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TECHNOLOGICAL INNOVATIONS

Crotamine, a cell-penetrating peptide, is able to translocate parthenogenetic and in vitro fertilized bovine embryos but does not improve exogenous DNA expression

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Abstract
Purpose Crotamine is capable of penetrating cells and embryos and transfecting cells with exogenous DNA. However, no studies are available regarding its uptake by parthenogenetic (PA) embryos or its use for transfection in in vitro fertilized (IVF) embryos. This study aimed to determine the translocation kinetics of crotamine into PA and IVF bovine embryos and assess its effect over in vitro development of PA embryos. Moreover, crotamine-DNA complexes were used to test the transfection ability of crotamine in bovine IVF zygotes.
Methods PA and IVF embryos were exposed to labeled crotamine for four interval times. Embryo toxicity was assayed over PA embryos after 24 h of exposure to crotamine. Additionally, IVF embryos were exposed to or injected with a complex formed by crotamine and pCX-EGFP plasmid.
Results Confocal images revealed that crotamine was uptaken by PA and IVF embryos as soon as 1 h after exposure. Crotamine exposure did not affect two to eight cells and blastocyst rates or blastocyst cell number ($p > 0.05$) of PA embryos. Regarding transfection, exposure or injection into the perivitelline space with crotamine-DNA complex did not result in transgene-expressing embryos. Nevertheless, intracytoplasmic injection of plasmid alone showed higher expression rates than did injection with crotamine-DNA complex at days 4 and 7 ($p < 0.05$).
Conclusions Crotamine is able to translocate through zona pellucida (ZP) of PA and IVF embryos within 1 h of exposure without impairing in vitro development. However, the use of crotamine does not improve exogenous DNA expression in cattle embryos, probably due to the tight complexation of DNA with crotamine.

Keywords Cattle · Peptide uptake · Gene delivery · Preimplantation embryo · Transgene expression

Capsule Crotamine is able to translocate through zona pellucida of PA and IVF embryos, but did not improve the efficiency of transgene expression in cattle embryo.

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Introduction

Crotamine is a highly cationic cell-penetrating peptide (CPP) isolated from the venom of South American rattlesnake *Crotalus durissus terrificus*. It has been demonstrated to have membrane translocation capacity and to carry DNA inside of cells [1]. Despite the majority of CPPs not displaying any cell specificity, crotamine is specifically uptaken by actively proliferating cells in vivo and is also able to penetrate into cells from several lineages in vitro [2, 3].

Crotamine penetration into mammalian embryos was first reported in mouse morulas, demonstrating its capability for translocating through the zona pellucida (ZP) [2]. More recently, it was observed that crotamine is also able to translocate the ZP of bovine in vitro fertilized (IVF) zygotes without impairing embryo development [4]. Despite these facts, no studies are available so far regarding the uptake of crotamine by parthenogenetic embryos. Parthenogenetic activation (PA)

is a widespread method used for the study of imprinting mechanisms in mammals [5, 6]. Moreover, PA is an essential step to assist two techniques usually applied for the production of transgenic animals: somatic cell nuclear transfer (SCNT) [7] and intracytoplasmic sperm injection (ICSI) [8].

Crotamine also shows high positive electric charge, turning it capable of stably binding to DNA molecules. For these reasons, it has been successfully used for cell transfection both in vitro and in vivo [9]. These characteristics turn crotamine into an interesting candidate for foreign DNA delivery into preimplantation embryos. However, the gene delivery capability of crotamine has not been studied in mammalian embryos.

To date, the delivery of foreign DNA into farm animals can be achieved by several methods, including pronuclear microinjection [10], sperm-mediated gene transfer [11, 12], electroporation [13], somatic cell nuclear transfer employing genetically modified donor cells [14], and gene transfer mediated by retroviruses [15] or cationic molecules [16]. However, the efficiency of these techniques still remains low. In order to improve efficiency in farm animals, some vector modification-based approaches, such as transposon [17] and CRISPR/Cas9 systems [18], gained increasing interests in recent years. However, efficient tools for the delivery of vector DNA still need more studies.

In this regard, the use of cationic molecules, such as liposome, polymers, and CPPs, has become a useful tool for gene transfection, due to its low toxicity and relative effectiveness to deliver foreign DNA into somatic cells in vitro [13, 16]. These biomaterials are able to readily conjugate nucleic acids and to deliver DNA molecules into a wide variety of mammalian somatic cells [19–21].

