Small Ruminant Research 113 (2013) 353–359

Contents lists available at SciVerse ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

# *In vitro* development of ovine preantral follicles and oocyte cleavage rate are not affected by long-term ingestion of detoxified castor meal

L.M. Silva[a,*], C.H.A. Oliveira[a], A.M. Silva[a], C.M.G. Silva[b], S.V. Castro[b], A.A. Carvalho[b], A.B.G. Duarte[b], E.C. Costa[b], C. Feltrin[c], J.R. Figueiredo[b], D. Rondina[a]

[a] *Laboratory of Ruminant Production and Nutrition, LANUPRUMI, Faculty of Veterinary Medicine, PPGCV, State University of Ceará, Fortaleza, CE, Brazil*
[b] *Laboratory of Manipulation of Oocytes and Preantral Follicles, LAMOFOPA, Faculty of Veterinary Medicine, PPGCV, State University of Ceará, Fortaleza, CE, Brazil*
[c] *University of Fortaleza, UNIFOR, Fortaleza, CE, Brazil*

ARTICLE INFO

*Article history:*
Received 13 July 2012
Received in revised form
20 November 2012
Accepted 7 March 2013
Available online 12 April 2013

*Keywords:*
Ewe
Reproduction performance
*In vitro* culture
Detoxified castor meal

ABSTRACT

In the present study, we evaluated the effect of long-term ingestion of detoxified castor meal on *in vitro* development of sheep preantral follicles as well as on the developmental competence of oocytes from antral follicles. Ovarian fragments were cultured for one or seven days and further analyzed by histology and fluorescence microscopy (experiment 1). The cumulus oocyte complexes (COCs) obtained from antral follicles were matured *in vitro* and activated by parthenogenesis or fertilization *in vitro* (experiment 2). Even after 1 day of culture, in both tested groups, a significant reduction in the percentage of primordial follicles and a concomitant increase in the percentage of intermediate follicles were observed when compared to non cultured tissue. After 7 days of culture, the percentage of primary follicles in both tested groups was significantly higher when compared to the non cultured tissue or the tissue cultured for 1 day ($P < 0.05$). The number of *in vitro* embryos produced was similar between the tested groups. However, for those animals fed with detoxified castor meal, the parthenogenesis method resulted in a higher number of embryos (morulae) when compared to *in vitro* fertilization (IVF) activation. In conclusion, detoxified castor meal can be used as an alternative protein source without affecting the ovine preantral and antral folliculogenesis.



## 1. Introduction

The castor oil plant (*Ricinus communis* L.) is one of the most traditional crops produced in the semi-arid region of Brazil. Its cultivation has great economic and social importance, with direct practical applications in the cosmetics and automobile industries, and it is one of the main oils used for biodiesel production (Diniz et al., 2010). However, there are major environmental concerns regarding the large amount of byproducts generated by the

* Corresponding author at: Programa de Pós-Graduação em Ciências Veterinárias (PPGCV), Laboratório de Nutrição e Produção de Ruminantes (LANUPRUMI), Universidade Estadual do Ceará (UECE), Av. Paranjana, 1700, Campus do Itaperi, Fortaleza, CE 60740-000, Brazil.
Tel.: +55 85 3101 9858; fax: +55 85 3101 9858.
*E-mail addresses:* liligomesvet@hotmail.com, lmsilvavet@gmail.com (L.M. Silva).

0921-4488
http://dx.doi.org/10.1016/j.smallrumres.2013.03.005

biodiesel industry during castor oil manufacture (European Food Safety Authority, 2008). For each ton of castor oil, 1.31 ton of husks and 1.13 ton of meal are produced (Lima et al., 2011). This byproduct is a potentially environmental contaminant containing toxic and allergenic elements, including ricin, ricinine and the allergen complex CB-1 (Burdock et al., 2006).

The ricin consists of a two-subunit protein (subunit A and B) bound by disulfide linkages (Barnes et al., 2009). According to the model proposed by Audi et al. (2005), the ricin B subunit binds to the plasma membrane glycoproteins and glycolipids and penetrates the cytosol by endocytosis. Once in the cytosol, ricin is translocated by vesicular transport into endosomes and then to the Golgi apparatus and endoplasmic reticulum, the site of subunit A and B cleavage. The presence of subunit A in the cytosol promotes protein synthesis inactivation by removal of adenine from the 60S ribosomal subunit. Furthermore, ricinine and CB-1 can trigger, respectively, bleeding and respiratory disorders in humans (Schieltz et al., 2011). Despite the presence of toxic compounds, there is a growing interest in converting castor byproducts to alternative feed sources for ruminants due to the nutritional content of these products.

Several studies have been conducted to establish efficient methods for castor meal detoxification and, thus, make it suitable for animal consumption (Godoy et al., 2009). Currently, there are many chemical and mechanical methods (Anandan et al., 2005) used to detoxify castor meal. However, until now, the effects of castor meal feeding on livestock and livestock reproduction have not been well understood. Moreover, no previous research has investigated the possible toxic effects of a long-term castor meal diet on the folliculogenesis of sheep.

