

ARTÍCULO ORIGINAL – ORIGINAL ARTICLE

Analysis of the activity of oncocalyxone A (*Auxemma oncocalyx*) and doxorubicin on the *in vitro* development of porcine oocytes

Análisis de la actividad de oncocalyxone A (*Auxemma oncocalyx*) y doxorubicina sobre el desarrollo *in vitro* de ovocitos porcinos

Johanna Leiva Revilla¹, Carolina Maside¹, Luis Vieira¹, Jesús Cadenas¹, Ana Clara Ferreira Acioly¹, Victor Macedo Paes¹, Francisco Leo Agiar¹, Juliana Jales de Hollanda Celestino², Benner Geraldo Alves¹, Otilia Deusdenia Loiola Pessoa³, Ricardo Toniolli⁴, Ana Paula Rodrigues¹, José Ricardo de Figueiredo¹

¹ Universidade Estadual do Ceará, Laboratory of Manipulation of Oocytes and Preantral Follicles (LAMOFOPA). Fortaleza-CE, Brazil.

² Universidade da Integração Internacional da Lusofonia Afro-Brasileira, Institute of Health Sciences. Acarape-CE, Brazil.

³ Universidade Federal do Ceará, Department of Organic and Inorganic Chemistry., Centro de Ciências. Fortaleza-CE, Brazil.

⁴ Universidade Estadual do Ceará, Laboratory of Swine Reproduction and Semen Technology. Fortaleza-CE, Brazil.

Autor de correspondencia: jrf.lamofopapapers@gmail.com

DOI: <https://doi.org/10.32480/rscp.2019-24-2.274-292>

Recibido: 20/08/2019. Aceptado: 3/12/2019.

Abstract: Most anticancer drugs like doxorubicin (DXR) have low specificity that results in undesirable effects especially when it comes to collateral effects on reproduction. Plants are excellent sources when searching for new drugs. *Auxemma oncocalyx* (*A. oncocalyx*) and its main component Oncocalyxone A (onco A) have anti-tumoral activity and are less toxic than DXR in reproductive parameters. However, there are no studies on the action of these drugs regarding the porcine *in vitro* oocyte competence and embryo development. The aim of this study was to evaluate the effect of *A. oncocalyx* and onco A exposure during *in vitro* maturation (IVM) of oocytes (Experiment 1) or *in vitro* embryo culture (IVC) (Experiment 2) on the oocyte developmental competence. For experiment 1, COCs were distributed in IVM medium alone (control) or supplemented with DXR (0.3 µg/mL), *A. oncocalyx* (1.2 µg/mL) and onco A (1 µg/mL). Then, oocytes were submitted to *in vitro* fertilization (IVF) and *in vitro* embryo culture. For experiment 2, zygotes were cultured with DXR, *A. oncocalyx* and onco A for 7 days. Viability, maturation, fertilization and embryo developmental parameters were evaluated in both



experiments. In experiment 1; DXR, *A. oncocalyx* and onco A reduced ($P < 0.05$) oocyte viability and IVM efficiency. Onco A increased ($P < 0.05$) the meiotic resumption. After IVF, all drugs reduced ($P < 0.05$) viability, IVF efficiency and percentage of cleaved embryos, nevertheless, only DXR decreased ($P < 0.05$) the percentage of blastocyst. In experiment 2; all drugs reduced ($P < 0.05$) the percentage of penetration, but only DXR and onco A decreased ($P < 0.05$) IVF efficiency. DXR and *A. oncocalyx* decreased ($P < 0.05$) the percentage of cleaved embryo, but had no effect on blastocyst formation. In conclusion, the addition of DXR during IVM or IVC negatively affected the IVF efficiency and cleavage rate. In addition, the exposure of COCs to DXR only during IVM was more detrimental to oocyte viability and blastocyst formation than *A. oncocalyx* and onco A.

Keywords: *Auxemma oncocalyx*; Oncocalyxone A; Doxorubicin; *in vitro* maturation; *in vitro* fertilization; COCs; embryo development

Resumen: La mayoría de los medicamentos contra el cáncer como la doxorubicina (DXR) tienen una baja especificidad que produce efectos indeseables, especialmente cuando se trata de efectos colaterales en la reproducción. Las plantas son excelentes fuentes cuando se buscan nuevos medicamentos. *Auxemma oncocalyx* (*A. oncocalyx*) y su componente principal Oncocalyxone A (onco A) tienen actividad antitumoral y son menos tóxicos que DXR en los parámetros reproductivos. Sin embargo, no hay estudios sobre la acción de estos medicamentos con respecto a la competencia de los ovocitos *in vitro* porcina y el desarrollo embrionario. El objetivo de este estudio fue evaluar el efecto de la exposición a *A. oncocalyx* y onco A durante la maduración *in vitro* (IVM) de los ovocitos (Experimento 1) o el cultivo de embriones *in vitro* (IVC) (Experimento 2) en la competencia del desarrollo de los ovocitos. Para el experimento 1, los COC se distribuyeron en medio IVM solo (control) o suplementado con DXR (0.3 $\mu\text{g} / \text{mL}$), *A. oncocalyx* (1.2 $\mu\text{g} / \text{mL}$) y onco A (1 $\mu\text{g} / \text{mL}$). Luego, los ovocitos se sometieron a fertilización *in vitro* (FIV) y cultivo embrionario *in vitro*. Para el experimento 2, los cigotos se cultivaron con DXR, *A. oncocalyx* y onco A durante 7 días. Se evaluaron los parámetros de viabilidad, maduración, fertilización y desarrollo embrionario en ambos experimentos. En el experimento 1; DXR, *A. oncocalyx* y onco A redujeron la viabilidad de los ovocitos ($P < 0.05$) y la eficiencia de IVM. Onco A aumentó ($P < 0.05$) la reanudación meiótica. Después de la FIV, todos los medicamentos redujeron la viabilidad ($P < 0.05$), la eficacia de la FIV y el porcentaje de embriones clivados, sin embargo, solo DXR disminuyó el porcentaje de blastocistos. En el experimento 2; todos los medicamentos redujeron ($P < 0.05$) el porcentaje de penetración, pero solo DXR y onco A disminuyeron ($P < 0.05$) la eficacia de la FIV. DXR y *A. oncocalyx* disminuyeron