Among the few CPPs explored for gene transfer, crotamine appears as a promising delivery tool. Based on these facts, we hypothesize that crotamine could facilitate the delivery of different types of nucleotides into both PA and IVF bovine embryos. Therefore, the objectives of this study were (i) to verify the capability of crotamine to translocate the ZP of PA and IVF bovine embryos after different times of exposure, (ii) to assess the effect of crotamine over in vitro development of PA bovine embryos, and (iii) to evaluate the efficiency of transfection mediated by crotamine in bovine IVF embryos as assessed by means of heterologous expression of a reporter gene.

Materials and methods

All chemicals were from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise indicated.

Experimental design

Three experiments were performed. In experiment 1, PA and IVF embryos were exposed for different times (1, 6, 17, and 24 h) to rhodamine B (RhoB) labeled-crotamine and its translocation into embryos was evaluated by confocal microscopy. In experiment 2, the toxicity of the peptide over the in vitro development of PA embryos were determined through embryo exposure to the highest concentration of unlabeled crotamine (10 μM) and time (24 h) as determined to bovine IVF zygotes in a previous study [4]. To assess in vitro development, two to eight cells and blastocyst production rates as well as total number of cells *per* blastocyst were determined, using PA embryos as control group. In experiment 3, crotamine-mediated transfection was evaluated, determining conditions for stable crotamine-plasmid DNA complex formation and efficiency of transfection in bovine IVF zygotes. Crotamine-DNA complexes were prepared in the solution (2.5:1; wt/wt), transferred to synthetic oviductal fluid (SOF) or PBS media, and maintained for 1 and 24 h. The formation of crotamine-DNA complexes was confirmed by electrophoretic mobility shift on agarose gel. Bovine zygotes were treated with crotamine-DNA complexes or DNA alone in three different ways: incubation in culture medium, perivitelline injection, and intracytoplasmic injection.

Crotamine isolation

Pure crotamine was kindly provided by Dr. Eduardo Brandt, Faculty of Medicine, University of São Paulo (Ribeirão Preto, Brazil). The method employed for crotamine purification was previously described as an established protocol [2]. Briefly, the venom was diluted with ammonium formate buffer and the bulk of crotoxin, the major venom component, was eliminated by slow-speed centrifugation. Tris-base was added and the solution was applied to a CM-Sepharose FF column (1.5 × 4.5 cm; Amersham-Biosciences, Buckinghamshire, UK). Afterward, the column was washed and crotamine was recovered, dialyzed, lyophilized, and stored at room temperature until use.

Labeling of crotamine

Crotamine was labeled with RhoB dye at the N-terminus as previously described [22]. Briefly, linear crotamine made by Fmoc chemistry was N-deblocked and RhoB coupled by means of N, N′-diisopropylcarbodiimide (10-fold molar excess of both) in dichloromethane. Full deprotection and resin cleavage was carried out and the labeled peptide purified by reverse phase on a C18 column according to previous report [23]. The correct molecular weights of RhoB-crotamine fractions were confirmed by mass spectrometry analysis. Then, the fractions were pooled and lyophilized. Finally, the oxidative peptide folding was achieved with a diluted solution of labeled-crotamine (5–10 μM) in the presence of reduced (GSH) and oxidized (GSSG) glutathione (1:100:10 peptide/GSH/GSSG molar ratio) and the

fluorescent-labeled peptide repurified by HPLC and lyophilized. Dried RhoB-crotamine was maintained at −20 °C until further use.

DNA construction

The plasmid used was pCX-EGFP that contains the enhanced green fluorescent protein (*EGFP*) gene under the chimeric cytomegalovirus-IE-chicken β-actin enhancer-promoter control (a strong and early expression promoter for mammals) in circular form [24]. The plasmid was kindly provided by Dr. Masaru Okabe (Osaka University, Osaka, Japan).

Crotamine-DNA complex formation and stability assay

Crotamine-DNA complex was performed as previously described [9]. Crotamine-plasmid DNA (CrP) solution was formed by mixing 2.5 μg of crotamine and 1.0 μg of plasmid in 150 mM NaCl buffer for 15 min. Plasmid alone (P) was used as positive control. CrP or P solution was diluted 1:10 in SOF (with 2.5 % of FBS) or in PBS (without FBS) media. Both solutions were incubated in 50-μL droplets under mineral oil at 38.5 °C for 1 or 24 h (SOF 1 h, SOF 24 h, PBS 1 h, and PBS 24 h groups). Moreover, solutions that were not incubated (CrP and P) were used as positive controls. SOF and PBS media were used as negative controls. After incubations, 20 μL of the mixtures were recovered and evaluated by electrophoresis mobility shift assay in an agarose gel (1 %, wt/vol), using Tris borate-EDTA buffer. Mixtures were stained in gel with ethidium bromide and visualized under ultraviolet (UV) light.

