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Disulphide-less crotamine is effective for formation of DNA–peptide complex but is unable to improve bovine embryo transfection

Vicente J.F. Freitas[1], Iana S. Campelo[1], Mirelly M.A.S. Silva[1], Camila M. Cavalcanti[1], Dárcio I.A. Teixeira[1], Luiz S.A. Camargo[2], Luciana M. Melo[1,3] and Gandhi Rádis-Baptista[4]

[1]Laboratory of Physiology and Control of Reproduction, Faculty of Veterinary, State University of Ceará (UECE), Fortaleza, Brazil; [2]Embrapa Dairy Cattle, Juiz de Fora, Brazil; [3]Molecular Genetics Research Unit, University Center Fametro (UNIFAMETRO), Fortaleza, Brazil and [4]Laboratory of Biochemistry and Biotechnology, Institute of Marine Science, Federal University of Ceará (UFC), Fortaleza, Brazil

Research Article

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Authors for correspondence:
Vicente J.F. Freitas. State University of Ceará, Av. Dr. Silas Mungusba, 1700 – Fortaleza, CE 60714-903, Brazil. Tel: +55 85 3101 9861. E-mail: vicente.freitas@uece.br; Gandhi Rádis-Baptista. Federal University of Ceará, Av. da Abolição, 3207 – Fortaleza – CE 60165-081, Brazil. Tel: +55 85 3366 7000, E-mail: radisbra@yahoo.com

Summary

This study aimed to investigate the ability of disulphide-less crotamine (dLCr) to complex DNA and to evaluate whether the DNA–dLCr complex is capable of improving transfection in bovine embryos. Three experiments were performed to: (i) evaluate the formation and stability of the DNA–dLCr complex; (ii) assess the dLCr embryotoxicity by exposure of bovine embryos to dLCr; and (iii) assess the efficiency of bovine embryo transfection after microinjection of the DNA–dLCr complex or green fluorescent protein (GFP) plasmid alone (control). DNA complexation by dLCr after 30 min of incubation at 1:100 and 1:50 proportions presented higher efficiency ($P < 0.05$) than the two controls: native crotamine (NCr) 1:10 and lipofectamine. There was no difference between DNA–dLCr 1:25 and the controls. The DNA–dLCr complexation was evaluated at different proportions and times. In all, at least half of maximum complexation was achieved within the initial 30 min. No embryotoxicity of dLCr was verified after exposure of *in vitro* fertilized embryos to different concentrations of the peptide. The effectiveness of dLCr to improve exogenous gene expression was evaluated by microinjection of the DNA–dLCr complex into *in vitro* fertilized zygotes, followed by verification of both embryo development and GFP expression. From embryos microinjected with DNA only, 4.6% and 2.8% expressed the GFP transgene at day 5 and day 7, respectively. The DNA–dLCr complex did not increase the number of GFP-positive embryos. In conclusion, dLCr forms a complex with DNA and its application in *in vitro* culture is possible. However, the dLCr peptide sequence should be redesigned to improve GFP expression.

Introduction

Transgenic technology in animals has been extensively used for many purposes. The use of the traditional methods to obtain transgenic mammals dates back to the 1970s (Jaenisch and Mintz, 1974) and led to the production of the first transgenic mice by pronuclear microinjection (Gordon *et al.*, 1980). Later, transgenic animals were produced from different species such as rabbit, sheep, pig (Hammer and Pursel, 1985), cattle (Krimpenfort *et al.*, 1991) and goat (Ebert *et al.*, 1991). However, this technique presents several drawbacks, ranging from low transfection efficiency to high cost of equipment and the need for skilled personal (Kues and Niemann, 2011). To overcome these disadvantages several strategies have been developed such as sperm-mediated gene transfer (Chang *et al.*, 2002), gene transfer mediated by cationic lipids (Carballada *et al.*, 2002), transfection of donor cells followed by nuclear transfer (Salamone *et al.*, 2006), gene transfer mediated by retroviruses (Xu *et al.*, 2013) and, more recently, genome editing using the CRISPR/Cas9 system (Li *et al.*, 2018).

For continuous improvement of transgenic technology, it is necessary to develop simple, affordable and efficient methods. Therefore, in recent decades, studies concerning the use of cell-penetrating peptides (CPPs) in transgenesis have been investigated (Rádis-Baptista *et al.*, 2017). CPPs are represented by short cationic or amphipathic oligopeptides that translocate across the plasma membrane and penetrate eukaryotic cells (Pooga and Langel, 2015). These peptides can usually carry different categories of therapeutic substances into the cells, including pharmaceuticals, fluorescent probes, proteins, DNA and RNA (Stewart *et al.*, 2008).

