the detection and prevention of these effects.

One of the miRs involved in the upregulation of ovarian cell functions is miR-152. Its analog (a miR-152 mimic), which increases the expression of this miR, promotes proliferation and the release of progesterone and reduces apoptosis and the release of estradiol by human ovarian granulosa cells [12,13]. Furthermore, miR-152 mimics promoted viability and proliferation, as well as the release of progesterone, IGF-I, oxytocin, and prostaglandin E2, but inhibited apoptosis and estradiol output by cultured porcine ovarian granulosa cells. The miR-152 inhibitor had the opposite effect [14]. These data demonstrated that miR-152 can promote ovarian cell functions. It has been suggested that miR-152 can not only stimulate ovarian cells but also prevent the inhibitory action of the environmental contaminant toluene on these cells.

To verify this hypothesis, we studied the effects of toluene, miR-152 mimics and the combination of toluene + miR-152 mimics on the basic functions of cultured porcine ovarian granulosa cells. The following markers and regulators of cell functions were analyzed: viability; 5-bromo-2′-deoxyuridine (BrdU, a marker of cell proliferation; [15]; PCNA, a marker and promoter of S-phase of mitosis; [16]; cyclin B1, a marker and promoter of M-cell and G-phase of mitosis; [15,17]; bax, a caspase 3, a marker and promoter of cytoplasmic/ mitochondrial apoptosis; [18]; progesterone, testosterone; and estradiol, which are regulators of ovarian cell proliferation, apoptosis, ovarian follicullogenesis, oogenesis and luteogenesis [19,20].

Materials and Methods

Oligonucleotides

MiR-152 mimics (double-stranded RNAs that mimic mature endogenous miR-152 and enhance miRNA activity, represented by a gain-of-function assay) and their respective negative controls (NCs) labeled with fluorescein (to control their presence in the cells) (Table 1) were synthesized by GenePharma Co., Ltd. (Shanghai, China). After synthesis, the oligonucleotides were purified by using high-performance liquid chromatography. According to the manufacturer's data, more than 97 % purity was observed by mass spectrometry.

Table 1. The sequences of miRNA mimics and miRNA inhibitor.

Oligonucleotides		Sequence
miR-152 mimics	Sense	5′-UCAGUGCAU-GACAGAACUUGG-3′
	Antisense	5′-AGUUCUGUC-AUGCACUGAUU-3′
miR-NC (negative control) mimics	Sense	5′-UUCUCCGAA-CGUGUCACGUTT-3′
	Antisense	5′-ACGUGACAC-GUUCGGAGAATT-3′

Preparation, transfection, culture, and processing of ovarian granulosa cells

Eighteen porcine ovaries were collected from prepubertal Landrace gilts (6-8 months of age) at the slaughterhouse of Chovmat F.U. in Rastislavice (Slovak Republic). Ovarian granulosa cells were aspirated with a syringe from ovarian follicles 4.5-6.5 mm in diameter without visible signs of atresia (including weak vascularization, thin follicular walls, and pale follicular fluid) *via* aspiration with a syringe. After aspiration and isolation *via* centrifugation for 10 min at 1500 rpm, the granulosa cells were washed in sterile DMEM/F12 1:1 medium (BioWhittakerTM; Lonza, Verviers, Belgium) and resuspended in the same medium supplemented with 10 % fetal calf serum (BioWhittakerTM) and 1 % antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA). The cells were counted using an automated cell counter (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the concentration was adjusted to the required volume (10^6 cells/ml medium). The cell suspension was dispensed in 24-well culture plates (NuncTM, Roskilde, Denmark; 1 ml suspension/well) for enzyme-linked immunosorbent assay (ELISA) and reverse transcription-quantitative PCR (RT-qPCR), in 96-well (100 µl/well) culture plates for the BrdU (bromodeoxyuridine) test, or onto 16-well chamber slides (Nunc, Inc., International, Naperville, IL, USA; 200 µl/well) for quantitative immunocytochemistry. The cells were precultured in medium at 37.5 °C in 5 % CO_2 until an 80 % confluent monolayer was formed (2-3 days). Thereafter, the experimental cells were transfected with miR-152 mimics and their respective negative controls (NCs) (both at a final concentration of 25 nM) by using

Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The control groups included nontransfected cells or cells that were transfected with NC.