Oocyte collection and in vitro maturation

Ovaries from slaughtered cows were collected and transported to the laboratory. *Cumulus*-oocyte complexes (COCs) from follicles of 2 to 8 mm of diameter were aspirated using an 18-ga needle attached to a syringe. Only COCs covered with at least three layers of cumulus cells were selected for in vitro maturation (IVM). The COCs were washed in HEPES-buffered Tyrode's albumin lactate pyruvate (H-TALP) medium. Groups of up to 20 COCs were placed into 100-μL droplets of maturation medium, which consisted of bicarbonate-buffered TCM-199 (11150-059; Gibco, Grand Island, NY, USA) containing 10 % (*v*/*v*) FBS (013/07; Internegocios, CABA, Argentina), 2-μg/mL follicle stimulating hormone (NIH-FSH-P1; Folltropin; Bioniche, Melbourne, Australia), 0.1 mM sodium pyruvate (S8636), 100 μM cysteamine (M9768), and 2 % (*v*/*v*) antibiotic-antimycotic (15240-096; Gibco) under mineral oil (0121-1; Fisher Scientific, Pittsburgh, PA, USA). IVM was performed at 38.5 °C for 22 to 24 h in a humidified atmosphere of 6.5 % CO_2.

Parthenogenetic activation

After IVM, cumulus cells were removed from COCs by vortexing for 2 min in 1-mg/mL hyaluronidase (H4272). Oocytes with an extruded first polar body were immediately treated with 5 μM ionomycin (I24222; Invitrogen, Carlsbad, CA, USA) in H-TALP for 4 min, followed by an incubation in 1.9 mM 6-dimethylaminopurine (6-DMAP; D2629) in maturation medium for 3 h. Afterwards, oocytes were thoroughly washed in H-TALP and randomly assigned to different experimental groups according to the assay.

In vitro fertilization

Frozen semen was thawed in a water bath at 37 °C for 30 s. Sperm were then centrifuged twice (490×*g* for 5 min) in a Brackett-Oliphant (BO) medium [25] supplemented with 5 mM caffeine (C4144) and 20 IU/mL heparin (H3149). Sperm concentration was adjusted to 32×10^6 spermatozoa/mL and subsequently diluted with BO medium containing 20-mg/mL bovine serum albumin (A7906) to half concentration. Matured COCs were washed with H-TALP and incubated in 100-μL droplets of sperm suspension covered with mineral oil at 38.5 °C in 6.5 % CO_2. After 5 h of incubation, presumptive zygotes were denuded by vortexing for 2 min in 1 mg/mL hyaluronidase (H4272) and subjected to different experimental groups according to the experiment.

Translocation assay

For assessment of crotamine translocation, embryos were exposed to RhoB-crotamine immediately postactivation (for PA) or postfertilization (for IVF) at final concentration of 10 μM in synthetic oviductal fluid (SOF) medium [26, 27] for 1, 6, 17, and 24 h at 6.5 % CO_2 and 38.5 °C. After exposure, embryos were washed in H-TALP and ZP was removed by treatment with 1.5-mg/mL pronase (P8811). Embryos were fixed in 4 % (*v*/*v*) paraformaldehyde (F1635) for 20 min and washed for 30 min in PBS. Nuclear DNA was counterstained with 0.2 mg/mL of DAPI (D9542) for 10 min and washed in PBS for 5 min. Embryos were mounted between coverslips in 70 % (*v*/*v*) glycerol. RhoB-crotamine was detected by confocal microscopy on a Zeiss LSM 510 Meta Confocal microscope (Zeiss, Oberkochen, Germany). DAPI and RhoB fluorescence was detected with excitation wavelengths of 405 and 543 nm, respectively. Complete Z series of 12 optical sections at 4–5-μm intervals were acquired from each embryo, and three-dimensional images were constructed using the LSM Image Browser software (Zeiss). The number of evaluated embryos was 9, 10, 9, and 11, for the PA group, and 11, 13, 6, and 15, for the IVF group, at 1, 6, 17, and 24 h, respectively. Three PA and IVF embryos were used as

negative and positive controls for RhoB-crotamine (red) and DAPI (blue) fluorescence, respectively.

Crotamine exposure

PA embryos were exposed to unlabeled crotamine in 50-μL droplets composed of 5 μL of crotamine solution and 45 μL of SOF medium under mineral oil at 38.5 °C and 6.5 % CO_2 for 24 h. PA embryos not subjected to crotamine exposure were used as controls.