A few years ago, our group started studies with crotamine, one of these CPPs, for potential use in animal transgenesis (Campelo *et al.*, 2016a, 2016b), exploring its ability to stable binding to nucleic acids by electrostatic interaction and forming DNA–peptide complex. Crotamine was first reported back in 1947 as one of the predominant components in the venom of South

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American rattlesnake *Crotalus durissus terrificus* (Gonçalves and Polson, 1947). More recently, crotamine was described as a novel CPP capable of translocation and be located in the cytoplasm of different eukaryotic cells (Kerkis *et al.*, 2004; Nascimento *et al.*, 2007) and preimplantation embryos such as mouse morulas (Kerkis *et al.*, 2004) and bovine zygotes (Campelo *et al.*, 2016a). It was also demonstrated that this venom-derived peptide is able to successfully transfect cells in murine model (Nascimento *et al.*, 2007). However, when using bovine as a model for gene delivery, crotamine did not improve gene transfer possibly due to its high affinity for DNA molecules (Campelo *et al.*, 2016b).

Therefore, crotamine structural derivatives and analogues have been designed and investigated for their efficiency of cell penetration and delivery of molecules. In this scenario, short crotamine-derived peptides (named NrTPs) have been evaluated for cellular uptake and retained some desirable properties of crotamine such as efficient cell uptake, nuclear homing and cellular labelling. Noteworthy, other advantageous properties (short size, nucleolar targeting, facility for zip-code localization of therapeutic protein) were improved in comparison with the native peptide (Rádis-Baptista *et al.*, 2008; Rádis-Baptista *et al.*, 2012). Therefore, the aim of the present study was to investigate the feasibility of using another variation of crotamine structure, i.e. a disulphide-less (linear) crotamine (dLCr) that could deliver nucleic acids (genes) and facilitate the DNA–peptide intracellular dissociation, aimed at the transfection of bovine embryos. With such purpose, the present work evaluated: (i) the ability of dLCr to form complex, under several *in vitro* conditions, with a plasmid harbouring the reporter gene for green fluorescent protein (GFP); (ii) the toxicity of dLCr toward *in vitro* fertilized (IVF) bovine embryos; and (iii) the effectiveness of dLCr to improve the expression of GFP transgene in embryos derived from *in vitro* fertilized bovine zygotes injected with DNA–dLCr.

Material and methods

Experimental design

This study consisted of three different experiments. In experiment 1, DNA–dLCr complex formation and stability were initially evaluated under different *in vitro* conditions to establish the parameters for further embryo transfection. In experiment 2, dLCr embryo toxicity was assessed by exposure of bovine embryos to dLCr. Finally, in experiment 3, expression of the GFP reporter gene was used to assess the efficiency of bovine embryo transfection after microinjection of the DNA–dLCr complex or GFP plasmid alone (control).

Disulphide-less and native versions of crotamine

Native crotamine (NCr) was purified from the crude venom of rattlesnakes kept in the serpentarium of São Paulo University (Ribeirão Preto, Brazil). The method for NCr preparation was as described previously (Kerkis *et al.*, 2004). dLCr was prepared commercially by solid peptide synthesis (China Peptides Co., Shanghai, China) using the designed sequence C-YKQSHKK GGHAbuFPKEKIAbuLPPSSDFGKMDAbuRWRWKAbuAbuK-KGSG (MW 4886.73). This new version of crotamine structure does not form intrachain or interchain disulphide bonds due to the replacement of cysteine residues for 2-aminobutyric acid (Abu). Lyophilized dLCr was stored at −20°C until use.

Plasmid DNA preparation

The circular plasmid pEGFP-N1 (Clontech Laboratories Inc., Mountain View, CA, USA), comprised of a reporter gene encoding GFP driven by the cytomegalovirus (CMV) promoter (GenBank accession no. U55762) was used. The plasmid was cloned and propagated in *Escherichia coli* and purified using the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions.

Experiment 1: Conditions of DNA–dLCr complex formation

Stoichiometry and kinetics of complex formation

To determine the stoichiometry of DNA–dLCr complex, a fixed concentration of pEGFP-N1 (25 ng/µl) was combined with increasing concentrations of dLCr (0.25, 0.625, 1.25 or 2.5 µg/µl) resulting in DNA:peptide mass ratios of 1:10, 1:25, 1:50 and 1:100, respectively. Both DNA–NCr complex and DNA-lipofectamine lipoplex were positive controls. A DNA–NCr complex was formed by mixing pEGFP-N1 at 25 ng/µl with NCr at 0.25 µg/µl (1:10; DNA:peptide; wt:wt). The lipoplex was prepared using Lipofectamine 2000 Reagent (Invitrogen Co.) and pEGFP-N1 as indicated by the manufacturer. All complex formations were measured after 30 min of incubation. In another assay, the kinetics of complex formation was monitored at 0, 5, 30, 60 and 90 min. DNA–peptides complexes were prepared as described above to achieve DNA:peptide mass ratios of 1:10, 1:25 and 1:50 for DNA–dLCr, and 1:10 for the DNA–NCr (positive control). For both assays, the complexes were prepared in 150 mM NaCl solution at approximately 26°C. The percentage of complexed DNA was measured indirectly using an 'in-lab' developed methodology based on the fluorophore exclusion assay and the Qubit dsDNA High Sensitivity (HS) Assay Kit (Life Technologies, Eugene, OR, USA), using a Qubit 2.0 fluorimeter (Invitrogen Co.) for readings, as previously described (Freitas *et al.*, 2014). Negative controls were prepared using DNA at the same concentrations described previously in the absence of peptide and were used for fluorescence signal normalization. Each assay was repeated three times and groups were performed in duplicate within repetitions.

