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Effects of steroidal glycoalkaloids from potatoes (*Solanum tuberosum*) on in vitro bovine embryo development☆

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Abstract

α-Solanine and α-chaconine are two naturally occurring steroidal glycoalkaloids in potatoes (*Solanum tuberosum*), and solanidine-*N*-oxide is a corresponding steroidal aglycone. The objective of this research was to screen potential cyto-toxicity of these potato glycoalkaloids using bovine oocyte maturation, in vitro fertilization techniques and subsequent embryonic development as the in vitro model. A randomized complete block design with four in vitro oocyte maturation (IVM) treatments (Experiment 1) and four in vitro embryo culture (IVC) treatments (Experiment 2) was used. In Experiment 1, bovine oocytes ($n = 2506$) were matured in vitro in medium supplemented with 6 μM of α-solanine, α-chaconine, solanidine-*N*-oxide or IVM medium only. The in vitro matured oocytes were then subject to routine IVF and IVC procedures. Results indicated that exposure of bovine oocytes to the steroidal glycoalkaloids during in vitro maturation inhibited subsequent pre-implantation embryo development. Potency of the embryo-toxicity varied between these steroidal glycoalkaloids. In Experiment 2, IVM/IVF derived bovine embryos ($n = 2370$) were cultured in vitro in medium supplemented with 6 μM of α-solanine, α-chaconine, solanidine-*N*-oxide or IVC medium only. The results showed that the pre-implantation embryo development is inhibited by exposure to these glycoalkaloids. This effect is significant during the later pre-implantation embryo development period as indicated by fewer numbers of expanded and hatched blastocysts produced in the media containing these alkaloids. Therefore, we conclude that in vitro exposure of oocytes and fertilized ova to the steroidal glycoalkaloids from potatoes inhibits pre-implantation

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embryo development. Furthermore, we suggest that ingestion of *Solanum* species containing toxic amounts of glycoalkaloids may have negative effects on pre-implantation embryonic survival. Published by Elsevier B.V.

Keywords: Bovine embryo development; α-Chaconine; Solanidine-*N*-oxide; α-Solanine; *Solanum tuberosum*

1. Introduction

Solanum tuberosum is commonly cultivated for human consumption throughout the world, and exposures occur daily. These plants are known to contain several types of steroidal glycoalkaloids that are toxic (Baker et al., 1991). Glycoalkaloids from potatoes and other *Solanum* species cause various types of poisoning in livestock and man from GI disturbance to teratogenesis and in certain circumstances contribute to public health concerns (Jadhav et al., 1981; Gaffield and Keeler, 1996a). Toxicity is dependent on factors such as cultivar, growing conditions, light, plant part ingested and type and concentration of glycoalkaloid present (Phillips et al., 1996). Recently, plant selection for insect resistance has increased the alkaloid content of some species (Yencho et al., 1998). Obviously, the glycoalkaloids impart some protective mechanism to the plant against herbivory. Therefore, it is important to increase our understanding and determine what potential toxicity problems may occur by ingestion of these potato glycoalkaloids.

Poisoning has been attributed to glycoalkaloids, primarily α-solanine and α-chaconine (Phillips et al., 1996; Stapleton et al., 1991). α-Solanine (SOL) and α-chaconine (CHA) are two naturally occurring steroidal glycoalkaloids in the green potato tuber (*S. tuberosum*), vines or sprouts, and solanidine-*N*-oxide (SNO) is a corresponding steroidal aglycone. Oral administration of the steroidal alkaloid glycosides, α-solanine and α-chaconine, and their aglycone solanidine is shown to induce craniofacial malformations (exencephaly, encephalocele, and anophthalmia) in Syrian hamsters (Gaffield and Keeler, 1996a). During investigation of potato-induced teratogenesis, it was noted that feeding pregnant dams sufficient plant material caused cranial and facial defects in developing fetuses and sometimes resulted in the death of the dams as well (Baker et al., 1991). The embryo toxicities of two major potato glycoalkaloids, α-chaconine and α-solanine, were examined individually and in mixtures using the frog embryo teratogenesis assay. Some combinations exhibited strong synergism. The results indicated that synergistic mortality or malformation could result from the mixture of α-chaconine and α-solanine (Rayburn et al., 1995).

Embryotoxicities caused by steroidal glycoalkaloids in the potato tuber have been reported and experimental investigation indicated that the developmental toxicity of these compounds is governed by their structural features (Friedman et al., 1992). The evaluation of the embryotoxicity of *Solanum* glycoalkaloids is complicated by maternal toxicosis in mammalian model systems (Baker et al., 1991). Bovine embryos were, therefore, used as the in vitro models to investigate the potential cyto-toxic effects. Using in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC) techniques provides an analytical approach for obtaining information on the potential toxicological effects of chemicals during three important stages of pre-placentation embryogenesis, i.e., oocyte maturation, fertilization and embryo growth (Bavister et al., 1992; Panter et al., 2004). By obtaining

slaughterhouse ovaries, an abundant supply of oocytes for IVM, IVF and IVC can be used for biological assessment (Bavister, 1995; Cetica et al., 1999). The objective of this study was to determine the effects of these steroidal glycoalkaloids on bovine pre-implantation embryo development using in vitro fertilization procedures.