($P < 0.05$) el porcentaje de embriones clivados, pero no tuvieron efecto sobre la formación de blastocistos. En conclusión, la adición de DXR durante IVM o IVC afectó negativamente la eficiencia de IVF y la tasa de clivaje. Además, la exposición de los COCs a DXR solo durante la IVM fue más perjudicial para la viabilidad de los ovocitos y la formación de blastocistos que *A. oncocalyx* y onco A.

Palabras clave: *Auxemma oncocalyx*, Oncocalyxone A, Doxorubicina, maduración *in vitro*, fertilización *in vitro*, COC, desarrollo embrionario.

1. INTRODUCTION

Cancer is a target of research for the development or discovery of new forms of treatments (1). Many drugs used in cancer chemotherapy, like doxorubicin (DXR), a widely used drug for different cancer types (2), have low specificity that results in undesirable effects. Studies show that DXR cause toxicity in both primordial follicles and growing ovarian follicles, triggering follicular and oocyte apoptosis (3), and eventually affect human fertility. Therefore, plants are excellent sources of raw material when searching for new anticancer drugs (4).

Auxemma oncocalyx (*A. oncocalyx*) is a common tree found in the state of Ceará in Northeast Brazil (5). It has been widely used in folk medicine as an adjunctive treatment of injuries such as wounds and cuts (5, 6). Some studies have suggested that this plant has biological activities such as analgesic, antioxidant, antitumor and anti-inflammatory effects (6-9). Oncocalyxone A (onco A) is *A. oncocalyx*'s active compound. Onco A has high antioxidant activity (6) and an anti-proliferative effect on tumor cell cultures (10). Studies have suggested onco A as a possible anticancer compound since it presents antitumor and cytotoxic activity in human leukemia cells, and other cell cancer lines, without causing genotoxicity (11) like most anticancer drugs.

Little is known about reproductive toxicity of *A. oncocalyx* and onco A in mammals. In recent pioneer studies conducted by our group with caprine preantral follicles cultured *in vitro* enclosed in ovarian cortical tissue, *A. oncocalyx* and onco A affected *in vitro* caprine early folliculogenesis in a concentration-dependent manner (12). However, no toxic effect of *A. oncocalyx* and onco A was observed on *in vitro* development of late caprine isolated secondary follicles. In contrast, these drugs affected the cumulus-oocyte complexes (COCs) viability after *in vitro* maturation but not the metaphase II rates (Leiva-Revilla et al., 2016 b - under review). In both studies, DXR was used as positive (toxic) control and presented a more toxic

effect than *A. oncocalyx* and onco *A*. These results suggest that *A. oncocalyx* and onco *A* despite of having anticancer effects they are, apparently, less harmful to reproductive parameters than commercial drugs, such as DXR.

Normal embryonic development is preceded by a sequence of coordinate events during maturation and fertilization. The mechanism of oocyte maturation encompasses interactions between the oocyte and its surrounding cumulus cells, which synchronizes meiosis with structural and molecular changes in the ooplasm, enabling the oocyte to support proper fertilization and subsequent embryo development (13). Consequently, it is of high significance to study the toxic effect of new drugs over *in vitro* maturation, fertilization and embryo development.

To the best our knowledge, there is no information about the influence of *A. oncocalyx* and onco *A* on the *in vitro* embryo development in mammals, including pigs. Compared to the other species, the porcine seems to be a suitable animal model for humans, due to the ovarian similarities (14). In addition, the advantage of using pig ovaries is that the ovaries are from animals at similar age, breed and controlled nutrition. Thus, the porcine specie has been quite used as a model for human oocytes in toxicity tests (15).

A. oncocalyx and onco *A* are being studied as possible anticancer agents, most anticancer treatments are known to have harmful effects on reproduction. The aim of this study is to analyze the effects of *A. oncocalyx* and onco *A* on some reproductive parameters and to compare whether it is the same, less or more harmful than a known anticancer (DXR). Therefore, we evaluate the effect of *A. oncocalyx*, onco *A* and DXR exposure during *in vitro* maturation of oocytes (Experiment 1) or *in vitro* embryo culture (Experiment 2) on the oocyte developmental competence, investigating the following end points: oocyte viability, maturation rates and efficiency, *in vitro* fertilization parameters and percentage of cleaved embryos and blastocyst formation.

2. METHODOLOGY

2.1. Culture media

All chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. The medium used for the collection of cumulus-oocyte complexes (COCs) and for washing was Dulbecco's phosphate-buffered saline (DPBS) medium composed of 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄ and 1.46 mM CaCl₂·2H₂O supplemented with 4 mg/mL bovine serum albumin (BSA), 0.34 mM sodium pyruvate, 5.4 mM D-glucose and 70 µg/mL kanamycin (mDPBS). The oocyte

maturation medium was modified-TCM 199 supplemented with 150 μM cysteamine and 10 ng/ml Lepidermal growth factor (TCM-199+) (15). The basic medium used for fertilization was essentially the same as that used by Abeydeera and Day (16). This medium, designated as a modified Tris-buffered medium, consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 20 mM Tris (crystallized free base), 11 mM glucose and 5 mM sodium pyruvate supplemented with 2 mM caffeine and 0.2% BSA. The embryo culture medium was a sequential medium based on North Carolina State University (NCSU)-23 Medium supplemented with 0.4% BSA (15).