Complex stability in embryo culture medium

The stability of the DNA–dLCr complex was evaluated in synthetic oviductal fluid (SOF) medium in the absence of fetal calf serum, albumin and amino acids (SOF^{-saa}). Therefore, the DNA–dLCr complex was previously prepared by 30 min incubation of dLCr mixed with pEGFP-N1 at a DNA:peptide mass ratio of 1:25 (the best condition defined in the kinetics assay). Samples were recovered at 0, 180 and 360 min after DNA–dLCr dilution in SOF^{-saa} medium (1:10; v:v) to measure the fluorescence signal. The concentration of complexed DNA was evaluated using the Qubit dsDNA HS Assay Kit and a Qubit 2.0 fluorimeter as previously. NCr was used as the positive control at a 1:10 DNA: peptide mass ratio as described before. The experimental groups were performed in duplicate in each repetition and the assay was repeated three times.

Experiment 2: Evaluation of dLCr embryotoxicity

Oocyte collection and in vitro maturation (IVM)

Bovine ovaries were collected from a local slaughterhouse and transported on ice to the laboratory in 0.9% (w:v) NaCl solution containing antibiotics (Pentabiótico; Fort Dodge, Campinas, Brazil). Cumulus–oocyte complexes (COCs) from follicles of

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2–8 mm in diameter were aspirated using an 18-gauge needle attached to a 10 ml syringe. Only COCs with homogeneous cytoplasm surrounded by at least three layers of compact cumulus cells were selected for further IVM. After collection, the COCs were washed in manipulation medium composed of TCM-199 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1% (v:v) antibiotic–antimycotic solution (Sigma-Aldrich), 0.1 mM sodium pyruvate (Sigma-Aldrich), 10% (v:v) fetal calf serum (FCS; Gibco, Grand Island, NY, USA) and 25 mM HEPES (Sigma-Aldrich). Groups of up to 50 COCs were placed in 500 µl of maturation medium composed of TCM-199 containing 10% (v:v) FCS, 0.1 mM sodium pyruvate, 10 ng/ml epidermal growth factor (Sigma-Aldrich), 100 µM cysteamine (Sigma-Aldrich), 20 µg/ml FSH/LH (Pluset; Hertape Calier, Barcelona, Spain), 1 µg/ml 17β-estradiol (Sigma-Aldrich), 1% (v:v) antibiotic–antimycotic solution, and 1 mM L-glutamine (Sigma-Aldrich). IVM was performed at 38.5°C for 23–24 h in an humidified atmosphere of 5% CO_2.

In vitro *fertilization (IVF)*

Oocytes were submitted to IVF with frozen–thawed semen of a bull with proven fertility. The semen was thawed for 5 s in air and 25 s in a water bath at 37°C. The semen was separated in 4 ml of Percoll gradient (Sigma-Aldrich; 55%/90%) by centrifugation at 700 *g* for 25 min. The Percoll-separated semen was washed twice with 2 ml of BO medium added with 5 mM caffeine (Sigma-Aldrich) and 20 UI/ml heparin (Calbiochem, Merck, Darmstadt, Germany). After washing, the semen was diluted with BO added with 10 mg/ml bovine serum albumin (BSA; Sigma-Aldrich) to achieve a final concentration of 20×10^6 spermatozoa/ml. Finally, the COCs subjected to IVM were washed in manipulation medium and co-incubated with a suspension of semen in droplets of 100 µl covered with mineral oil (Sigma-Aldrich) at 38.5°C in a humidified atmosphere of 5% CO_2. At 6 h post-IVF, presumptive zygotes were denuded by vortexing in manipulation medium and distributed randomly for respective groups of exposure or microinjection experiments.

Exposure to dLCr

Zygotes were exposed to dLCr for 6 h and after that they were transferred to droplets composed of 5 µl of dLCr solution and 45 µl of SOF^{-saa} and maintained at 38.5°C in an atmosphere of 5% CO_2, 5% O_2, 90% N_2. dLCr solution was prepared in NaCl 150 mM to achieve concentrations of 0 µM (vehicle), 5 µM (0.025 µg/µl) and 12.5 µM (0.0625 µg/µl) in the culture medium. After exposure, the embryos were washed extensively in manipulation medium and subjected to embryo culture as described ahead. A group not exposed to dLCr (IVF group) was performed as the negative control. In addition, the exposure of zygotes to higher concentrations of dLCr: 25 µM (0.125 µg/µl) and 50 µM (0.25 µg/µl) was evaluated.