2. Materials and methods

2.1. Production of bovine embryos in vitro

Ovaries were collected from a local abattoir. Oocytes were aspirated from small antral follicles (3–8 mm in diameter) as described by Hawk and Wall (1994). Cumulus oocyte complexes (COCs) with evenly granulated ooplasm and surrounded by at least three layers of compact cumulus cells were selected for use according to the oocyte grading system of Hawk and Wall (1994). Oocytes were washed three times with Hepes-TALP solution (Parrish et al., 1988) and once with maturation medium. In vitro maturation of oocytes followed the procedure of Sirard et al. (1988) and Bavister et al. (1992) with minor modification. The maturation medium consisted of M-199 plus 10% (v/v) fetal bovine serum (FBS, A-1111, HyClone Laboratories Inc., Logan, UT, USA), 25 mM HEPES, 2 mM glutamine, 0.25 mM sodium pyruvate, 0.5 μg/ml ovine FSH (F-4520, Sigma Chemical Company, St. Louis, MO, USA), 5.0 μg/ml ovine LH (L-5269, Sigma) and 1.0 μg/ml estradiol (E-2258, Sigma). Polystyrene plastic four-well culture petri dishes (Nunclon7, Nunc Inc., Naperville, IL, USA) were used for IVM culture. Each well contained 500 μl IVM medium covered with paraffin oil (6358, Mallinckrodt Inc., Port, KY, USA). Approximately 40–65 oocytes were transferred to the IVM medium per well and cultured in a humidified 5% CO_2 atmosphere at 39 °C for 24 h.

Cryopreserved bovine semen was used for in vitro fertilization (IVF). Live sperm were separated by Percoll (P-4937, Sigma) gradients (45% and 90% on the upper and lower layers, respectively) and centrifuged at 500 × *g* for 30 min. Motile spermatozoa were added to the fertilization medium (Fert-TALP, Parrish et al., 1988) to provide a final concentration of 1.5×10^6 per ml. Capacitation of spermatozoa occurred in Fert-TALP containing 10 μg heparin/ml and 0.6% (w/v) fatty acid free bovine serum albumin. IVM-matured oocytes were added to Fert-TALP containing spermatozoa and cultured in plastic four-well petri dishes under paraffin oil in a humidified 5% CO_2 atmosphere at 39 °C for 17 h. Each well contained 500 μl Fert-TALP and approximately 40–65 oocytes.

Cumulus and corona cells were removed from ova by vortexing in Hepes-TALP supplemented with 0.3% (w/v) bovine serum albumin for 3 min. The presumptive zygotes were then cultured in plastic four-well petri dishes under paraffin oil at 39 °C in a humidified 5% CO_2 atmosphere. A modified CR2 medium (Wang et al., 1997) comprising 108.3 mM NaCl, 2.9 mM KCl, 24.9 mM $NaHCO_3$, 2.5 mM hemicalcium lactate, 0.5 mM sodium pyruvate, BME amino acids (B-6766, Sigma), MEM nonessential amino acids (M-7145, Sigma), 0.5 mM glycine, 0.5 mM alanine, 1.0 mM glutamine, 1.0 mM glucose, and antibiotics was used to culture embryos. Each well contained 500 μl CR2 medium with approximately 35–55 oocytes. During culture, medium was changed every other day so that it contained 5% (v/v) fetal bovine serum on Day 1 (Day 0 = IVF), 10% on Day 3, 15% on Day 5 and 20% on Day 7 of culture (Zhang et al., 1992).

2.2. Alkaloid preparation

α-Solanine, α-chaconine and the steroidal aglycone, solanidine-*N*-oxide, were extracted from *S. tuberosum* sprouts and isolated and purified according to Gaffield and Keeler (1996a). Briefly, dried ground potato sprouts were extracted using organic solvents and separated into active fractions as tested in a hamster teratology bioassay. The purity of these alkaloids was determined by chromatographic (TLC) and spectroscopic (GC/MS) analysis following re-crystallization after repeated chromatographic separations on silica gel (Gaffield and Keeler, 1996a). The alkaloids used in this study were essentially pure.