After transfection for 24 h, the granulosa cells were cultured in the presence or absence of toluene (0, 10, and 100 ng/ml; AppliChem GmbH, Darmstadt, Germany). These doses were comparable to the doses of toluene that were effective in previous similar *in vitro* experiments [5-7]. Immediately before administration to cells, toluene was added to culture medium. The control groups were composed of nontransfected or transfected cells not treated with toluene.

After culture during 2 days, the medium from the 24-well culture plates was aspirated and stored at -20 °C until further analysis *via* ELISA. The cells from the 96-well plates were immediately processed for the Trypan blue and BrdU assays and RT-qPCR. On 16-well chamber slides, 4 % paraformaldehyde in PBS was added for 10 min to fix a monolayer of cells, which were then stored at +4 °C until immunocytochemistry analysis.

Cell viability test

Cell viability was determined by using the Trypan blue exclusion test (0.4 %), as described previously [21]. Briefly, the medium was removed from the culture plates after the granulosa cells were incubated. Subsequently, the cell monolayer was subjected to Trypan blue staining (Sigma-Aldrich) for 15 min. After Trypan blue treatment, the cells were fixed for 30 min in 4 % paraformaldehyde. After fixation, the plates were washed with a physiological solution and subjected to microscopic inspection (magnification: 400×). The ratio of dead (stained) cells to the total cell count was calculated.

BrdU assay

Cell proliferation, based on the measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporation during DNA synthesis, was determined by using a colorimetric cell proliferation ELISA (Roche Diagnostics GmbH, Roche Applied Science, Germany) according to the manufacturer's instructions. The reaction products were quantified by measuring the absorbance at 450 nm using an ELISA reader (Thermo Fisher Scientific).

Immunocytochemical analysis of proliferation and apoptosis markers

The markers of proliferation (PCNA and cyclin B1) and intrinsic (cytoplasmic/mitochondrial) apoptosis (bax and caspase 3) were detected *via* immunocytochemistry as previously described [14] by using primary mouse monoclonal antibodies against PCNA, cyclin B1, bax, or caspase 3 (dilution of 1:500 in PBS; Santa Cruz Biotechnology, Inc.) and secondary swine antibody against mouse IgG (dilution of 1:1000; Santa Cruz Biotechnology, Inc.) labeled with horseradish peroxidase (Servac, Prague, Czech Republic). Cells labeled with horseradish peroxidase were stained with 3,3'-diaminobenzidine (DAB) substrate (Roche Diagnostics GmbH, Mannheim, Germany). Cells treated without the primary antibody were used as negative controls. In addition, the number of stained cells and the location of intracellular molecules were determined based on the brown coloration of DAB peroxidase by using a light microscope (Leica Microsystems, Wetzlar, Germany). The ratio of stained cells to the total number of cells was determined.

RT-qPCR for miR-152

After treatment with miR-152 mimics or negative control for 48 h, total RNA was extracted from the transfected granulosa cells by using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. The concentration and quality of total RNA were measured by using a UV spectrophotometer (Bio-Rad, Inc.; Hercules, CA, USA). The expression levels of mature miR-152 were quantified by using the Hairpin-it-miRNAs qPCR Kit (GenePharma Co., Ltd.) on an ABI 7500 Fast Instrument (Thermo Fisher Scientific, Inc.). The amplification thermocycling procedure was as follows: initial denaturation at 95 °C for 3 min; 40 cycles of 95 °C for 10 s, annealing and elongation at 60 °C for 10 s, and 60 °C for 60 s. U6 small nuclear RNA (snRNA) was used as an internal control, and relative gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method of [22]. The sequences of the utilized primers (Table 2) were designed and synthesized by GenePharma Co., Ltd. All of the samples were analyzed in triplicate from the same RNA preparation, and the mean values were calculated.