Experiment 3: Assess the efficiency of bovine embryo transfection with DNA–dLCr

Cytoplasmic microinjection of DNA–dLCr complex

To assess the ability of dLCr to improve exogenous DNA expression, DNA–dLCr complexes were first microinjected into the cytoplasm of IVF presumptive zygotes. The complex was prepared with pEGFP-N1, which encodes a red shifted variant of the wild type GFP reporter gene, and dLCr in 150 mM NaCl for at least 30 min. The DNA:peptide mass ratio for complex formation was 1:25 (as defined in experiment 1). Also, a positive control group was performed by microinjection of a solution composed of only pEGFP-N1 plasmid (25 ng/µl). Next, 5 µl of solution were backfilled in microinjection capillaries (FemtoTip II; Eppendorf, Hamburg, Germany) with an internal tip diameter of approximately 0.5 µm. Presumptive zygotes were transferred to 50 µl droplets of manipulation medium in a 60 mm dish and covered with mineral oil. Zygotes were fixed by suction to a holding pipette, meanwhile the capillary was pushed through the embryo's zona pellucida and cytoplasm membrane. DNA–dLCr and DNA alone solutions were injected using a FemtoJet (Eppendorf) set as follows: injection pressure = 300 hPa, compensation pressure = 10 hPa, injection time = 0.5 s. Embryo micromanipulation was performed in a Narishige hydraulic micromanipulator (Narishige Science, Tokyo, Japan) mounted on a Nikon Eclipse TE2000 inverted microscope (Nikon, Tokyo, Japan). The microinjection procedure is described in Supporting Information. After injection, the zygotes were transferred into droplets of culture medium. In addition, an IVF control group (not microinjected) was performed.

Embryo culture and in vitro *development assessment*

Embryo culture was performed in 50 µl droplets of SOF medium supplemented with 0.8% (w:v) BSA and 2.5% (v:v) FCS under mineral oil at 5% CO_2, 5% O_2, 90% N_2 and 38.5°C in high humidity. Embryo cleavage rate was assessed at day 2 post-IVF and blastocyst formation rates were evaluated at day 7 (D7) and day 8 (D8). At D8, hatching/hatched embryos were counted to assess embryo quality by means of hatching rate. GFP expression was evaluated under ultraviolet (UV) light using the Nikon Eclipse TE2000 microscope (excitation filter at 488 nm and emission filter at 530 nm) at day 5 (D5) and D7 of *in vitro* culture.

Data and statistical analysis

Statistical analyses were performed using Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA). For stoichiometry assay, complexed DNA rate was assessed by one-way analysis of variance (ANOVA) multiple comparisons. In the kinetics assay, complexed DNA rate was evaluated by multiple *t*-test for differences between groups at the same time and by one-way ANOVA repeated measurements for differences between times at the same group. In complex stability assay, the complexed DNA rate was assessed by multiple *t*-test for differences between groups at the same time and by two-way ANOVA multiple comparisons for differences between times at the same group. The statistical analysis of cleavage, blastocyst, hatching and GFP expression rates was assessed using non-parametric Fisher's exact test. For all tests, a P-value < 0.05 was considered to be statistically significant.

Results

Experiment 1: Stoichiometry and kinetics of complex formation

Figure 1 shows that proportions of 1:100 and 1:50 DNA–dLCr reached higher ($P < 0.05$) complexation efficiency than DNA–NCr 1:10 and lipofectamine. In addition, similar rates of complexed DNA ($P > 0.05$) were observed between those proportions, which were able to complex the maximal amount of DNA (over 90%) within 30 min of incubation. However, there was no difference when 1:25 DNA–dLCr and both controls ($P > 0.05$)

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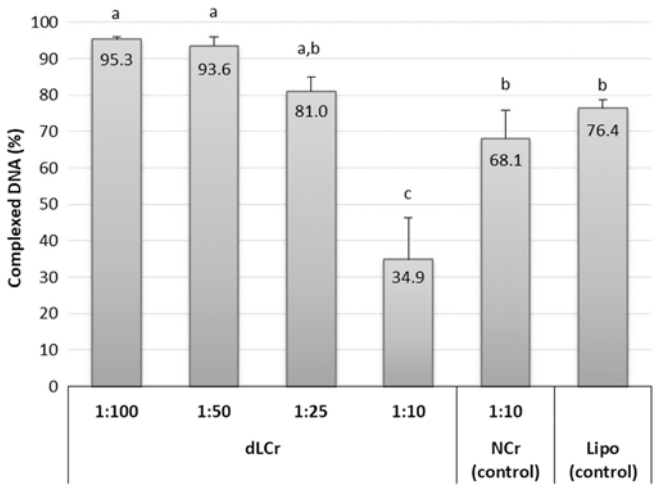

Figure 1. Stoichiometry of DNA–disulphide-less crotamine (dLCr) complex formation assessed by fluorophore exclusion assay. A DNA–dLCr complex was formed at 1:100, 1:50, 1:25, and 1:10 DNA:peptide mass ratios. Native crotamine (NCr) at 1:10 (DNA:peptide; wt:wt) and lipofectamine 2000 (Lipo) were used as positive controls. [a–c]Different lowercase letters indicate statistical differences ($P < 0.05$).