2.2. Isolation of onco A from A. oncocalyx

The obtaining of the A. oncocalyx and onco A has previously been described by Pessoa et al. (11). Briefly, A. oncocalyx was collected and identified by Dr. Maria Iracema B. Loiola of the Department of Biology of Federal University of Ceará. Onco A (C17H18O5) was extracted from woody parts of A. oncocalyx (Boraginaceae) by phytochemical extraction methods using organic solvents, and was isolated and purified by crystallization and recrystallization. It is noteworthy that the fraction of A. oncocalyx contains 80 % of onco A (9), therefore the concentration of A. oncocalyx was in equal proportion of onco A. A. oncocalyx and onco A were diluted with DMSO as a vehicle. The concentrations of A. oncocalyx and onco A were chosen based on previous studies performed in our laboratory (17).

2.3. Experimental design

Experiment 1: Effect of A. oncocalyx and onco A on IVM of porcine COCs and subsequent embryo development.

In this experiment, we evaluated the effect of DXR, A. oncocalyx and onco A on in vitro maturation (IVM) of porcine COCs and subsequent embryo development (Figure 1). Immediately after oocyte recovery, COCs in vitro matured in four treatments: I) TCM-199+ alone (control), or supplemented with II) 0.3 $\mu\text{g}/\text{mL}$ DXR; III) 1.2 $\mu\text{g}/\text{mL}$ A. oncocalyx or IV) 1 $\mu\text{g}/\text{mL}$ onco A. After IVM, oocyte chromatin configuration and viability were assessed. Moreover, in vitro fertilization (IVF) was performed and 18 hours post insemination (hpi) we assess fertilization parameters, and finally, after 7 (168 hpi) days we evaluated embryo development.

Experiment 2: Effect of A. oncocalyx and onco A on the in vitro culture of porcine embryos.

COCs were submitted to IVM and IVF as described for experiment 1 (control group), then, zygotes were randomly allocated into four treatments for in

vitro embryo culture (IVC): I) NCSU-23 alone (control) or supplemented with II) 0.3 µg/mL DXR; III) 1.2 µg/mL A. oncocalyx or IV) 1 µg/mL onco A. The presumptive zygotes were cultured for 18 hpi to assess fertilization parameters, and for 7 days (168 hpi) to evaluate embryo development (Figure 1).

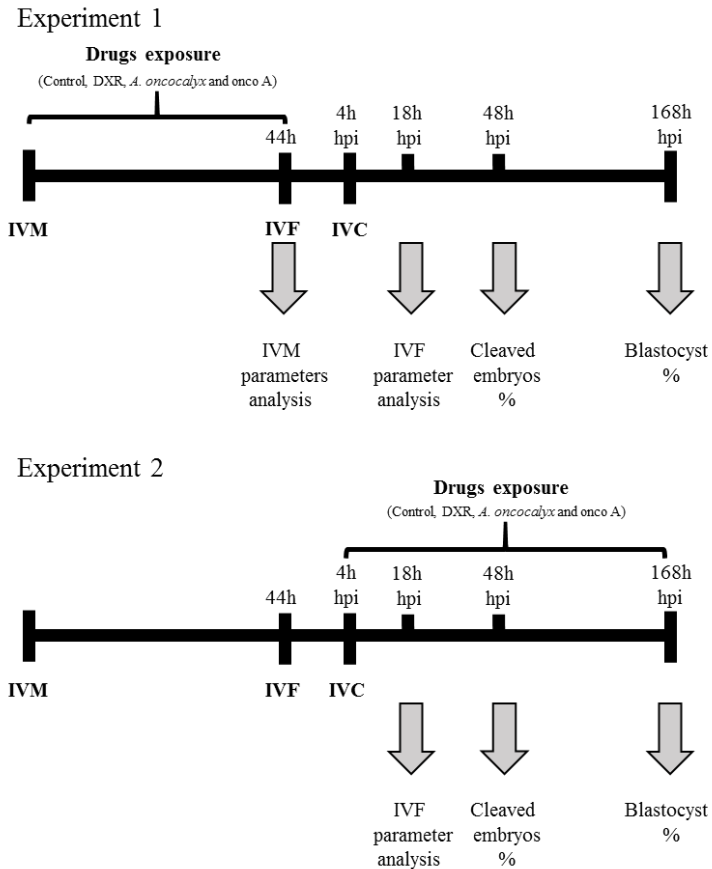


Figure 1: Experimental design and endpoints of experiment 1 and 2.

Abbreviations: *In vitro* maturation (IVM), *in vitro* fertilization (IVF), *in vitro* embryo culture (IVC), hours post insemination (hpi).

2.4. Oocyte collection and IVM

Ovaries were obtained from prepuberal gilts at a local slaughterhouse and

transported in 0.9% NaCl containing 70 µg/mL kanamycin, at 33°C within 1 h. In the laboratory, COCs were aspirated from medium-sized follicles (3 to 6 mm in diameter) using an 18-gauge needle connected to a 10-mL disposable syringe. Oocytes with a compact cumulus mass and a dark, evenly granulated cytoplasm were washed three times in maturation medium, and 50–60 oocytes were transferred into each well of a 4-well multidish (Nunc, Roskilde, Denmark) containing 500-µL of maturation medium supplemented with 10 IU/mL pregnant mare's serum gonadotropin and 10 IU/mL human chorionic gonadotropin for 20–22 h. The oocytes were then incubated for another 20–22 h in maturation medium without hormones. Oocyte maturation was carried out under mineral oil at 39°C in a humidified atmosphere of 5% CO₂ in air. After maturation, COCs were mechanically denuded (15).

