Zygote **24** (April), pp. 277–285. © Cambridge University Press 2015
doi:10.1017/S0967199415000179 First Published Online 17 June 2015

Amburana cearensis leaf extract maintains survival and promotes *in vitro* development of ovine secondary follicles

R.S. Barberino[2], V.R.P. Barros[2], V.G. Menezes[2], L.P. Santos[2], V.R. Araújo[3], M.A.A. Queiroz[4], J.R.G.S. Almeida[5], R.C. Palheta Jr[6] and M.H.T. Matos[1,2]
Federal University of San Francisco Valley, Petrolina, PE, Brazil; and State University of Ceara, Fortaleza, CE, Brazil

Date submitted: 02.12.2014. Date revised: 04.03.2015. Date accepted: 15.04.2015

Summary

The antioxidant properties of *Amburana cearensis* extract may be a useful substitute for standard cell culture medium. Thus, the aim of this study was to evaluate the effect of this extract, with or without supplementation, on *in vitro* survival and development of sheep isolated secondary follicles. After collection of the ovaries, secondary follicles were isolated and cultured for 18 days in α-MEM$^+$ supplemented with bovine serum albumin, insulin, transferrin, selenium, glutamine, hypoxanthine and ascorbic acid (control medium) or into medium composed of different concentrations of *A. cearensis* extract without supplements (Amb 0.1; 0.2 or 0.4 mg/ml) or *A. cearensis* extract supplemented with the same substances described above for α-MEM$^+$ supplementation. The *A. cearensis* supplemented medium was named Amb 0.1$^+$; 0.2$^+$ or 0.4$^+$ mg/ml. There were more morphologically normal follicles in Amb 0.1 or Amb 0.4 mg/ml than in the control medium (α-MEM$^+$) after 18 days of culture. Moreover, the percentage of antrum formation was significantly higher in Amb 0.1 or Amb 0.2 mg/ml than in α-MEM$^+$ and Amb 0.1$^+$ mg/ml, and similar to the other treatments. All *A. cearensis* extract media induced a progressive and significant increase in follicular diameter throughout the culture period. In conclusion, this study showed that 0.1 mg/ml of this extract, without supplementation, maintains follicular survival and promotes the development of ovine isolated secondary follicles *in vitro*. This extract can be an alternative culture medium for preantral follicle development.

Keywords: Culture, Medicinal plant, Oocyte, Sheep, Umburana

Introduction

In recent years, much attention has focused on optimizing *in vitro* culture medium composition for small ovarian follicles as they can act as a source of fertilizable oocytes for further *in vitro* embryo production (Gupta *et al.*, 2007). Although acceptable rates of follicular viability and growth have been obtained with various media, Minimal Essential Medium alpha modified (α-MEM) has emerged as the most commonly used medium for culture of oocytes and follicular cells in caprine (Chaves *et al.*, 2012) and ovine (Esmaielzadeh *et al.*, 2013; Santos *et al.*, 2014) species. However, for improving the developmental competence of oocytes, α-MEM needs a constant addition of supplements, such as antioxidants, hormones and growth factors (Abedelahi *et al.*, 2010; Andrade *et al.*, 2012), which can make follicular cell culture more expensive. Therefore, the natural compounds

[1]All correspondence to: M.H.T. Matos. Universidade Federal do Vale do São Francisco (UNIVASF). Campus de Ciências Agrárias. Colegiado de Medicina Veterinária. Laboratório de Biologia Celular, Citologia e Histologia. Rodovia BR 407, Km 12, Lote 543 – Projeto de Irrigação Nilo Coelho – S/N, C1. CEP: 56300–990, Petrolina, PE, Brasil. Tel: +55 87 2101 4839. E-mail: helena.matos@univasf.edu.br
[2]Nucleus of Biotechnology Applied to Ovarian Follicle Development, Federal University of San Francisco Valley, Petrolina, PE, Brazil.
[3]Faculty of Veterinary Medicine, Laboratory of Manipulation of Oocytes and Preantral Follicles, State University of Ceara, Fortaleza, CE, Brazil.
[4]Laboratory of Bromatology and Animal Nutrition, Federal University of San Francisco Valley, Petrolina, PE, Brazil.
[5]Nucleus for Studies and Research on Medicinal Plants, Federal University of San Francisco Valley, Petrolina, PE, Brazil.
[6]Laboratory of Pharmacology, Federal University of San Francisco Valley, Petrolina, PE, Brazil.

presented in medicinal plants are a promising strategy for alternative sources of basic culture medium. Among the several extracts of medicinal plants, *Amburana cearensis* noteworthy for its numerous cytoprotective characteristics.