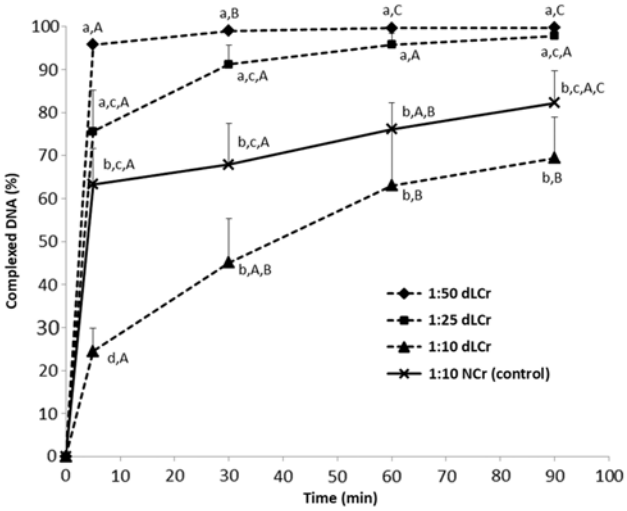

Figure 2. Kinetics of DNA–disulfide-less crotamine (dLCr) complex formation assessed by fluorophore exclusion assay. A DNA–dLCr complex was formed at 1:50, 1:25, and 1:10 (DNA:peptide; wt:wt). Complexed DNA was measured at 0, 5, 30, 60 and 90 min of complexation. Native crotamine (NCr) was used as a positive control at 1:10 DNA:peptide mass ratio. Different lowercase letters (a–d) indicate statistical differences between groups at different times and different uppercase letters (A–C) indicate statistical differences between times within the same group ($P < 0.05$).

were compared. Conversely, the proportion of 1:10 DNA–dLCr, resulted in the lowest ($P < 0.05$) complexation (less than 35%).

According to kinetics data, the DNA complexation by dLCr seems to occur very fast (Fig. 2). Therefore, for 1:50 or 1:25 DNA–dLCr more than 75% of the DNA was complexed by the peptide within 5 min of incubation. For all DNA–dLCr groups, the efficiency of the complexation was higher or similar than the 1:10 DNA–NCr group within 30 min of incubation. At 60 min incubation, the 1:50 DNA–dLCr group achieved a complexation rate of 99.6%, which was similar to 1:25 (95.8%) group, although higher ($P < 0.05$) than the 1:10 DNA–dLCr (63.0%) and

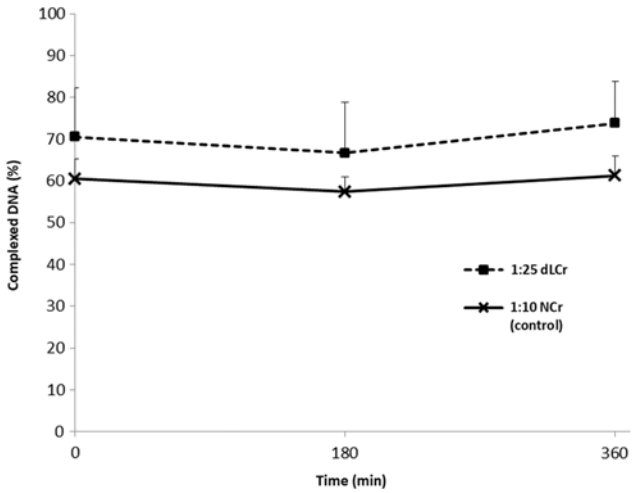

Figure 3. Stability of DNA–disulphide-less crotamine (dLCr) complex in embryo culture medium (SOF^{-saa}) assessed by fluorophore exclusion assay. DNA–dLCr complex were formed at 1:25 DNA:peptide mass ratio for 30 min, diluted in SOF^{-saa}, and then measured at 0, 180 and 360 min of incubation. Native crotamine (NCr) was used as positive control at 1:10 DNA:peptide mass ratio. There was no statistical difference between groups at diverse times and between times within the same group ($P > 0.05$).

1:10 DNA–NCr (76.1%) groups. The 1:10 dLCr group seems to have the slowest complexation, reaching less than 25% DNA complexation at 5 min and less than 65% at 60 min.

Experiment 1: Complex stability in embryo culture medium

To evaluate the stability of DNA–dLCr complexes in a DNA-free culture medium, complexes were incubated in SOF^{-saa} medium and analyzed at 0, 180 and 360 min intervals (Fig. 3). No statistical difference was observed when comparing DNA–dLCr and DNA–NCr at the same time of incubation nor when comparing the different times within the same group ($P > 0.05$). Therefore, it was observed that there was no additional complexation or degradation of complexes previously formed in NaCl solution.