ELISA

The concentrations of progesterone and 17β-estradiol were determined in 25 µl aliquots of the incubation medium by using an ELISA according to the manufacturer's instructions (LDN Immunoassays and Services, Nodhorn, Germany). The characteristics of these assays are presented in Table 3. This ELISA was validated for use with culture medium samples by using dilution tests.

Table 2. The sequences of gene primers for RT-qPCR.

Gene symbol	Primer sequence	
miR-152	Forward	5′-TGGTTCGCT-CAGTGCATGA-3′
	Reverse	5′-TATGGTTGTTCA-CGACTCCTTCAC-3′
U6	Forward	5′-CTCGCTTCG-GCAGCACA-3′
	Reverse	5′-AACGCTTCA-CGAATTTGCGT-3′

Statistical analysis

The data from this study are reported as the means of values that were obtained in three separate experiments performed on separate days with different groups of granulosa cells, each obtained from at least six ovaries. Each experimental group included four culture wells containing ovarian granulosa cells. For the immunocytochemical analyses, the proportion of cells containing antigen, at least 1000 cells per well, was calculated. The optical density of the signal was calculated as a percentage of the control according to the instructions of the kit manufacturer. For the ELISA, blank control values of medium cultured without the cells were subtracted from the corresponding values to exclude any nonspecific background (less than 10 % of the total values). The rates of hormone secretion were calculated per 10^6 viable cells/day. Significant differences between the groups were determined by using the Shapiro-Wilk normality test and Student's *t*-test, as well as one-way ANOVA followed by Tukey's test, with SigmaPlot 11.0 (Systat Software, GmbH, Erkrath, Germany). Differences were considered to be significant at P values less than 0.05 ($P<0.05$).

Table 3. Characteristics of the immunoassays used in experiments.

Substance assayed	Specificity of assay (cross-reactivity of antiserum)	Sensitivity of assay (ng/ml)	Maximal coefficient of variation (%)	
			Intra-assay	Inter-assay
Progesterone	≤1.1 % with 11-desoxycorticosterone, ≤0.35 % with pregnenolone, ≤0.30 % 17α-OH with progesterone, ≤0.20 % with corticosterone, <0.10 % with estriol, 17β-estradiol, testosterone, cortisone and 11-desoxycortisol, <0.02 % with DHEA-S and cortisol	0.045	5.4	5.59
Testosterone	≤3.3 % to 11β-hydroxyestosterone and 19-nortestosterone, ≤0.9 % to androstenedione, ≤0.8 % to 5α-dihydrotestosterone, <0.1 % to 17α-methyltestosterne, epitestosterone, oestradiol, progesterone, cortisol, oestrone and danazol	0.083	4.16	4.73
17β-estradiol	≤9.5 % with fulvestrant, ≤4.2 % with estrone, ≤3.8 % with E2-3-glucuronide, ≤3.6 % with E2-3-sulphate, ≤0.4 % with estriol, <0.1 % with androstenedione, 17-hydroxyprogesterone, corticosterone, pregnenolone, E2-17-glucuronide, progesterone, and testosterone	0.0062	6.4	4.5

Results

Efficiency of transfection with miR-152 mimics

RT-PCR analysis revealed that transfection of cells with miR-152 mimics increased the relative expression of miR-152 (Fig. 1). No significant differences in any of the measured parameters were found between the cells not subjected to treatment (control, C) and those subjected to transfection with the negative control (NC) (Fig. 2A-I).