2.5. In vitro fertilization and embryo culture.

Groups of 30 denuded oocytes were placed in 50-µL drops of fertilization medium in a 35x10-mm Petri dish under mineral oil and held at 38.5°C in an atmosphere of 5% CO₂ in air. Pool of freshly ejaculated semen, diluted in extender from three boars from a local breeding station, was washed three times by centrifugation at 1900 x g for 3 min in mDPBS. The resulting pellet was re-suspended in fertilization medium, and after the appropriate dilution, 50 µL was added to a 50µL drop of fertilization medium containing the oocytes (2000:1 spermatozoa:oocyte ratio). The gametes were co-incubated at 38.5°C in a humidified atmosphere of 5% CO₂ in air for approximately 4 h.

Presumptive zygotes were removed from the fertilization medium and washed three times in pre-equilibrated embryo culture medium. The zygotes were then transferred to a 4-well multidish (30 zygotes per well), with each well containing 500 µL of the same medium under mineral oil, and were cultured at 38.5°C in a humidified atmosphere of 5% CO₂ in air. Presumptive zygotes were cultured for the first 2 days (Day 0 = day of fertilization) in glucose-free NCSU-23 supplemented with 0.33 mM pyruvate and 4.5 mM lactate and then in fresh NCSU-23 medium containing 5.5 mM glucose until day 7.

2.6. Assessment of oocyte chromatin configuration, viability, sperm penetration and embryo development

To evaluate maturation and fertilization parameters, denuded oocytes and presumptive zygotes were washed in PBS-BSA, and incubated in 500 µL droplets containing 4 µM calcein-AM, 2 µM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany), 0.5% of glutaraldehyde and 10 µM Hoechst 33342 for 15 min.

The maturation rate was assessed at 44h of IVM. The chromatin configuration patterns were the following: abnormal chromatin configuration, germinal vesicle (GV) or meiotic resumption. Meiotic resumption was defined when the nucleus was in germinal vesicle break down (GVBD), metaphase I (MI) or in metaphase II (MII) stages. Maturation efficiency was calculated by MII/total oocytes cultured. Thereafter, oocytes were also examined under a fluorescence microscope (Nikon, Eclipse 80i, Tokyo, Japan) for evaluation of live/dead fluorescent staining. The emitted fluorescent signals of calcein-AM and ethidium homodimer-1 were collected at 488 and 568 nm, respectively. Oocytes were considered viable when the cytoplasm was stained positively with calcein-AM (green) and chromatin was not labelled with ethidium homodimer-1 (red) and they showed a normal chromatin configuration.

Fertilization parameters were evaluated at 18 hpi. The oocytes were considered penetrated when they contained one or more swollen sperm heads and/or male pronuclei, with their corresponding sperm tails, and two polar bodies. The fertilization parameters evaluated were penetration rate (number of oocytes penetrated/total matured), monospermy (number of oocytes containing only one male pronucleus/total penetrated), number of spermatozoa/oocyte (mean number of spermatozoa in penetrated oocytes), and efficiency of fertilization (number of monospermic oocytes/total inseminated).

At 2 and 7 days after IVF, the cleavage rate (number of oocytes divided to 2–4 cells/total) and blastocyst formation rate (number of blastocyst/total cleaved), respectively, were evaluated under a stereomicroscope. An embryo that had cleaved to the two-cell stage or beyond was counted as cleaved, and an embryo with a clear blastocele was defined as a blastocyst

2.7. Statistical analyses

Statistical analysis was carried out with Sigma Plot 11.0 (Systat Software Inc., USA). Variables presented as percentage were analysed by chi-square or Fisher's exact tests. Comparison of means among treatments (n° spermatozoa/oocyte) was evaluated by Kruskal-Wallis test. Data are presented as mean (\pm SEM) or percentage and $P < 0.05$ indicates significant difference.

3. RESULTS

3.1. Experiment 1. Effect of A. oncocalyx and onco A on IVM of porcine COCs and subsequent embryo development

The rates of oocyte viability and maturation after exposure to DXR, A.

oncocalyx and onco A are shown (Table 1). After IVM, the DXR, A. oncocalyx and onco A treatments showed a significant lower percentage of oocyte viability and maturation than control. Except for oocyte viability, A. oncocalyx and onco A showed similar results for the endpoints above being both higher ($P < 0.05$) than DXR. However, when only viable oocytes were considered to calculate the maturation rate no differences were observed among treated groups (DXR, A. oncocalyx and onco A). To determine the meiotic resumption and MI rates only viable oocytes were used. The percentage of meiotic resumption in the onco A treatment and rate of MI in the A. oncocalyx and onco A treatments were similar to DXR but higher ($P < 0.05$) than control.

The exposure of COCs to DXR, A. oncocalyx and onco A during IVM reduced significantly the IVF efficiency when compared to the control treatment (Table 2). However, when only matured oocytes were taken into account the penetration and monospermy rates as well as the number of spermatozoa/oocyte were similar ($P > 0.05$) among the treatments.

When the COCs were exposed to the three tested drugs during IVM (Figure 2), DXR, A. oncocalyx and onco A treatments showed lower ($P < 0.05$) cleaved rates compared to control. This endpoint was higher in the onco A treatment than DXR and A. oncocalyx treatments. With regard to blastocyst rate, lower ($P < 0.05$) values were observed in the DXR treatment.

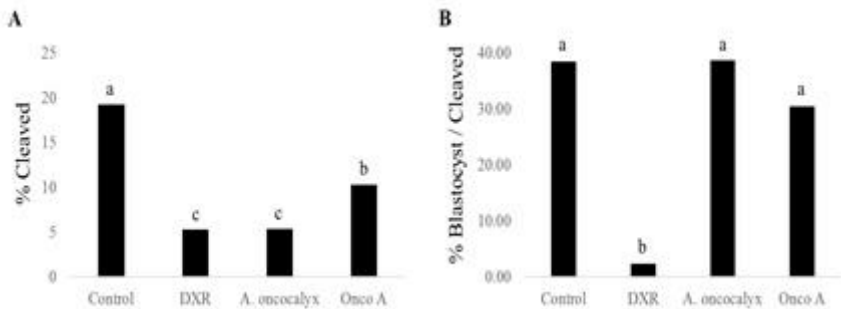


Figure 2: Experiment 1. Percentage of cleaved (A) and blastocyst/cleaved (B) after previous exposure (only during *in vitro* maturation) to DXR, A. oncocalyx or onco A. ^{a,b,c} Distinct letters represent significant differences among treatments ($P < 0.05$).