Crotamine-plasmid complex exposure and injection

For exposure treatment, IVF presumptive zygotes were transferred to 50-μL droplets composed of 5 μL of crotamine-plasmid complex solution (performed as described above) and 45 μL of SOF medium reaching a final concentration of 10 μM crotamine (CrP exposure group). Exposure was accomplished for 1 h (according to results of complex stability assay) under mineral oil at 38.5 °C and 6.5 % CO_2. A control treatment was performed incubating embryos with plasmid alone (P exposure group). For perivitelline and intracytoplasmic injection treatment, crotamine-plasmid complex (CrP perivitelline and CrP cytoplasm groups) and plasmid alone (P cytoplasm group) solutions were diluted in 10 % polyvinylpyrrolidone (PVP; 99219; Irvine Scientific, Santa Ana, CA, USA), containing 50 ng/mL of pCX-EGFP plasmid for both solutions. IVF presumptive zygotes were transferred to 100-μL droplets of H-TALP under mineral oil in 100 × 20-mm tissue culture dishes (430167; Corning, Corning, NY, USA) and micromanipulated using a Narishige hydraulic micromanipulator (Narishige Sci., Tokyo, Japan) mounted on a Nikon Eclipse E-300 microscope (Nikon, Tokyo, Japan). For intracytoplasmic injection (CrP cytoplasm and P cytoplasm groups), the oolemma was broken by aspiration with the injection pipette (inner diameter of 9 μm) and PVP containing the working solutions was injected into the cytoplasm of each zygote. For perivitelline injection (CrP perivitelline group), PVP containing the working solution was expelled on the perivitelline space, until observing an increase of the space between the ZP and the oolemma on the site of injection. Besides, an IVF control group was performed.

In vitro embryo development

After treatment with crotamine or crotamine-plasmid complex, the in vitro culture was performed in 50-μL droplets of SOF at 6.5 % CO_2 and 38.5 °C in high humidity with 20–30 embryos per droplet. The stage of two to eight cells was assessed at days 1 and 2 for PA and IVF embryos, respectively. Blastocyst production was evaluated at day 7 of culture for both PA and IVF groups. Assessment of EGFP fluorescence in IVF embryos was performed by a brief exposure to UV light (excitation at 488 nm and emission at 530 nm) and observed under an epifluorescence microscope (Eclipse TE300; Nikon), at days 4 and 7 after IVF. Moreover, PA and IVF blastocysts at day 7 were stained with Hoechst 33342 and mounted between coverslips to count the total number of cells under UV light.

Statistical analysis

Statistical analysis of two to eight cell rates, blastocysts production, and EGFP-expressing embryos were assessed by non-parametric Fisher's exact test for experiments 2 and 3. Total number of cells per blastocyst was evaluated by *t* test for experiment 2 and by one-way ANOVA for experiment 3. For all tests, a p value <0.05 was considered statistically significant, using Prism 6.01 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Experiment 1: crotamine translocation into PA and IVF embryos

In the first experiment, it was possible to record the fluorescence signal of RhoB-crotamine and DAPI at all time intervals assayed for both PA and IVF embryos. Control embryos showed no red fluorescence signal, while blue fluorescence signal was present for embryos of both groups. The pattern of fluorescence staining corresponding to RhoB-crotamine and DAPI in both groups of embryos and in all time intervals can be observed in Fig. 1. In addition, imaging projections of PA and IVF embryos exposed to RhoB-crotamine for 17-h time interval can be accessed as animation (Online Resource 1 and 2, respectively).

Experiment 2: embryo toxicity of crotamine over PA embryos

The results obtained from four replicates are summarized in Table 1. The treatment of PA embryos with 10 μM unlabeled crotamine during the initial 24 h of embryo culture did not result in significant differences ($p > 0.05$) in terms of in vitro development, measured as rate of progression to the two to eight cell stage (76.2 %) and blastocyst production (18.9 %), compared to the PA control group (74.6 and 24.6 %, respectively). Concerning the blastocyst quality, no significant differences were found ($p > 0.05$) for the total number of cells per blastocyst between crotamine (89.38 ± 28.34) and control group (89.50 ± 16.72).