Effect of toluene administration

The addition of toluene to cells not transfected with miR-152 mimics (10 or 100 ng/ml) affected the main measured parameters. Toluene significantly reduced cell viability (at all doses added; Fig. 2A), accumulation of PCNA (at dose 100 ng/ml; Fig. 2B) and cyclin B1 (at all doses; Fig. 2C), and cell proliferation, as measured by BrdU incorporation (at all doses; Fig. 2D). Furthermore, the addition of toluene at all doses increased the accumulation of bax (Fig. 2E) and caspase 3 (Fig. 2F). Finally, exogenous toluene given at all doses reduced the release of progesterone (Fig. 2G), testosterone (Fig. 2H) and estradiol (Fig. 2I).

Effect of transfection with miR-152 mimics

Transfection of cells with miR-152 mimics (toluene at 0 ng/ml + miR-152 mi) did not change cell viability (Fig. 2A) but significantly increased the accumulation of PCNA (Fig. 2B) and cyclin B1 (Fig. 2C) and cell proliferation, as measured by BrdU incorporation (Fig. 2D). Furthermore, transfection with miR-152 significantly diminished the accumulation of bax (Fig. 1E) and caspase 3 (Fig. 1F). Finally, compared with control cells, cells transfected with miR-152 mimics released more progesterone (Fig. 1G) but less testosterone (Fig. 1H) and estradiol (Fig. 1I).

Ability of miR-152 mimics to modify the effects of toluene

A comparison of the responses of cells transfected or not transfected with miR-152 mimics to the addition of toluene demonstrated the ability of miR-152 mimics to modify the response of cells to toluene. The cells transfected with miR-152 mimics, similar to control cells, responded to toluene addition by reducing their viability. The viability of the cells transfected with miR-152 mimics was significantly greater than that of the cells not transfected with miR-152 cultured with toluene (Fig. 1A).

Similarly, the administration of toluene reduced the accumulation of PCNA (Fig. 2B) and cyclin B1 (Fig. 2C) in cells transfected with miR-152 mimics, but these parameters were greater in toluene-treated untransfected cells. Cell proliferation, measured by BrdU incorporation, was reduced after benzene addition in both cells transfected or not transfected with miR-151 mimics, but toluene given at a dose of 100 ng/ml reduced BrdU incorporation by cells transfected with miR-152 less than by control cells (Fig. 2D).

Toluene increased the accumulation of bax (at a dose of 100 ng/ml; Fig. 2E) and caspase 3 (at all doses added; Fig. 2F) in cells transfected with miR-152 mimics, but these markers of apoptosis were significantly lower in the transfected cells than in the control cells.

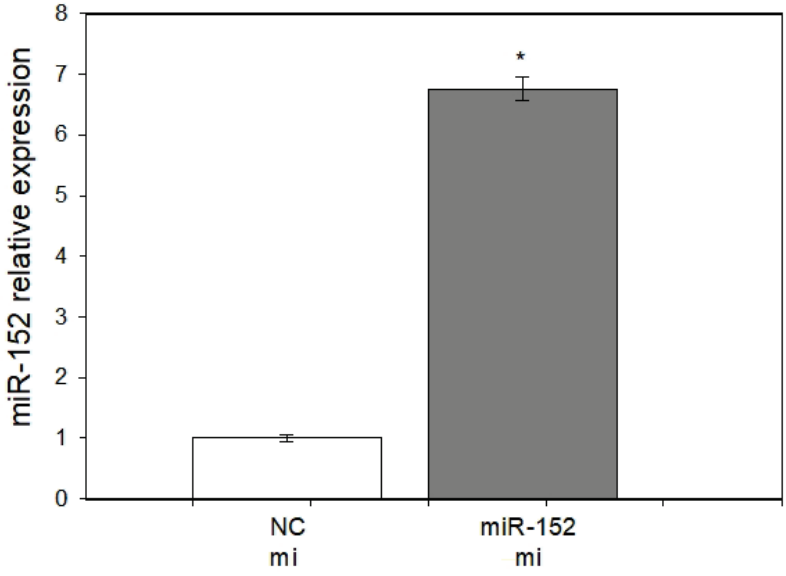

Fig. 1. Effect of transfection with miR-152 mimics on the expression of miR-152 in cultured porcine ovarian granulosa cells (RT-qPCR). * – significant ($P<0.05$) differences between the negative control cells transfected with noncoding oligonucleotide and cells transfected with miR-152 mimics.