Table 1: Rates of viable oocytes, germinal vesicle (GV), meiotic resumption and metaphase II (MII) rates, after *in vitro* maturation of porcine oocytes in control medium alone or supplemented with DXR, A. oncoalyx or onco A (experiment 1).

Treatments	Total (n)	Viable oocytes				Oocyte chromatin configuration					Maturation Efficiency	
		%	Meiotic resumption*	GVBD	MI	MI	MI	MI	MI	MI	MI	MI
Control	157	81.53 (128/157) A	7.81 (10/128) A	92.19 (118/128) B	2.34 (3/128)	26.56 (34/128) B	63.28 (81/128) A	51.59 (81/157) A				
DXR	187	12.83 (24/187) D	8.33 (2/24) A	91.67 (22/24) AB	-	45.83 (11/24) AB	45.83 (11/24) AB	5.88 (11/187) C				
A. oncoalyx	181	48.62 (88/181) C	7.95 (7/88) A	92.05 (81/88) B	-	59.09 (52/88) A	32.95 (29/88) B	16.02 (29/181) B				
Onco A	163	59.51 (97/163) B	1.03 (1/97) A	98.97 (96/97) A	-	64.95 (63/97) A	34.02 (33/97) B	20.25 (33/163) B				

A,B,C,D Distinct capital letters represent significant differences among treatments (P < 0.05).
 n Total number of analyzed oocytes per treatment.

* % Meiotic resumption

3.2. Experiment 2. Effect of *A. oncocalyx* and onco A on the *in vitro* culture of porcine embryos

In the experiment 2 (Table 3), there was no difference ($P > 0.05$) between control group and other treatments regarding to viability and maturation rates. However, DXR and onco A showed a lower percentage ($P < 0.05$) of viability than *A. oncocalyx*. All the tested treatments reduced ($P < 0.05$) the penetration rate compared to control treatment. Onco A reduced significantly the monospermy and IVF efficiency, and increased ($P < 0.05$) the number of spermatozoa/oocyte. The IVF efficiency was also reduced ($P < 0.05$) in the DXR group.

The exposure of presumptive zygotes to onco A did not change either the cleavage or blastocyst rates comparing control (Figure 3). The addition of DXR and *A. oncocalyx* only reduced ($P < 0.05$) the cleavage rate.

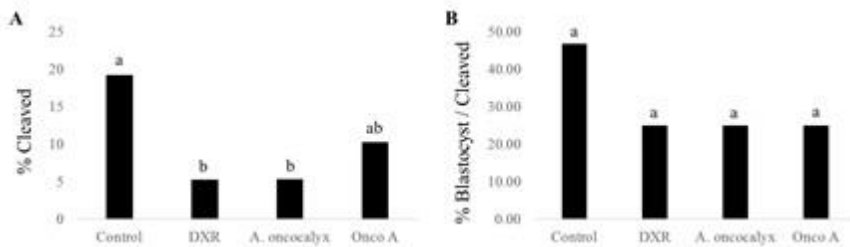


Figure 3: Experiment 2. Percentage of cleaved (A) and blastocyst / cleaved (B) after exposure (only during *in vitro* embryo culture) to DXR, *A. oncocalyx* or onco A. ^{a,b} Distinct letters represent significant differences among treatments ($P < 0.05$).

Table 2: Rates of viable oocytes, matured, penetrated, monospermy and efficiency rates and number of spermatozoa per oocyte after previous exposure (only during *in vitro* maturation) to DXR, A. *oncocalyx* or onco A(experiment 1).

Treatments	Total	Endpoints *						# SPZ / oocyte
		Viability	Matured / viable	Penetrated / matured	Monospermy / penetrated	IVF Efficiency (2pn/total)	%	
	(n)	%	%	%	%	%	%	
Control	251	78.49 (197/251) A	96.95 (191/197) A	67.02 (128/191) A	57.03 (73/128) A	29.08 (73/251) A	1.94±0.14 A	
DXR	170	38.82 (66/170) C	93.93 (62/66) A	77.42 (48/62) A	52.08 (25/48) A	14.71 (25/170) B	1.72±0.17 A	
A. <i>oncocalyx</i>	291	68.73 (200/291) B	77.50 (155/200) B	76.77 (119/155) A	45.38 (54/119) A	18.56 (54/291) B	2.15±0.13 A	
Onco A	252	66.67 (168/252) B	83.92 (141/168) B	69.50 (98/141) A	51.02 (50/98) A	19.84 (50/252) B	2.15 ± 0.2 A	

A,B,C Distinct capital letters represent significant differences among treatments (P < 0.05).

Table 3: Rates of viable oocytes, matured, penetrated, monospermy and efficiency rates and number of spermatozoa per oocyte after 18hpi exposure (only during *in vitro* embryo culture) to DXR, *A. oncocalyx* or onco A (experiment 2).

Treatments	Total (n)	Endpoints *						# SPZ / oocyte
		Viability	Matured / viable	Penetrated / matured	Monospermy / penetrated	Efficiency (2pn/total)	%	
Control	71	71.83 (51/71) AB	96.07 (49/51) A	85.71(42/49) A	54.76 (23/42) A	32.39 (23/71) A	1.43 ± 0.1 B	
DXR	87	59.7 (52/87) B	98.07 (51/52) A	64.7 (33/51) B	45.45 (15/33) AB	17.24 (15/87) BC	1.86±0.16 AB	
<i>A. oncocalyx</i>	88	75 (66/88) A	93.93 (62/66) A	69.35 (43/62) B	51.16 (22/43) AB	25 (22/88) AC	1.72±0.14 B	
Onco A	73	58.9 (43/73) B	97.67 (42/43) A	59.52 (25/42) B	28 (7/25) B	9.59 (7/73) B	2.51 ± 0.28 A	

^{A,B,C} Distinct capital letters represent significant differences among treatments (P < 0.05).