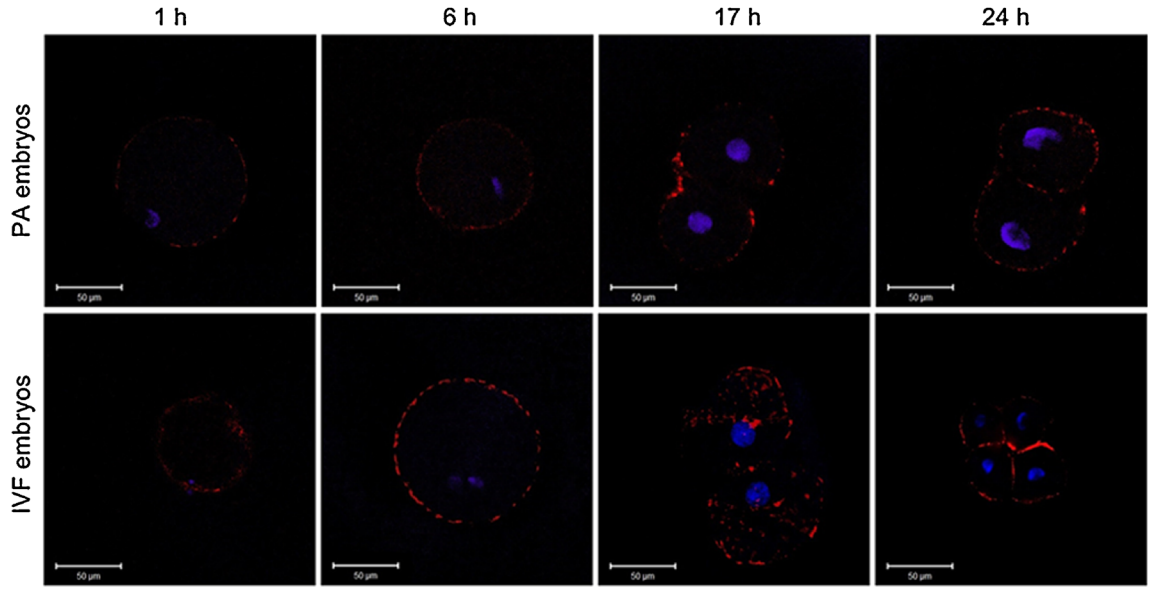

Fig. 1 Confocal microscopy imaging of crotamine translocation into PA and IVF bovine embryos after 1 h, 6 h, 17 h and 24 h of exposure to rhodamine B-labelled crotamine. Crotamine labelling (red) and nuclei counterstaining with DAPI (blue) were observed into fixed zygotes. Scale bars 50 μm

Experiment 3: transfection of IVF embryos with stable complex of crotamine-DNA

The stability of crotamine-plasmid DNA complex was confirmed by mobility shift in agarose gel (Fig. 2). The peptide-plasmid DNA complex formation occurred in the presence of SOF or PBS media (Fig. 2, lanes CrP + SOF and CrP + PBS). As expected, stable crotamine-plasmid complex (CrP) did not show mobility in gel, a phenomenon caused by neutralization of negative charges of DNA by crotamine complexation and consequently lack of electrophoretic mobility. DNA signal was not detected in the solution of plasmid alone, incubated in SOF for 24 h (P + SOF 24 h). In contrast, typical well-resolved DNA bands were evidenced in agarose gel with samples of plasmid incubated alone (without crotamine) in PBS for 1 and 24 h and SOF for 1 h. This latter condition (incubation in SOF medium for 1 h) was selected for further transfection assays with embryos. Negative controls (only PBS and SOF media) were loaded into the gel and no band was detected, as expected for samples that do not contain DNA.

To assess the efficiency of crotamine to transfect bovine embryos, early IVF zygotes were either exposed (CrP or P exposure groups) or microinjected into the perivitelline space (CrP perivitelline group) or into the cytoplasm (CrP or P cytoplasm groups) with the stable complex of crotamine and plasmid DNA. Results for in vitro embryo development and EGFP expression rates are shown in Table 2 and Fig. 3, respectively. According to in vitro development, two to eight cell rates did not differ ($p > 0.05$) between CrP exposure (68.4 %) and P exposure (76.6 %) groups. Meanwhile, two to eight cell rates were higher ($p < 0.05$) for CrP perivitelline (93.1 %), CrP cytoplasm (86.6 %), and P cytoplasm (93.1 %) groups, with no differences among them ($p > 0.05$). For the IVF control group, the two to eight cell rates (85.0 %) was similar to all groups assayed except for CrP perivitelline, which presented a higher rate ($p < 0.05$).

Concerning blastocyst production, both CrP exposure and CrP cytoplasm groups showed lower rates (18.0 and 22.3 %, respectively) than those observed for the CrP perivitelline (37.1 %) group ($p < 0.05$), which did not differ from the IVF control group (34.6 %, $p > 0.05$). In accordance, blastocyst

Table 1 Effect of exposure to 10 μM crotamine for 24 h over development of parthenogenetically activated bovine embryos

Treatment	Presumptive embryos	2-8 cells (%)	Blastocysts (%)[a]	Total number of cells per blastocyst mean ± SD (*n*)
PA crotamine	122	93 (76.2)a	23 (18.9)a	89.38 ± 28.34 (13)a
PA control	122	91 (74.6)a	30 (24.6)a	89.50 ± 16.72 (12)a

Experiment was replicated 4 times. Different lowercase letters within the same column differ significantly ($p < 0.05$)