In the cells transfected with miR-152 mimics, toluene reduced progesterone release, but this release was substantially greater than that in toluene-treated cells not transfected with miR-152 mimics and even in control cells (Fig. 2G).

The effect of miR-152 on the action of toluene on testosterone output depended on the toluene dose added; miR-152 mimics promoted the effect of toluene at a dose of 10 ng/ml but suppressed the action of toluene at 100 ng/ml (Fig. 2H).

MiR-152 did not modify the suppressive effect of toluene at 100 ng/ml on estradiol output but slightly promoted this effect at 10 ng/ml (Fig. 2I).

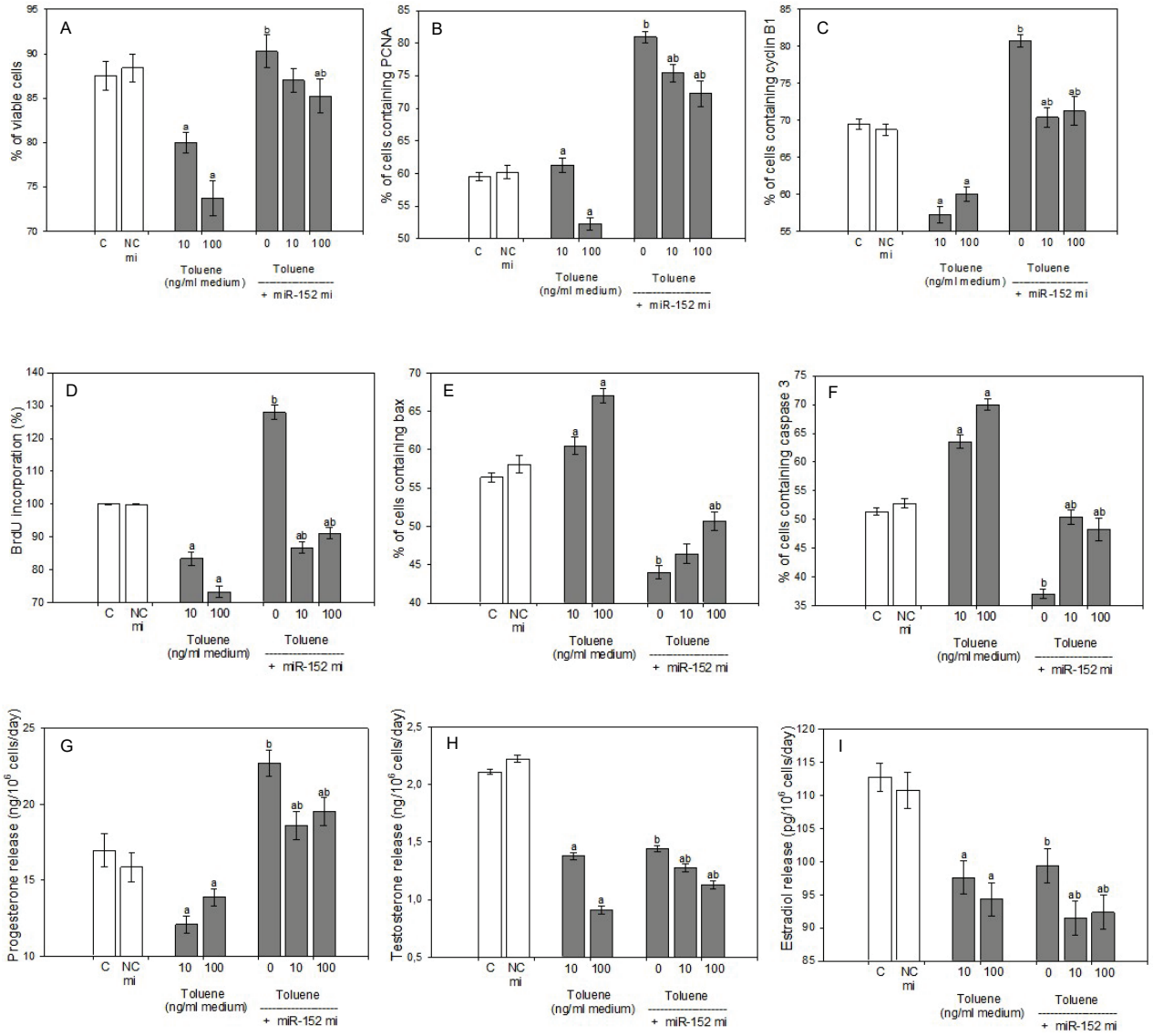

Fig. 2. Effects of the addition of toluene on viability (**A**, Trypan blue test), the accumulation of PCNA (**B**, quantitative immunocytochemistry), cyclin B1 (**C**, quantitative immunocytochemistry), proliferation (**D**, BrdU incorporation), the accumulation of bax (**E**, quantitative immunocytochemistry), and caspase 3 (**F**, quantitative immunocytochemistry), and the release of progesterone (**G**, ELISA), testosterone (**H**, ELISA) and estradiol (**I**, ELISA) by cultured porcine ovarian granulosa cells not transfected or transfected with miR-152 mimics (miR-152 mi). C, control (no treatment); NC, mi, negative control (transfection with negative control oligonucleotide). The results show (a) the effects of toluene and (b) the effect of miRNA mimics, with a significant ($P<0.05$) difference between the cells treated and not treated with these molecules. The results are expressed as the mean ± SEM.

Discussion

Effect of toluene administration

In the present study, the addition of toluene reduced the expression of markers of proliferation (accumulation of PCNA and cyclin B1 and BrdU incorporation) in cultured porcine ovarian granulosa cells. The inhibitory effects of toluene on