4. DISCUSSION AND CONCLUSION

To our knowledge, the present study demonstrated for the first time the effect of *A. oncocalyx* and its isolated compound, onco A, on the *in vitro* maturation of porcine oocytes and subsequent *in vitro* embryo development.

When the three tested drugs exposure occurred only during IVM (Experiment 1), DXR, *A. oncocalyx* and onco A had a detrimental effect on the oocyte viability, being DXR the most toxic with only 12.83 % viable oocytes after 44h of exposure. It is known that DXR acts on several levels by different molecular mechanisms including an interaction with iron, upsetting calcium homeostasis, altering the activity of intracellular or intra-mitochondrial oxidant enzymes, and binding to topoisomerases (TOPOs) promoting their dysfunction leading to DNA damage and apoptosis (18). Specifically, DXR acts by inhibiting Topoisomerase II (Topo II). In the female, Topo II is required for chromosome separation during oocyte meiotic maturation, but is dispensable for resumption of meiosis (19). On the other hand, *A. oncocalyx* and onco A also disrupt oocyte viability and caused damage in the chromatin configuration. Sbardelotto (20) showed that, in human promyelocytic leukemia line (HL-60) cells, onco A activates first the intrinsic apoptotic pathway by caspase 8, and then the extrinsic pathway by caspase 3 and 7. Contrary to DXR, onco A does not affect the TOPOs. However, in HL-60 cells, onco A cleaved poly (ADP-ribose) polymerase (PARP) (20). PARP binds and repair DNA-strand breaks generated by genotoxic agents. Likewise, PARP is implicated in the regulation of a wide range of important cellular processes including transcriptional regulation, chromatin modification, cellular homeostasis, and cell proliferation and death (21). Therefore, cleaved PARP results in an oocyte proapoptotic protein (22). A study conducted in porcine showed that the cleavage of PARP1 was strongly implicated in follicular development and atresia of fetal, neonatal, and adult porcine ovaries (21). In addition, PARP-1 synthesizes poly(ADP-ribose) (PAR), which is required for assembly and function of the bipolar spindle (23). PARP-1 also mediates the regulation of centrosome duplication and chromosomal stability. The inhibition of PARP-1 is associated with mislocalization of centromeric and centrosomal proteins, defective chromatin modifications and genomic instability characterized by loss of mitotic checkpoint integrity (24).

In the present study, the IVM efficiency was compromised in all treatments compared to control treatment. Interestingly, onco A increased the meiotic resumption rates. However, these oocytes were arrested at the MI stage. Anticancer drugs can cause double-strand breaks (DSBs). These DSBs do not arrest mouse oocytes in the G2/prophase but, instead, allow them to progress to the MI stage (25). The exposure of oocytes to genotoxic agents, such as

PARP inhibitors, causes failures in the spindle assembly checkpoint, leading to an oocyte meiotic arrest at MI (25). Another study showed that damage to the microtubules, main structural elements of the spindle, result in oocytes arrest at the MI stage during in vitro maturation in mouse (26). Our results suggest that onco A might be affecting the oocyte chromatin configuration, through PARP inhibition, leading to failures in the spindle assembly checkpoint and therefore causing this meiotic arrest at the MI stage.

In the present study, the exposure of COCs to the tested drugs only during IVM negatively affected the IVF efficiency. Even though DXR, A. oncocalyx and onco A reduced embryo cleavage rate, only DXR showed a more toxic effect on blastocyst development. DXR elicits apoptosis by various mechanisms in a variety of cells. DXR is capable to accumulate in both nucleus and mitochondria and induce chromosomal obliteration by inhibiting Topo-II. In oocytes, it can interfere with mitochondrial function and start the intrinsic pathway of apoptosis via the mitochondria by reducing the mitochondrial membrane potential and releasing cytochrome C (27). Impaired mitochondrial function lead to improper fertilization and a reduction of embryo development (28).

When DXR, A. oncocalyx and onco A were added only during the in vitro embryo culture (Experiment 2), all drugs negatively affected the penetration rate evaluated after 18 hpi. However, DXR and onco A showed a detrimental effect on IVF efficiency. Nonetheless, only onco A augmented the percentage of spermatozoa per oocyte. Ferreira et al. (29) showed that onco A was able to inhibit platelet aggregation by increasing the cyclic guanosine monophosphate (cGMP) levels in platelets by a synergistic mechanism, combining increased production and reduced degradation of cGMP (29). In porcine, cGMP activates cGMP-dependent protein kinase (PKG). This pathway plays an essential role in acrosome reaction, which enables the spermatozoa to penetrate the zona pellucida, and therefore, to fuse with the oocyte plasma membrane (30). Zhang (31) observed that when a cGMP analog, atrial natriuretic peptide (ANP), was added during IVF of frozen-thawed giant panda sperm with porcine salt-stored oocytes, it resulted in a higher proportion of oocytes with spermatozoa in the zona pellucida and perivitelline space, and a higher average number of spermatozoa/oocyte.