[a] Rates calculated at day 7 over embryos

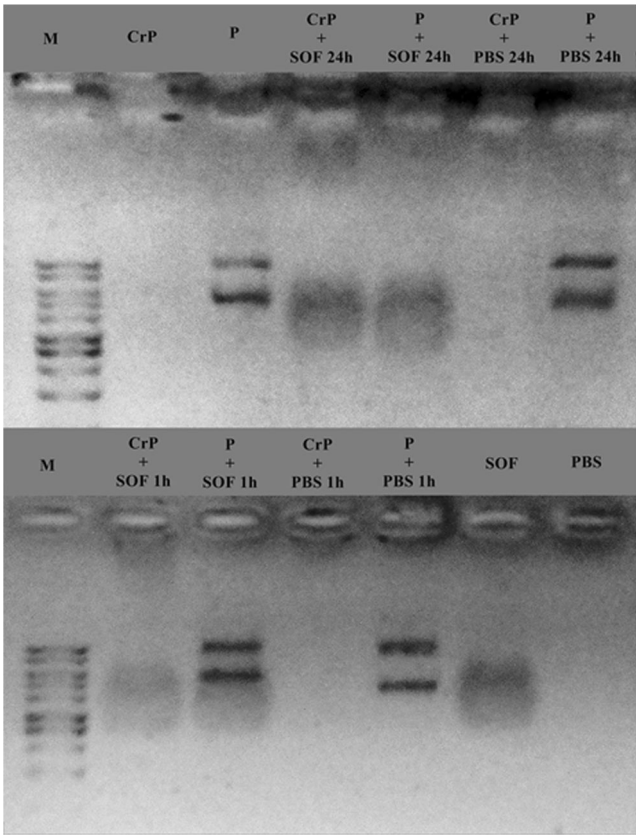

Fig. 2 Gel shift assay of crotamine-DNA complex stability examined by the alteration of electrophoretic mobility of the complex on agarose gel (1 %). Crotamine-DNA complex (CrP) and plasmid DNA alone (P) were incubated for 24 or 1 h in SOF or PBS

production rates did not differ ($p > 0.05$) between the P exposure (30.5 %) and P cytoplasm (39.5 %) groups, even when compared to the IVF control group.

The numbers of cells per blastocyst (mean ± SD) were 106.0 ± 27.1 for CrP exposure, 107.4 ± 25.5 for P exposure, 115.5 ± 32.5 for CrP perivitelline, 108.7 ± 37.7 for CrP cytoplasm, and 110.7 ± 32.3 for P cytoplasm groups. None of the treatments affected ($p > 0.05$) the mean number of cells per blastocyst when compared to the IVF control (109.1 ± 29.3).

With regard to the EGFP expression, neither CrP exposure nor P exposure or CrP perivitelline groups showed protein expression at days 4 and 7 of in vitro development. In contrast, the P cytoplasm group showed 56.1 and 48.9 % of EGFP-expressing embryos at days 4 and 7, respectively. These rates were higher ($p < 0.05$) than those observed for the CrP cytoplasm group (10.3 and 8.0 % for days 4 and 7, respectively). The expression pattern of EGFP in blastocysts at day 7 of in vitro culture for all exposed or microinjected groups are shown in Fig. 4.

Discussion

To the best of our knowledge, this is the first report to evaluate the kinetics of crotamine uptake by PA bovine embryos and its potential as an agent to induce transgene expression in IVF bovine embryos.

In the present work, we first monitored the kinetics of crotamine uptake by PA and IVF embryos (experiment 1). RhoB-crotamine translocated across the ZP of both types of embryos after 1 h of exposure. The accumulation of labeled crotamine was not observed in the zygote's cytoplasm even after 24-h exposure time in any of the groups. These results are consistent with a previous study, in which the migration of the labeled crotamine to the inner cell mass of intact mouse blastocysts was observed [2]. Unlike observations for murine and human in vitro cultured cells showing complete or partial overlap of crotamine fluorescence signal in the nuclei [2], our results suggest that crotamine lacks nuclear compartmentalization specificity in early bovine embryos. In addition, the localization pattern of crotamine in PA embryos was similar to previous observations for IVF bovine embryos [4].