Amburana cearensis (Allemão) A.C. Smith (Fabaceae) is a tree commonly found in northeastern Brazil, where it is popularly known as 'Cumaru', 'Amburana-de-cheiro' or 'Umburana' (Albuquerque *et al.*, 2007; Leal *et al.*, 2010), being widely used in traditional medicine for the treatment of a wide range of diseases including respiratory problems in general, influenza, cough, expectorant, thrombosis, hypertension, inflammations and healing (Cartaxo *et al.*, 2010). This plant has phenolic compounds, especially flavonoids and phenolic acids (isokaempferide, kaempferol, afromosin and quercetin) which can act as antioxidants through their ability to scavenge reactive oxygen species (ROS) (Leal *et al.*, 2005; Ruijters *et al.*, 2013; Wang & Shahidi, 2013). In addition, coumarin, also present in *A. cearensis*, reduces the lipid peroxidation activity in different types of rat cells (Neichi *et al.*, 1983; Martín-Aragón *et al.*, 1998).

It is thought that ovarian follicular atresia is initiated as a consequence of inadequate protection of cultured follicular cells from the damaging effects of ROS (Tilly & Tilly, 1995). Therefore, it could be speculated that the natural compounds of *A. cearensis*, especially those with antioxidant properties, would protect ovarian follicles from ROS during *in vitro* culture. Thus, *A. cearensis* extract may be a useful substitute for standard cell culture medium. In this sense, there were no reports in which *A. cearensis* extract has been used as a culture medium for ovine ovarian follicles. Thus, this study was conducted to evaluate the effect of this extract on *in vitro* survival and development of sheep isolated secondary follicles. Additionally, the effectiveness of the addition of supplements to *A. cearensis* extract was studied.

Materials and methods

Unless otherwise mentioned, culture media, supplements and chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Plant material and extract preparation

Fresh leaves of *A. cearensis* were collected in Petrolina (09°23′55″ South and 38–40°30′03″ West, Pernambuco, Brazil), from the Caatinga biome, and characterized in a semi-arid climate, with average annual temperature of 26.4ºC. A voucher specimen (5545) was deposited at the Herbário Vale do São Francisco (HVASF) of the Federal University of San Francisco Valley during the dry season, flowering and fructification stages of the plant. The leaves were dried in an oven at 40°C and pulverized and extracted at room temperature with 95% ethanol (Vetec, Duque de Caxias-RJ, Brazil) for 72 h. The extract was dried at 45°C using a rotavator and the yield was approximately 10%, obtaining the crude ethanolic extract of the leaves of *A. cearensis*, which was dissolved in 0.9% saline solution, corresponding to concentrations of 0.1, 0.2 or 0.4 mg/ml, which were keep at 4°C.

Source of ovarian tissue

This experiment was approved and performed under the guidelines of the Committee of Ethics and Deontology Studies and Research at the Federal University of San Francisco Valley (00508/2012).

Ovaries ($n = 50$) were collected at a local abattoir from 25 adult (1–3 years old) mixed-breed ovine (*Ovis aries*). Immediately post-mortem, pairs of ovaries were washed once in 70% alcohol and then twice in minimum essential medium buffered with HEPES (MEM-HEPES) and supplemented with antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin). Next, the ovaries were transported within 1 h to the laboratory in tubes containing MEM-HEPES with antibiotics at 4°C (Chaves *et al.*, 2008).

Isolation and selection of ovine secondary follicles

In the laboratory, the surrounding fatty tissues and ligaments were stripped from the ovaries; large antral follicles and corpora lutea were removed. Ovarian cortical slices (1–2 mm thick) were cut from the ovarian surface using a surgical blade under sterile conditions and subsequently placed in fragmentation medium consisting of MEM-HEPES with antibiotics. Ovine secondary follicles, approximately 200 μm in diameter without antral cavities, were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan; at x100 magnification) and mechanically isolated by microdissection using 26-gauge (26G) needles. These follicles were then transferred to 100 μl droplets containing basic culture medium for the quality evaluation. Only follicles that displayed the following characteristics were selected for culture: an intact basement membrane, two or more granulosa cells layers and a visible and healthy oocyte that was round and centrally located among the cells, without any dark cytoplasm. Isolated follicles were pooled and then randomly allocated to the treatment groups, with 30 follicles per group.

https://doi.org/10.1017/S0967199415000179 Published online by Cambridge University Press

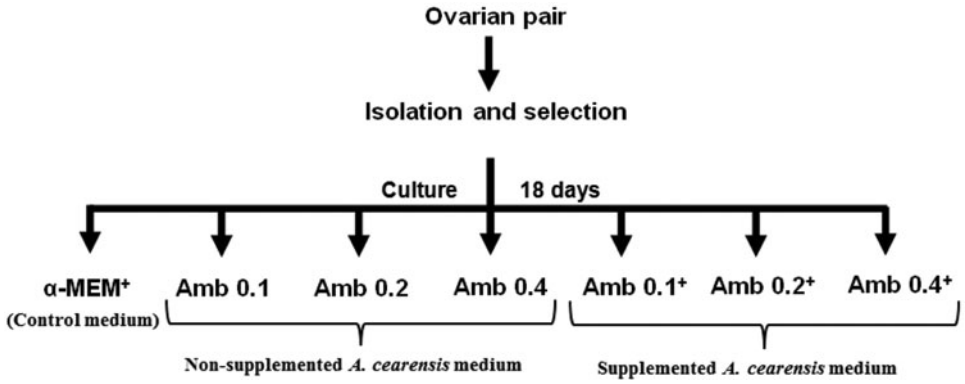

Figure 1 General experimental protocol for *in vitro* culture of ovine preantral follicles in α-MEM$^+$ or different concentrations of *Amburana cearensis* extract in the absence (Amb 0.1; 0.2 and Amb 0.4) or presence of supplements (Amb 0.1$^+$; Amb 0.2$^+$ and Amb 0.4$^+$).