2.3. In vitro experiments

A complete randomized block experimental design was applied to determine the in vitro effects of *Solanum* glycoalkaloids on bovine oocytes (Experiment 1), and IVM/IVF (in vitro matured/in vitro fertilized) derived embryos (Experiment 2). Experiment 1 (IVM) consisted of 10 replicates (total 2506 oocytes). In each of the replications, oocytes were from the same collection of abattoir ovaries. There were four IVM treatments (TRT). For TRT1, the IVM medium was supplemented with 6 μM α-solanine. For TRT2, 6 μM α-chaconine was added into IVM medium. For TRT3, the IVM medium was supplemented with 6 μM solanidine-*N*-oxide. For TRT 4 (control) the medium contained no *Solanum* glycoalkaloids but did contain the organic solvent used to solubilize alkaloids at the same concentration as treatments 1–3. The organic solvent used was the mixture of equal amount (v/v) of Pharmasolve (ISP Technologies Inc., Wayne, NJ) and ethanol. The matured oocytes were then fertilized and cultured in vitro as described above. Experiment 2 (IVC) consisted of 10 replications (total 2370 oocytes). The oocytes were in vitro matured and fertilized as described above. There were four in vitro embryo culture (IVC) treatments (TRT). For TRT1, the IVC medium was supplemented with 6 μM α-solanine. For TRT2, 6 μM α-chaconine was added into IVC medium. For TRT3, the medium was supplemented with 6 μM solanidine-*N*-oxide. Again, for TRT 4 (control), the medium contained no *Solanum* glycoalkaloids but contained the organic solvent at the same concentration as TRTs 1, 2 and 3. Oocyte cleavage rate was determined at 48 h after exposure of oocytes to spermatozoa. Embryo development was determined on Days 6, 8 and 10 of culture using an inverted microscope at 100×.

The oocytes, presumptive zygotes and embryos were cultured in media as described above in the presence of the alkaloids and under a cover of paraffin oil. Although these alkaloids contain a hydrophobic steroidal framework, the presence of polar functional groups in their structures would prohibit solubility into the paraffin oil cover.

2.4. Statistical analysis

In Experiments 1 and 2, percentage data were angularly transformed and analyzed by the use of a general linear model (GLM) ANOVA. The Fisher's least significant difference (LSD) at the 5% significant level ($P < 0.05$) was used to test the differences between treatment means. The SAS computer software package (SAS Inst. Inc., 1999) was used for all statistical calculations.

3. Results and discussion

Exposure of bovine oocytes to the steroidal glycoalkaloids during in vitro maturation inhibited the subsequent pre-implantation embryo development. Potency of the embryotoxicity varied between these steroidal glycoalkaloids. Cleavage rates in the medium containing steroidal glycoalkaloids (TRTs 1–3) were significantly less ($P < 0.05$) as compared with those for the control group (Table 1). The number of morula-stage embryos at Day 6 and blastocysts at Day 8 after IVC in SOL-containing medium (TRT1) was reduced ($P < 0.05$) compared with those from the control group. Embryos derived from oocytes exposed to SOL (TRT1) or to SON (TRT3) during maturation had lesser ($P < 0.05$) yields of expanded and hatched blastocyst compared with those from the control group. Furthermore, oocytes matured in SOL-containing medium (TRT1) and CHA-containing medium (TRT2) had lesser ($P < 0.05$) cleavage rates than SON-containing medium (TRT3). The morula production in SOL was less ($P < 0.05$) compared with that for SON. The blastocyst yields in SOL appeared to decrease compared with treatment with other alkaloids.

The results in Table 1 further demonstrate that pre-implantation embryo development is adversely affected by treatment with SOL, SON and CHA glycoalkaloids during embryo culture. This effect is most significant during the later pre-placentation embryo development stage as indicated by the reduced numbers of expanded and hatched blastocysts. There was

Table 1
The effects of steroidal alkaloids from the potato plant (*S. tuberosum*) on bovine oocyte maturation and pre-implantation embryo development in vitro

Alkaloid in medium (6 μM)	Number of oocytes	Cleavage Rate at 48 h[a], *n*(%)[b]	Morulae[c] at Day 6[d], *n* (%)[e]	Blastocysts at Day 8, *n* (%)	Expanded and hatched blastocysts at d 10, *n* (%)
Experiment 1: in vitro oocyte maturation					
α-Solanine	622	418 (67.2)a	122/377 (32.4)a	59 (14.1)a	53 (12.7)ab
α-Chaconine	636	430 (67.6)a	153/385 (39.7)b	82 (19.1)b	68 (15.8)bc
Solanidine-*N*-oxide	622	470 (75.6)b	178/420 (42.4)b,c	85 (18.1)a,b	57 (12.1)b
Control	626	522 (83.4)c	219/474 (46.2)c	115 (22.0)b	100 (19.2)c
Experiment 2: in vitro embryo culture					
α-Solanine	582	448 (77.0)	170/356 (47.8)	68 (15.2)a	41 (9.2)a
α-Chaconine	598	426 (71.2)	169/331 (51.1)	83 (19.5)a,b	51 (12.0)ab
Solanidine-*N*-oxide	589	457 (77.6)	182/359 (50.7)	109 (23.9)b	59 (12.9)b
Control	601	482 (80.2)	200/376 (53.2)	113 (23.4)b	92 (19.1)c

Values with different letters (a–c) are significantly different ($P < 0.05$).