The possible detrimental effect caused by this type of feed on pre-antral folliculogenesis can be evaluated using *in vitro* follicular culture, particularly culture employing primordial follicles, which represent the main resting stockpile of germ cells in the mammalian ovary (Figueiredo et al., 2011). Similarly, techniques used for oocyte activation, such as *in vitro* fertilization and parthenogenesis, can be employed to investigate oocyte developmental competence from antral follicles to produce embryos *in vitro*. Therefore, the aforementioned techniques can aid in studies of reproductive toxicology. Thus, the aim of this study was to verify if the long-term ingestion of detoxified castor meal affects the activation, viability and growth of ovine preantral follicles cultured *in vitro* as well as the developmental potential of oocytes from antral follicles to produce embryos *in vitro* after activation by parthenogenesis or *in vitro* fertilization.

## 2. Materials and methods

This study was submitted and approved by the Ethics Committee for experimental use of animals of the State University of Ceará (CEUA-UECE) with the protocol number 09230950-0/74.

### 2.1. Source of animals and dietary treatments

In this work, 12 adult (2–4 years) multiparous Santa Inês breed sheep were used for the study. During 15 months (March 2010 to June 2011), the animals were randomly divided into two experimental groups: soybean meal (control), $n = 6$; and detoxified castor meal, $n = 6$. The group of animals fed with soybean meal (control) received Tifton hay and concentrated meal (80% corn, 15% soybean meal, and 5% minerals). The animals fed with the detoxified castor meal received Tifton hay and concentrated meal (80% corn, 15% castor meal, and 5% minerals). Both diets were isoenergetic (74% of NDT) and isoproteic (14% gross protein) following the nutritional requirements of maintenance (NRC, 2007). Each experimental group was kept in a collective stall with mineral salt and water *ad libitum*.

### 2.2. Chemical treatment of castor meal and quantification of ricin

The detoxification was performed as described by Anandan et al. (2005). For each 1 kg of castor meal, 60 g of calcium oxide was added for detoxification. The treated samples were left overnight (8 h). Subsequently, the sun-dried lime-treated samples were used for analysis. After this process, ricin elimination was detected by electrophoresis in a 12% polyacrylamide gel under non-denaturing conditions (native-PAGE) performed in a vertical mini-gel Bio-Rad PowerPac Basic Supply system. The gel was stained with Coomassie Blue R250, and ricin quantification was accomplished by determination of total protein in the castor meal samples using the Bradford method (1976); bovine serum albumin (BSA) was the standard for normalization. The polyacrylamide gel images were visualized by an ImageScanner™ from GE Healthcare (ImageMaster software compatible) and were analyzed with the Image Master Platinum program. The protein bands were quantified into volume units (area × intensity) following the methodology described by Meunier et al. (2005).

### 2.3. Chemical source and ovarian recovery

Unless otherwise mentioned, all chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, MO, USA). The ovaries of six animals from each experimental group *i.e.*, soybean meal (control) and detoxified castor meal, were collected immediately *postmortem*. The ovaries were washed once in 70% alcohol for 10 seconds and twice in Minimum Essential Medium (MEM) supplemented with HEPES and antibiotics (100 μg/mL penicillin and 100 μg/mL streptomycin) and finally transported within 1 h to the laboratory in MEM at 4 °C (Chaves et al., 2008).

### 2.4. Experiment 1. Effect of long-term ingestion of detoxified castor meal on the activation, growth and viability of ovine preantral follicles

#### 2.4.1. Ovine preantral follicle culture

In the laboratory, ovarian cortical tissue samples from each ovarian pair of both experimental groups *i.e.*, soybean meal (control) and detoxified castor meal, were cut into slices (approximate size 3 mm × 3 mm, with a thickness of 1 mm) using a needle and scalpel under sterile conditions. The tissue slices were then immediately analyzed (non cultured tissue) or placed in culture for one or seven days randomly. Before and after culture period the fragments were analyzed by classical histology and fluorescence microscopy.

The ovine tissue slices destined for *in vitro* culture were transferred to 24-well culture dishes containing 1 mL of culture media. The basic culture medium was α-MEM (pH 7.2–7.4) supplemented with ITS (insulin 10 μg/mL, transferrin 5.5 μg/mL and selenium 5 ng/mL), 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/mL BSA and 50 ng/mL recombinant follicle-stimulating hormone (rFSH). The culture medium was incubated for 1 h prior to use and replenished every other day. The culture was performed at 39 °C in 5% $CO_2$ in a humidified incubator. In this particular experiment, the animal represents the experimental unit.

#### 2.4.2. Morphological analysis and assessment of in vitro follicular growth

Before (non cultured tissue) and after one or seven days in culture, the pieces were fixed in Carnoy's solution for 4 h and then dehydrated in increasing concentrations of ethanol. After paraffin embedding (Synth, São Paulo, Brazil), the ovine tissue slices were cut into 7-μm sections, and each section was mounted on glass slides and stained with hematoxylin–eosin. The follicular development stages and survival rates were assessed microscopically in serial sections. Coded, anonymized slides were examined using a microscope (Nikon, Japan) under 400× magnification.

The developmental stages of follicles were classified as either primordial (1 layer of flattened granulosa cells around the oocyte) or growing