In experiment 2, DXR, A. oncocalyx and onco A negatively affected the cleavage rate. Wang et al. (32) showed that DXR blocked pre-implantation development in early mouse embryos by altering apoptosis-related gene expression, Bcl211 and Casp3, and inactivating DNA repair by PARP. In the same study, the authors found out that DXR arrested zygotes at the 1-cell stage by disruption of DNA and of the cytoskeleton. In addition, it is known

that during blastocyst formation, the inhibition PARP suppresses selective autophagic degradation of ubiquitinated proteins, which contributes to apoptosis (33). Thus, the interaction between PARP and autophagy influences the quality of in vitro produced embryos in porcine (34). Due to the fact that onco A affects PARP in HL-60 cells (20), and that A. oncocalyx contains 80% of onco A in its composition (9), both drugs could be affecting embryonic competition by this pathway. Moreover, a study evaluating different concentrations (1 to 100 µg/mL) of a quinone fraction of A. oncocalyx in sea urchin eggs reported that the cleavage of eggs was inhibited in a concentration-dependent manner (35). Despite the fact that DXR, A. oncocalyx and onco A reduced porcine embryo cleavage rate, they did not affect blastocyst development, showing that porcine blastocyst tend to be more resistant to toxic agents (36).

As expected DXR caused deleterious effects on the evaluated reproductive parameters; in brief, the addition of DXR during IVM or IVC negatively affected the IVF efficiency and cleavage rate. Even though both, A. oncocalyx and onco A, impair in vitro porcine oocyte developmental competence, they were less detrimental than DXR in oocyte viability and blastocyst formation.

In conclusion, although A. oncocalyx and onco A showed toxic behavior, they are less harmful than DXR in the reproductive parameters studied. Therefore, more studies are required to determine if it really would be a less toxic treatment alternative than conventional anticancer in terms of reproductive harm.

REFERENCES

1. Cragg GM, Grothaus PG, Newman DJ. New Horizons for Old Drugs and Drug Leads. *J Nat Prod.* 2014;77:703–23.
2. Oktem O, Oktay K. Quantitative assessment of the impact of chemotherapy on ovarian follicle reserve and stromal function. *Cancer* [Internet]. 2007 Nov 15 [cited 2014 Oct 15];110(10):2222–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17932880>
3. Kropp J, Roti ECR, Ringelstetter A, Khatib H, Abbott DH, Salih SM. Dexrazoxane Diminishes Doxorubicin- Induced Acute Ovarian Damage and Preserves Ovarian Function and Fecundity in Mice. *PLoS One.*
4. Graham JG, Quinn ML, Fabricant DS, Farnsworth NR. Plants used against cancer – an extension of the work of Jonathan Hartwell. *J Ethnopharmacol.* 2000;73:347–77.
5. Braga R. Plantas do Nordeste, especialmente do Ceará. 3rd ed. Mossoró; 1976. 103 p.

6. Ferreira MAD, Nunes ODRH, Leal LKAM, Pessoa ODL, de Lemos TLG, Viana GSDB. Antioxidant effects in the quinone fraction from *Auxemma oncocalyx* TAUB. *Biol Pharm Bull.* 2003;26(5):595–9.
7. Pessoa C, Mendes CS, Pessoa LO, Sabino SH, Lemos T., Moraes MO. Avaliação da atividade antitumoral de *Auxemma oncocalyx* Taub. (Pau Branco). In: VII Annual Meeting of the Federação de Sociedades de Biologia Experimental, Caxambu, MG, Brazil. 1992. p. 26–9.
8. Lino CS, Pessoa ODL, Lemos TLC, Viana GSB. Estudo da atividade analgésica e antiedematogênica do extrato hidroalcoólico de *Auxemma oncocalyx* e *oncocalyxona A*. In: Simpósio De Plantas Medicinais Do Brasil. 1996. p. 95.
9. Ferreira MAD, Nunes ODRH, Fontenele JB, Pessoa ODL, Lemos TLG, Viana GS. Analgesic and anti-inflammatory activities of a fraction rich in *oncocalyxona A* isolated from *Auxemma oncocalyx*. *Phytomedicine.* 2004;11(4):315–22.
10. Costa-lotufo LV, Cunha GMA, Farias PAM, Viana GSB, Cunha KMA, Pessoa C, et al. The cytotoxic and embryotoxic effects of kaurenoic acid , a diterpene isolated from *Copaifera langsdorffii* oleo-resin. *Toxicon.* 2002;40:1231–4.
11. Pessoa C, Vieira FMAC, Lemos TG, Moraes MO, Lima PDL, Rabenhorst SHB, et al. *Oncocalyxona A* from *Auxemma oncocalyx* lacks genotoxic activity in phytohemagglutinin-stimulated lymphocytes. *Teratog Carcinog Mutagen* [Internet]. 2003 Jan [cited 2014 Oct 14];Suppl 1:215–20. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12616611>
12. Leiva-revilla J, Lima LF, Castro SV, Campello CC, Araújo VR, Celestino JJH, et al. Fraction of *Auxemma oncocalyx* and *Oncocalyxona A* affects the in vitro survival and development of caprine preantral follicles enclosed in ovarian cortical tissue. *Forsch Komplementarmed.* 2016;in press.
13. Schoevers EJ, Fink-Gremmels J, Colenbrander B, Roelen BAJ. Porcine oocytes are most vulnerable to the mycotoxin deoxynivalenol during formation of the meiotic spindle. *Theriogenology* [Internet]. 2010;74(6):968–78. Available from: <http://dx.doi.org/10.1016/j.theriogenology.2010.04.026>
14. Santos RR, Schoevers EJ, Roelen BA. Usefulness of bovine and porcine IVM/IVF models for reproductive toxicology. *Reprod Biol Endocrinol* [Internet]. 2014;12(1):117. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25427762>
15. Gil M, Abeydeera L, Day B, Vazquez J, Roca J, Martinez E. Effect of the volume of medium and number of oocytes during in vitro fertilization on embryo development in pigs. *Theriogenology.* 2003;60:767–76.