Experiment 2: In vitro *development of embryos exposed to dLCr*

In this experiment, the potential embryotoxicity of dLCr was assessed by evaluating the *in vitro* development of IVF bovine embryos exposed to dLCr for 6 h. The effects were recorded by means of embryo cleavage rate, blastocyst formation at D7 and D8 rates and hatching rate of groups exposed to 5 or 12.5 µM dLCr. One control group consisted of embryos exposed to SOF^{-saa} medium with vehicle solution and without dLCr for 6 h (0 µM dLCr), and another consisted of embryos not exposed to vehicle solution and dLCr (IVF control). *In vitro* embryo development is shown in Table 1.

Cleavage, blastocyst productions and blastocyst hatching rates were similar among groups exposed to dLCr, 0 µM dLCr and IVF control ($P > 0.05$). In addition, higher dLCr concentrations (25 and 50 µM, i.e., 0.125 µg/µl and 0.25 µg/µl, respectively, for 6 h) also did not affect the development of *in vitro* fertilized bovine embryos (Table 2). Therefore, exposure of zygotes for 6 h to up to 50 µM dLCr had no detrimental effect on *in vitro* embryo development.

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Table 1. Effect of low concentrations of disulphide-less crotamine (dLCr) on development of *in vitro* fertilized (IVF) bovine embryos

dLCr (μM)	Zygotes	Cleavage (%)	Blastocysts (%)		Hatching (%)*
			Day 7	Day 8	
12.5	161	129 (80.1)[a]	69 (42.9)[a]	71 (44.1)[a]	18 (25.4)[a]
5	162	126 (77.8)[a]	60 (37.0)[a]	62 (38.3)[a]	15 (24.2)[a]
0 (vehicle)	119	87 (73.1)[a]	50 (42.0)[a]	49 (41.2)[a]	13 (26.5)[a]
IVF control	118	93 (78.8)[a]	54 (45.8)[a]	51 (43.2)[a]	8 (15.7)[a]

Experiment was replicated four times.
[a]Similar superscripts within the same column mean statistical similarity ($P < 0.05$).
*Calculated from total number of blastocysts at day 8.

Table 2. Effect of high concentrations of disulphide-less crotamine (dLCr) on development of *in vitro* fertilized (IVF) bovine embryos

dLCr (μM)	Zygotes	Cleavage (%)	Blastocyst (%)		Hatching (%)*
			Day 7	Day 8	
50	171	107 (62.6)[a]	46 (26.9)[a]	43 (25.1)[a]	15 (34.9)[a,b]
25	170	113 (66.5)[a]	51 (30.0)[a]	52 (30.6)[a]	19 (36.5)[a]
0 (vehicle)	120	75 (62.5)[a]	30 (25.0)[a]	32 (26.7)[a]	15 (46.9)[a]
IVF control	120	83 (69.2)[a]	42 (35.0)[a]	42 (35.0)[a]	7 (16.7)[b]

Experiment was replicated four times.
[a,b]Different superscripts within the same column differ significantly ($P < 0.05$).
*Calculated from total number of blastocysts.

Experiment 3: Transfection of embryos by microinjection of DNA–dLCr complex

Microinjection of the 1:25 DNA–dLCr complex was compared with microinjection of DNA alone by means of *in vitro* development and emission of GFP fluorescence. Also, an IVF control group was performed to assess the viability of *in vitro* embryo culture (Table 3). For embryo cleavage and blastocyst production (D7 and D8), microinjected groups had lower rates ($P < 0.05$) than the IVF control. Nevertheless, embryo development parameters were similar ($P > 0.05$) among groups of embryos microinjected (DNA–dLCr and DNA alone). To determine the quality of blastocysts formed, hatching rate was evaluated for all groups and no difference ($P > 0.05$) was found among these (Table 3).

The rate of GFP expression is shown in Table 3. In total, 4.6% and 2.8% of cleaved embryos was observed to express fluorescence at D5 and D7, respectively, after microinjection of plasmid alone. Use of the DNA–dLCr complex for microinjection did not increase the number of GFP-positive embryos when compared with plasmid DNA alone ($P > 0.05$). Additionally, there was no detectable qualitative transgene expression on embryos microinjected with DNA–dLCr complex in comparison with microinjection of DNA plasmid alone.

Discussion

To date, this is the first study to describe the use of a synthetic version of disulphide-less (linear) crotamine for DNA complex formation and gene transfer. The native peptide is rich in positive amino acid residues (nine lysine and two arginine residues) and six cysteine. The positive residues conferred a net positive charge to this molecule, i.e. a basic characteristic and cysteine make up three precisely arranged disulphide bonds (Nicastro *et al.*, 2003; Fadel *et al.*, 2005). In addition, the presence of two histidine residues could provide an extra positive charge to these molecules in physiological pH and, especially, in the pH of the medium (Marinovic *et al.*, 2016).