PCNA (a marker and promoter of S-phase cells in the cell cycle) [16] and cyclin B1 (a marker and promoter of M- and G-phase cells in mitosis) [15,17] suggest that toluene can suppress cell proliferation by downregulating the expression of intracellular promoters of both S- and M/G-phases in the cell cycle.

Furthermore, the addition of toluene at all doses increased the accumulation of bax and caspase 3 (markers and promoters of cytoplasmic/mitochondrial apoptosis) [18]. This observation suggested that toluene can promote cytoplasmic/mitochondrial apoptosis in ovarian cells. A reduction in ovarian cell proliferation and promotion of apoptosis could be the causes of impaired viability in cells treated with toluene.

Finally, in the present study, the administration of toluene reduced the release of the steroid hormones progesterone, testosterone and estradiol. These hormones are considered stimulators of ovarian cell proliferation and viability and inhibitors of apoptosis [19,20]. Therefore, toluene may affect these processes *via* the downregulation of ovarian steroid hormone release.

These findings are in line with previous reports concerning the ability of toluene to directly inhibit porcine ovarian cell proliferation and release hormones to promote ovarian cell apoptosis and reduce ovarian cell viability [4-7]. These data demonstrate the female reproductive toxicity of toluene and the mechanism of its suppressive action on female reproductive processes in various species [1-3].

Effect of transfection with miR-152 mimics

In the present study, transfection of porcine ovarian cells with miR-152 mimics increased the relative expression of miR-152, which was associated with increased accumulation of PCNA and cyclin B1 and enhanced cell proliferation. These findings suggest that miR-152 stimulates ovarian cell proliferation *via* the upregulation of both PCNA (a promoter of S-phase during mitosis) [16] and cyclin B1 (a promoter of M- and G-phases during the cell cycle) [15,17].