Results from experiment 2 showed that, under the conditions tested, crotamine had no deleterious effects over embryonic development, taking the in vitro development of

Table 2 Comparison of embryo development of exposed and injected bovine IVF zygotes with crotamine-plasmid complex (+) or plasmid alone (−)

Treatment	Crotamine	Presumptive zygotes	Two to eight cells (%)	Blastocysts (%)
Intracytoplasmic injection	+	112	97 (86.6)ac	25 (22.3)bc
	−	114	98 (86.0)ac	45 (39.5)a
Perivitelline injection	+	116	108 (93.1)c	43 (37.1)a
Exposure	+	133	91 (68.4)b	24 (18.0)b
	−	141	108 (76.6)ab	43 (30.5)ac
IVF control	na	133	113 (85.0)a	46 (34.6)a

Experiment was replicated at least three times for injection groups and five times for exposure groups and IVF control. Different lowercase letters within the same column differ significantly ($p < 0.05$). Blastocyst rate was calculated at day 7 over zygotes

na not applicable

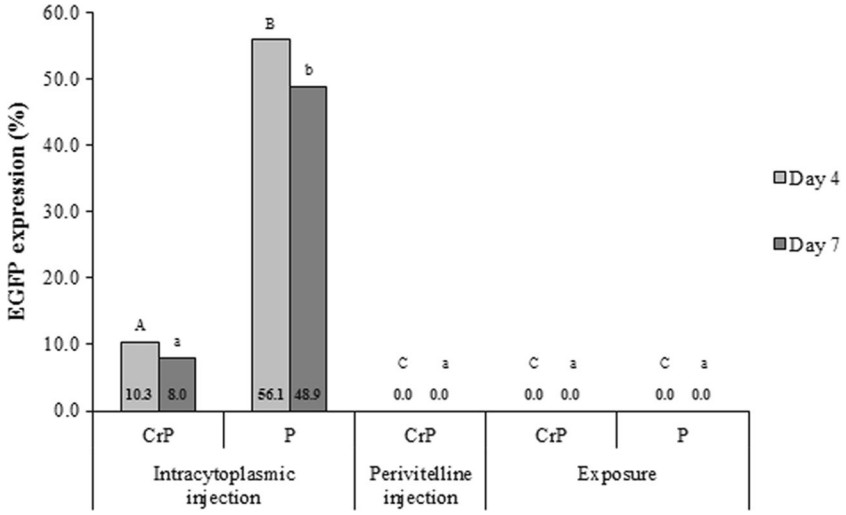

Fig. 3 Comparison of EGFP expression after exposure or injection of bovine IVF zygotes with crotamine-DNA complex (CrP) or plasmid DNA alone (P). Percentages were calculated over two to eight cell zygotes for day 4 and over blastocysts for day 7. Significant differences ($p < 0.05$) between groups are presented in uppercase letters for day 4 and in lowercase letters for day 7 of in vitro embryo culture. Experiment was replicated at least three times for injection groups and five times for exposure groups

embryos and the total number of cells of produced blastocysts as parameter. These results are in agreement with previous observations for IVF bovine embryos [4].

In experiment 3, covalently closed circular plasmids were used for complex formation since its linearized form could be more liable to degradation by nucleases present in the culture medium, or even in embryo cytoplasm before its delivery into the nucleus [28]. In this experiment, crotamine-DNA complexes were demonstrated to remain stably formed from a few minutes after their initial incubation until 24 h later in PBS medium. However, longer incubation in SOF medium (24 h) resulted in some degree of DNA degradation. In a previous report, the proteolytic stability of the crotamine-DNA complex was assayed against trypsin and proteinase K [29]. This study revealed that crotamine-DNA complex was resistant to proteinase K degradation; meanwhile, only 17 % of the complex was stable after 12 h of proteolytic exposition. Herein, we tested the stability of the crotamine-DNA complex against nucleases present in different media for up to 24 h. Given that there were no differences in plasmid stability between SOF and PBS medium for 1 h of incubation and that SOF is a more appropriate medium for embryo culture, further embryo exposures were performed in SOF medium for the shortest time (1 h).

Delivery of exogenous genes into preimplantation embryos is usually hampered by their surrounding ZP, independent of the transfection method employed. Nevertheless, the introduction of plasmid DNA and mRNA into murine preimplantation embryos has been achieved by electroporation of zona-weakened [30] and zona-intact [31] embryos, suggesting that even macromolecules of high molecular weight can enter into the embryonic cytoplasm through ZP. Those findings lead us to investigate whether crotamine could deliver exogenous DNA through ZP of intact bovine preimplantation embryos.