In the present study, the ability of a new synthetic disulphide-less crotamine, i.e. the linear version free of the three S–S bonds, was evaluated for its ability to form a complex with DNA molecules. It is known that crotamine binding to nucleic acids seems to rely on electrostatic interaction; nucleic acids have a net negative charge while crotamine possesses an overall positive surface. One region, in particular, was suggested due to being potentially involved in the interaction with DNA. The region is comprised of the sequence RWRWK (Arg–Trp–Arg–Trp–Lys) and corresponds to residues 31–35 of NCr (Chen *et al.*, 2012). In the linear disulphide-less peptide, this sequence was not altered, therefore preserving the ability of dLCr to bind to DNA. The rational to use disulphide-less crotamine as the delivery tool for embryo transfection instead of the native disulphide-bond folded crotamine relied on the fact that CPPs should make a complexation between the peptide and DNA complex that was strong enough to permit stable uptake through the zona pellucida and/or cell membrane translocation, but should be sufficiently weak to permit the release of DNA though dissociation into the cell cytoplasm to enable it to traffic to the nucleus. In our previous studies (Campelo *et al.*, 2016a, 2016b), bovine embryos were used as a model for gene transfer with DNA–NCr complexes. However, the use of the DNA–NCr complex did not improve the transgene expression rate, supposedly because of strong DNA–peptide binding within embryo cells (Campelo *et al.*, 2016b). Therefore, it was desirable to evaluate whether modified crotamine peptides, such as dLCr could reduce the binding strength of DNA–peptide and circumvent such physicochemical behaviour.

In fact, it was observed here that the capacity of complexation with DNA of dLCr was reduced when compared with NCr. In experiment 1, stoichiometry of DNA–dLCr and DNA–NCr complexes at the same proportion (1:10) was analyzed and complexed DNA was significantly reduced (34.9% vs 68.1%). The DNA–dLCr complex achieved similar complexation for 1:10 DNA–NCr (81.0% vs 68.1%) only at the 1:25 proportion, i.e. using approximately 2.5 times higher dLCr than NCr peptide mass for the same mass of plasmid DNA. It was expected that changes in the primary sequence, by substitution of cysteine residues in crotamine with 2-aminobutyric acid residues (Abu), would not alter membrane translocation, but facilitate peptide:DNA dissociation and preserve the accumulation patterns in cell cytoplasm.

Nevertheless, linear peptides usually have lower stability due to higher liability to proteases when compared with more compact disulphide-bond-stabilized structures, as for native crotamine (Marinovic *et al.*, 2016). Despite this concern, it was verified that the DNA–dLCr complex was highly stable in embryo culture medium and any degradation in peptide–DNA was observed even after incubation for 6 h (61.3% at 360 min vs 60.5% at 0 min). The stability of a given CPP in complete culture medium and in the presence of serum is a highly useful property in gene delivery (Rádis-Baptista *et al.*, 2017). Here, the potential toxic effect of dLCr on IVF bovine embryos was also evaluated and a significantly higher concentration of dLCr (12.5 μM) did not cause any deleterious effect upon IVF embryo development. Even at higher concentrations of dLCr (25 and 50 μM), toxicity in post-IVF early bovine embryos was undetectable. NCr has been extensively tested

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Table 3. Embryo development and enhanced green fluorescent protein (EGFP) expression of microinjected *in vitro* fertilized (IVF) bovine embryos with DNA-disulphide-less crotamine (DLCr) complex (+) or plasmid DNA alone (–)

Treatment	dLCr	Zygotes	Cleavage (%)	Blastocysts (%)*		Hatching (%)**	Number of embryos expressing EGFP (%)***	
				Day 7	Day 8		Day 5	Day 7
Intracytoplasmic microinjection	+	189	101 (53.4)^a	32 (16.9)^a	33 (17.5)^a	7 (21.2)^a	0 (0.0)^a	0 (0.0)^a
	–	191	108 (56.5)^a	33 (17.3)^a	32 (16.8)^a	8 (25.0)^a	5 (4.6)^a	3 (2.8)^a
IVF control	NA	210	182 (86.7)^b	85 (40.5)^b	84 (40.0)^b	9 (10.7)^a	NA	

Experiment was replicated five times.
NA, not applicable.
*Rates calculated over zygotes.
**Rates calculated from total number of blastocysts at day 8.
***Rates calculated over cleaved embryos.
^{a,b}Different superscripts within the same column differ significantly ($P < 0.05$).

on eukaryotic cells such as mouse embryonic stem cells, human carcinoma HCT116 cells and CHO-K1 cells (Rádis-Baptista and Kerkis, 2011). Specifically, mammalian embryos were also used as model to detect possible toxic effects of crotamine. However, for murine and bovine species (Kerkis *et al.*, 2004; Campelo *et al.*, 2016a) no embryotoxicity was noticed when using native crotamine ranging from 1 μM to 10 μM, for up to 24 h. Overall, in agreement with these data and reports, native and linear crotamine are harmless to embryo cells in micromolar concentrations (< 50 μM for bovine and < 10 μM for mice).