Furthermore, the association between miR-152 overexpression and reduced accumulation of bax and caspase 3 (markers and promoters of cytoplasmic/ mitochondrial apoptosis) [18] suggests the antiapoptotic action of miR-152. The ability of miR-152 to increase ovarian cell viability observed in the present study could be explained by the ability of miR-152 to upregulate proliferation and downregulate apoptosis in these cells and, therefore, to increase the proliferation:apoptosis rate.

In the present study, miR-152 mimics promoted the release of progesterone and reduced testosterone and estradiol outputs. These findings indicate that miR-152 could physiologically promote ovarian cell luteinization, which is characterized by an increase in progestogen levels, a decrease in androgen and estrogen production, enhanced ovarian cell proliferation and decreased ovarian cell death/apoptosis [20]. Furthermore, steroid hormones are well-known regulators of ovarian cell proliferation, apoptosis, viability, ovarian follicular development and resulting fecundity [20]. The associations between miR-152-induced changes in ovarian cell steroidogenesis, proliferation, apoptosis and viability indicate that miR-152 can affect ovarian cell proliferation and apoptosis through changes in ovarian steroid hormones.

These findings are in line with previous reports concerning the ability of miR-152 mimics to promote proliferation and the release of progesterone; reduce cytoplasmic/mitochondrial apoptosis; increase the output of estradiol by cultured human ovarian granulosa cells [12,13]; and promote viability, proliferation and release of progesterone but suppress apoptosis and estradiol output by cultured porcine ovarian granulosa cells [14].

The stimulatory influence of miR-152 on animal and human ovarian cells indicates the potential usefulness of miR-152 for the diagnosis, prediction and promotion of female reproductive processes in animal production, biotechnology and assisted reproduction.

Ability of miR-152 mimics to modify the effects of toluene

A comparison of the effects of toluene and miR-152 on ovarian cells revealed antagonistic effects of these molecules on the majority of the measured parameters (viability, PCNA, cyclin B1, BrdU incorporation, bax, caspase 3 and progesterone), although toluene and miR-152 had similar (inhibitory) influences on testosterone and estradiol release. The first indicator is that miR-152 can oppose the toxic effect of toluene on ovarian cells.

The second indicator could be the ability of miR-152 to modify toluene effects. The character of toluene action was retained in the presence of miR-152. miR-152 mimics not only opposed but also mitigated and even prevented the effects of toluene on the majority of the analyzed cell parameters (viability, PCNA, cyclin B1, BrdU incorporation, bax, caspase 3 and progesterone). The effect of miR-152 on the action of toluene on testosterone and estradiol output depended on the toluene dose. Taken together, these findings showed that miR-152 was able to mitigate or prevent the adverse effects of toluene on 8 of the 10 measured parameters, while the

effect of miR-152 on the 2 remaining parameters depended on the dose of toluene.

The present observations suggest that the response of the ovary to environmental contaminants and the drug toluene could be influenced by endogenous miR-152. It is not to be excluded, that miR-152 could be a marker and predictor of such a response. Furthermore, these findings indicate that endogenous or exogenous miR-152 could not only stimulate the female reproductive system but also protect against the adverse effects of toluene. However, this hypothesis requires validation with further *in vivo* experiments.

The results of the present study performed on cultured porcine granulosa cells indicate that toluene can suppress basic ovarian cell functions and that toluene mainly stimulates miR-152. The present results demonstrate that miR-152 can protect ovarian cells from the harmful influence of toluene on ovarian cell viability, proliferation and apoptosis. Furthermore, these results provide the first evidence that miR-152 could be potentially applicable for the diagnosis, prediction, mitigation and prevention of the toxic effects of toluene on the female reproductive system.

Conflict of Interest

There is no conflict of interest.

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