16. Abeydeera LR, Day BN. In vitro penetration of pig oocytes in a modified Tris-buffered medium: effect of BSA, caffeine and calcium. *Theriogenology*. 1997;48(4):537-44.
17. Leiva-Revilla J, Cadenas J, Vieira LA, Campello CC, Celestino JJ de H, Pessoa ODL, et al. Toxicity effect of Auxemma oncocalyx fraction and its active principle oncocalyxone A on in vitro culture of caprine secondary follicles and in vitro oocyte maturation. *Semin Ciências Agrárias* [Internet]. 2017 Jun 13;38(3):1361. Available from: <http://www.uel.br/revistas/uel/index.php/semagrarias/article/view/25905>
18. Mitry MA, Edwards JG. Doxorubicin induced heart failure: Phenotype and molecular mechanisms. *Int J Cardiol Hear Vasc*. 2016;10:17-24.
19. Li X, Yu C, Wang Z, Zhang Y, Liu X, Zhou D, et al. DNA Topoisomerase II Is Dispensable for Oocyte Meiotic Resumption but Is Essential for Meiotic Chromosome Condensation and Separation in Mice. *Biol Reprod*. 2013;89(5):118.
20. Sbardelotto AB. ESTUDO DO MECANISMO DE CITOTOXICIDADE DA ONCOCALIXONA-A EM LEUCEMIA PROMIELOCÍTICA HUMANA - LINHAGEM HL-60. 2013.
21. Wei Q, Ding W, Shi F. Roles of poly (ADP-ribose) polymerase (PARP1) cleavage in the ovaries of fetal, neonatal, and adult pigs. *Reproduction*. 2013;146:593-602.
22. Chen Z, Kang X, Wang L, Dong H, Wang C, Xiong Z, et al. Rictor / mTORC2 Pathway in Oocytes Regulates Folliculogenesis , and Its Inactivation Causes Premature Ovarian Failure. *J Biol Chem*. 2015;290(10):6387-96.
23. Johannes JW, Almeida L, Daly K, Ferguson AD, Grosskurth SE, Guan H, et al. Discovery of AZ0108 , an orally bioavailable phthalazinone PARP inhibitor that blocks centrosome clustering. *Bioorg Med Chem Lett*. 2015;25(24):5743-7.
24. Weaver AN, Yang ES. Beyond DNA repair : additional functions of PARP-1 in cancer. *Front Oncol*. 2013;3(November):1-11.
25. Marangos P, Stevense M, Niaka K, Lagoudaki M, Nabti I, Jessberger R, et al. DNA damage-induced metaphase I arrest is mediated by the spindle assembly checkpoint and maternal age. *Nat Commun*. 2015;6(May):1-10.
26. Jiang M-X, Shi Y, Sun Z-G, Zhang Z, Zhu Y. Inhibition of the Binding between RGS2 and β -Tubulin Interferes with Spindle Formation and Chromosome Segregation during Mouse Oocyte Maturation In Vitro. *PLoS One*. 2016;11(7).
27. Bar-Joseph H, Ben-Aharon I, Rizel S, Stemmer SM, Tzabari M, Shalgi R. Doxorubicin-induced apoptosis in germinal vesicle (GV) oocytes. *Reprod Toxicol* [Internet]. 2010 Dec [cited 2014 Oct 12];30(4):566-72. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/20656019>

28. Wakefield SL, Lane M, Mitchell M. Impaired Mitochondrial Function in the Preimplantation Embryo Perturbs Fetal and Placental Development in the Mouse. *Biol Reprod*. 2011;84:572–80.
29. Ferreira MAD, do Nascimento NRF, de Sousa CM, Pessoa ODL, de Lemos TLG, Ventura JS, et al. Oncocalyxone A inhibits human platelet aggregation by increasing cGMP and by binding to GP Ib-alpha glycoprotein. *Br J Pharmacol* [Internet]. 2008 Jul [cited 2014 Oct 14];154:1216–24. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2483390&tool=pmcentrez&rendertype=abstract>
30. Zhang M, Hong H, Zhou B, Jin S, Wang C, Fu M, et al. The expression of atrial natriuretic peptide in the oviduct and its functions in pig spermatozoa. *J Endocrinol*. 2006;189:493–507.
31. Zhang M, Tang H, Shen G, Zhou B, Wu Z, Peng Z, et al. Atrial natriuretic peptide induces an acrosome reaction in giant panda spermatozoa and enhances their penetration of salt-stored porcine oocytes. *Theriogenology*. 2005;64(6):1297–308.
32. Wang Q, Sun S, Han J, Kwak Y, Kim N, Cui X. Doxorubicin Induces Early Embryo Apoptosis by Inhibiting Poly (ADP ribose) Polymerase. *In Vivo* (Brooklyn). 2012;834:827–34.
33. Ran H, Hyoun D, Gyeong M, Sung J, Ho J, Taek H. Biochemical and Biophysical Research Communications The regulation of autophagy in porcine blastocysts : Regulation of PARylation-mediated autophagy via mammalian target of rapamycin complex 1 (mTORC1) signaling. *Biochem Biophys Res Commun*. 2016;473(4):899–906.
34. Lee HYERAN, Gupta MK, Kim DUKH, Hwang JHO, Kwon B, Lee HT. Poly (ADP-ribosyl) ation Is Involved in Pro-Survival Autophagy in Porcine Blastocysts. *Mol Reprod Dev*. 2016;83:37–49.
35. Costa-Lotufo LV, Ferreira MAD, Lemos TLG, Pessoa ODL, Viana GSB, Cunha GM. Toxicity to sea urchin egg development of the quinone fraction obtained from *Auxemma oncocalyx*. *Brazilian J Med Biol Res*. 2002;35(8):927–30.
36. Schoevers EJ, Santos RR, Fink-gremmels J, Roelen BAJ. Toxicity of beauvericin on porcine oocyte maturation and preimplantation embryo development. *Reprod Toxicol*. 2016;65:159–69.