[a] 0 h = the time when the in vitro matured oocytes were added to Fert-TALP containing spermatozoa.

[b] The percentage data were angularly transformed and analyzed by general linear model (GLM) ANOVA. The percentage of each treatment in this table represents 10 replications.

[c] Morula rates were based on nine replications for Experiment 1 and eight replications for Experiment 2.

[d] Day 0 = IVF.

[e] Percentage development of morulae/blastocysts/expanded blastocysts was calculated with respect to cleaved oocytes.

no difference ($P > 0.05$) in cleavage rate at 48 h after IVF and in morula production at Day 6 between treatments. Blastocyst yield in SOL was decreased compared with the SON-treatment and control groups. Blastocyst yield with CHA-treated groups appeared to decrease, but not significantly ($P > 0.05$) compared with other alkaloids. Embryos exposed to all steroidal glycoalkaloids during IVC had lower ($P < 0.05$) yields of expanded and hatched blastocysts compared to control.

Steroidal glycoalkaloids from *Solanum* have a variety of biological activities including affecting the active sodium transport in certain somatic cells, altering the membrane potential of embryos, modifying the relationship of phospholipid sterol/liposomes, etc. (Blankemeyer et al., 1992; McWilliams et al., 2000). It has been reported that feeding of potato alkaloids affects food consumption resulting in decreased body and liver weights in mice (Friedman et al., 1996). Studies of the toxicity of *Solanum* glycoalkaloids by the use of in vivo transgenic mice models showed that the mutation frequencies in the livers of the dams dosed with α-chaconine and α-solanine were three to four times greater than typical in the livers of this transgenic mouse strain (Crawford and Myhr, 1995).

We report here that embryos cultured in media containing either α-solanine (TRT 1) or α-chaconine (TRT 2) had a reduced rate of development to the blastocyst stage (Table 1). These results agree with other studies demonstrating that α-chaconine and α-solanine, the two major potato glycoalkaloids, are toxic to amphibian embryos also (Rayburn et al., 1995; Blankemeyer et al., 1992). Adverse effects on embryos were found by observing changes in membrane potentials using a di-4-ANEPPS fluorescent electrochromic dye (McWilliams et al., 2000). The frog embryo teratogenesis assay further showed that variable embryo toxicity is associated with specific steroidal alkaloid structural components (Friedman et al., 1992). Furthermore, α-solanine and α-chaconine exhibited a stronger synergistic effect when combined as compared with treatments with each alone in culture media with respect to the embryo-toxic effects manifest as mortality or malformation when closely examined (Rayburn et al., 1995). In the present experiments, the synergistic effects of α-solanine and α-chaconine was not determined and this could be important in mammals as ingestion of *Solanum* plants often results in absorption of multiple toxins together including the two glycoalkaloids mentioned above.

These results of in vitro embryo culture also demonstrate that solanidine-*N*-oxide (TRT 3) did not have any significant effects on cleavage rate or early embryo development, but did adversely affect development of later pre-implantation embryo development (expanded and hatched blastocysts). This observation supports the findings reported by Gaffield and Keeler (1996a), indicating that the *N*-oxide is less teratogenic than the glycosides. Biochemically, solanidine-*N*-oxide is a putative metabolite derived from potato steroidal glycoalkaloids by the *N*-oxidation pathway. This aglycone was supposedly nontoxic compared with the glycosides, however, increased malformations were observed in litters born to mothers dosed with relatively small amounts of both the nontoxic aglycone, solanidine and the derivative solanidine-*N*-oxide (Gaffield and Keeler, 1996b). These previous results demonstrate that terata induction by solanidanes is not due to maternal toxicity, nor is the oligosaccharide portion of the steroidal alkaloid glycosides required to facilitate passage of the teratogen to the fetus. Furthermore, this suggests that the *N*-oxidation pathway does not completely detoxify these glycoalkaloids as previously thought (Gaffield and Keeler, 1996b).

4. Conclusion

In conclusion, this research provides an example of an in vitro screening method to evaluate potential embryo-toxicity of *Solanum* glycoalkaloids using IVF-produced bovine embryos as the assay model. Furthermore, the present results suggest that these alkaloids may have a negative effect on early embryo development and survival in vivo when ingested by man or animals. Additionally, the differences between the glycosides and the aglycone in their embryonic impact further emphasize the importance of structure activity relationship in alkaloid toxicoses.

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