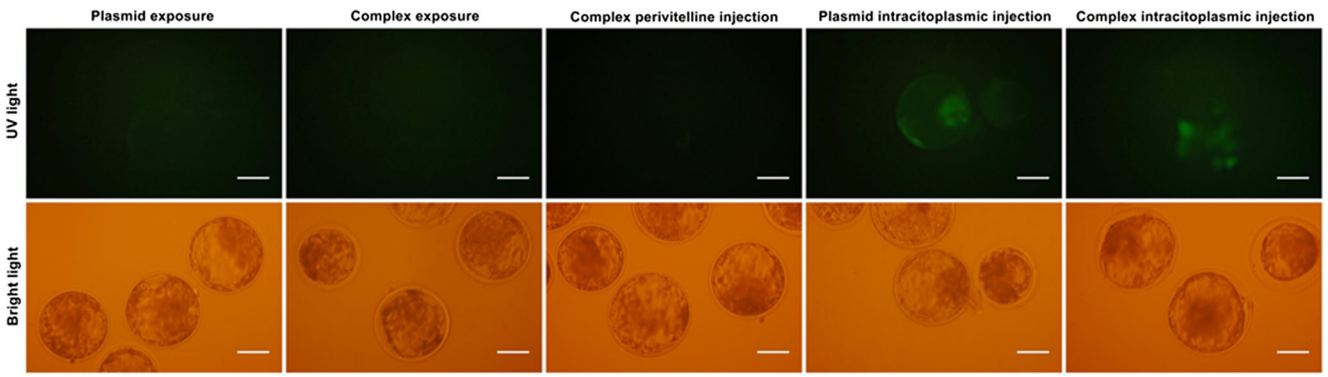

Fig. 4 EGFP expression in IVF bovine blastocysts after exposure or injection of zygotes with crotamine-plasmid complex or plasmid alone. Scale bars 100 μm

The results reported here indicate that none of the IVF embryos exposed to crotamine-DNA complex showed EGFP fluorescence. Even when embryos were directly microinjected into the perivitelline space with crotamine-DNA complex, EGFP-expressing blastocysts were not detected. Herein, we detected that the main localization of crotamine was in the cell membrane and subcortical area even after 24 h of exposure. It seems to indicate that crotamine has difficulties in arriving in the nuclear compartment and therefore has deprived DNA delivery after exposure or perivitelline injection.

Only embryos injected into the cytoplasm with crotamine-DNA complexes resulted in EGFP expression. However, EGFP expression rates were lower than those observed for the group injected with plasmid alone. Based on previous reports, it can be inferred that approximately 70 % of the total mass of plasmid was not complexed at the crotamine-DNA ratio used here (2.5:1; wt/wt) [29]. Therefore, the low expression rates achieved after intracytoplasmic injection of crotamine-DNA complex might be explained by the presence of plasmid not completely complexed with the peptide. These results are in contrast with a previous report in mouse embryonic stem cells USP 1 and human carcinoma cells HCT116, in which expression levels reached 20–30 and 98 %, respectively, after transfection with crotamine-plasmid complex of these in vitro cultured cells [9].

Herein, our report provides evidences that the delivery of exogenous DNA into the nucleus of bovine embryos is not improved by the use of crotamine, given the lower expression rates of reporter gene achieved. The high affinity and binding capacity of crotamine to DNA, due to the high number of cysteine residues in the peptide [32], might inhibit the release of the plasmid harboring the EGFP reporter gene from crotamine-DNA complex. Consequently, the availability of free plasmid for the action of zygote transcriptional machinery decreased, blocking or hindering EGFP expression. Also, introduced exogenous DNA tends to address a defense mechanism in mammalian cells, resulting in epigenetic changes of DNA, such as methylation, which may induce gene transcriptional inactivation [33].

Only embryos injected into the cytoplasm with plasmid alone reached high rates of expression (56.1 and 48.9 % at days 4 and 7, respectively), in agreement with previous studies [34, 35]. The proportion of EGFP-expressing blastocysts obtained by intracytoplasmic microinjection correlates with those from other delivery transgene strategies used previously, such as liposome-DNA complex injection [36, 37] and DNA-incubated vesicles (fragments of ooplasm without nucleus surrounded by oolemma) injection [38] into IVF bovine zygotes.

Although the use of crotamine may not be an immediate suitable strategy for improving the efficiency of transgenesis in bovine embryos, the ability of this peptide to rapidly translocate through the ZP of PA and IVF zygotes turns crotamine an interesting candidate for embryo biotechnologies. Crotamine is able to bind DNA and RNA molecules with high affinity, so de novo design and punctual modification of crotamine amino acid sequence might facilitate the release of nucleic acid cargos. It would be desirable to further evaluate modified crotamine peptides, with decreased binding capacity, in order to test its potential as a gene delivery tool.

Conclusions

In summary, crotamine is able to translocate across the ZP of PA and IVF bovine embryos within all evaluated time intervals, starting from 1 h of exposure. However, the application of stable crotamine-DNA complex using EGFP reporter gene did not improve the efficiency of transgene expression in cattle embryo, even by direct intracytoplasmic injection.

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Compliance with ethical standard

Conflict of Interest The authors declare that they have no conflict of interest.

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