Cytoplasmic microinjection of plasmids in zygotes is a simple alternative to drive ectopic expression of exogenous DNA in embryos (Iqbal *et al.*, 2009). The development of a simple and effective method that could allow the delivery of exogenous DNA into the zygote's cell membrane, through the zona pellucida, would be a great advantage to transgenic technologies, especially to the recent introduced engineered site-directed nucleases (ESDNs) methodologies, such as the CRISPR/Cas9 system (Kim, 2016). These new technologies may require the injection of high concentrations of the transgene construct. In this scenario, improvements, such as using DNA construction in association with substances that enable further increase in efficiency of cytoplasmic microinjection, are a great advantage. For example, injection of DNA–liposome lipoplexes into IVF zygotes increased the rate of GFP+ blastocysts to 12% in cattle (Vichera *et al.*, 2011). In accordance, here it was evaluated if enhancement in efficiency of cytoplasmic microinjection of DNA carrying a GFP reporter could be achieved by associating exogenous DNA with dLCr by a non-covalent interaction. *In vitro* development parameters and fluorescence for GFP of IVF bovine embryos was compared with microinjection of DNA alone and IVF control groups.

Cleavage and blastocyst production at D7 and D8 were lower in the microinjected group than in the IVF control group, demonstrating, as expected, a possible negative effect of microinjection upon embryo development. Nevertheless, embryo development parameters were similar between embryos microinjected with DNA–dLCr or with DNA alone. Therefore, no detrimental effect was detected by using dLCr complexed with DNA for microinjection of post-IVF bovine embryos. A study using the same GFP plasmid construct with a microinjection semi-automated system was carried out to determine the most appropriate microinjection time, concentration and volume in IVF and parthenogenetic pig embryos. In this study, microinjection also reduced the developmental competence of injected zygotes, even when using the best conditions defined by the study (Malaweera *et al.*, 2014). In contrast, a study in buffalo using an ApaL I linearized pEGFP-N1 plasmid, but with a manual microinjection system, resulted in cleavage and blastocyst development rates similar to those of non-injected IVF embryos (Meng *et al.*, 2015).

Concerning the expression of GFP, a low rate was achieved (4.6% at D5 and 2.8% at D7) for group of embryos microinjected with plasmid DNA alone. This reduced rate could be explained by the low chance and the efficiency by which embryos normally express an exogenous cassette. In a correlated work, no GFP-expressing IVF bovine embryos were obtained after sperm-mediated gene transfer treatment, when exogenous DNA quantification could be assessed by real-time PCR (Campos *et al.*, 2011). As in our study, this group used a pEGFP-N1 plasmid containing the CMV promoter. Previously, the CMV promoter was thought to present low efficiency (Rieth *et al.*, 2000) and, in this case, an additional CMV enhancer would be necessary to improve GFP expression. Moreover, injected DNA should be able to traffic to the nucleus and to be expressed even if DNA is integrated into the genome, randomly, and in a non-active transcription region of the chromosome (Hacker and Balasubramanian, 2016). This event is a significant drawback for genetic engineering regarding stable insertion of exogenous DNA in the genomes of farm animals, and an essential step for production of transgenic animal (Garrels *et al.*, 2012) that could be better achieved by CRISPR/Cas 9 technology.

When comparing embryos microinjected with plasmid alone or with the DNA–dLCr complex, the rate of DNA expression of exogenous DNA in bovine embryos was not comparatively improved. The high affinity for DNA and binding capacity of dLCr might again restrain the release of the GFP plasmid gene in the complex for protein expression, similar to found previously when using native crotamine to induce exogenous DNA expression in bovine embryos (Campelo *et al.*, 2016b). Therefore, it was hypothesized that dLCr remains complexed to exogenous DNA even inside the cytoplasm of embryo cells, and this possibly led to very low GFP expression and detectable fluorescence.

The results indicated that dLCr effectively formed a complex with DNA in a concentration- and time-dependent manner. Its application in embryo culture medium is possible for concentrations up to 50 μM for 6 h. However, transgene expression was not comparatively improved using dLCr complexed with a GFP reporter plasmid DNA. In conclusion, dLCr formed a stable complex with DNA, in the presence of complete embryo medium and was not embryotoxic. Substitution of the reporter gene will be under consideration for further utilization of dLCr as a tool to carry genes intracellularly and to improve heterologous gene expression.

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Additionally, the dLCr peptide sequence should be redesigned to improve GFP expression.

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Conflicts of interest. The authors have no conflicts of interest to declare.

Ethical standards. All animal experiments in this study were approved by the Ethics Committee on Animal Use of State University of Ceará (1672976/2017).

Supporting information. Supporting information of an MP4 video file is available for this paper.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0967199419000716

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