In vitro culture of secondary follicles

The basic control medium consisted of α-MEM (pH 7.2–7.4; Gibco, Invitrogen, Karlsruhe, Germany) supplemented with 3.0 mg/ml bovine serum albumin (BSA), 10 ng/ml insulin, 5.5 μg/ml transferrin, 5.0 ng/ml selenium, 2 mM glutamine, 2 mM hypoxanthine and 50 μg/ml ascorbic acid and then referred as α-MEM$^+$. To verify the influence of the plant, the follicles were randomly distributed in α-MEM$^+$ (control medium) or into medium composed of different concentrations of *A. cearensis* extract without supplements (Amb 0.1; 0.2 or 0.4 mg/ml) or *A. cearensis* extract supplemented with the same substances described above for α-MEM$^+$ supplementation. The *A. cearensis* supplemented medium was named Amb 0.1$^+$; 0.2$^+$ or 0.4$^+$ mg/ml (Figure 1). The follicles were individually cultured (one follicle per droplet) in 100 μl of culture medium under mineral oil in Petri dishes (60 × 15 mm; Corning, Sarstedt, Newton, NC, USA). Incubation was performed at 39°C and 5% CO_2 in air for 18 days. In all the treatments 60 μl of the culture media was replaced with fresh media in each droplet at every 6 days (Magalhães *et al.*, 2011).

Morphological evaluation of follicle development

The morphological aspects of all preantral follicles were assessed every 6 days using a pre-calibrated ocular micrometer in a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan; ×100 magnification). Only those follicles showing an intact basement membrane, with bright and homogeneous granulosa cells and an absence of morphological signs of degeneration, were classified as morphologically normal follicles. Follicular atresia was recognized when a darkening of the oocytes and surrounding cumulus cells, misshapen oocytes or decreased follicle diameter was noted. The rupture of the basement membrane was also observed and characterized as oocyte extrusion. The following characteristics were analyzed in the morphologically normal follicles: (i) antral cavity formation, defined as the emergence of a visible translucent cavity within the granulosa cell layers; (ii) follicle diameter, measured from the basement membrane, which included two perpendicular measures of each follicle; and (iii) daily follicular growth rate, calculated as the diameter variation during the culture period.

Statistical analysis

Data from morphologically normal follicles, extruded follicles and antrum formation after *in vitro* culture were expressed as percentages and compared by the chi-squared test. Follicular diameter and daily follicular growth rates were submitted to the Shapiro–Wilk test to verify normal distribution of residues and homogeneity of variances using PROC UNIVARIATE SAS 9.0 procedure. The residues that did not show a normal distribution were logarithmically transformed [log10(X + 1)] for normal distribution adjustment. Because requirements underlying the analysis of variance were present, for follicular diameter, ANOVA was executed using PROC MIXED SAS (2003) with REPEATED statement to account for autocorrelation between sequential measurements. The model included the effects of treatment (α-MEM and *A. cearensis* concentrations), time of culture (0, 6, 12 or 18 days) and their interactions. Data from follicular growth were analyzed in the same way, however, using the PROC GLM procedure. Due to the presence of heterogeneity of variances for data from follicular diameter and growth rate, the Kruskal–Wallis non-parametric test was used through the PROC NPAR1WAY SAS 9.0 procedure. These results were expressed as the means

https://doi.org/10.1017/S0967199415000179 Published online by Cambridge University Press

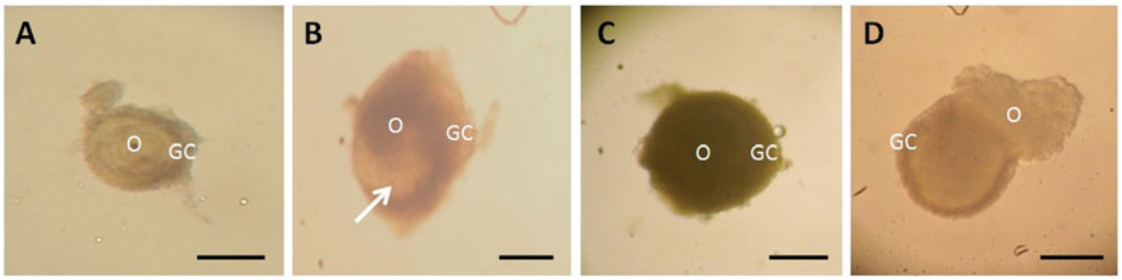

Figure 2 Morphologically normal secondary follicle at day 0 (*A*), antral (*B*), atretic (*C*), and extruded (*D*) follicle after 6 days of culture. GC: granulosa cell; O: oocyte. Arrow: antral cavity. Scale bar: 100 μm (× 100 magnification).

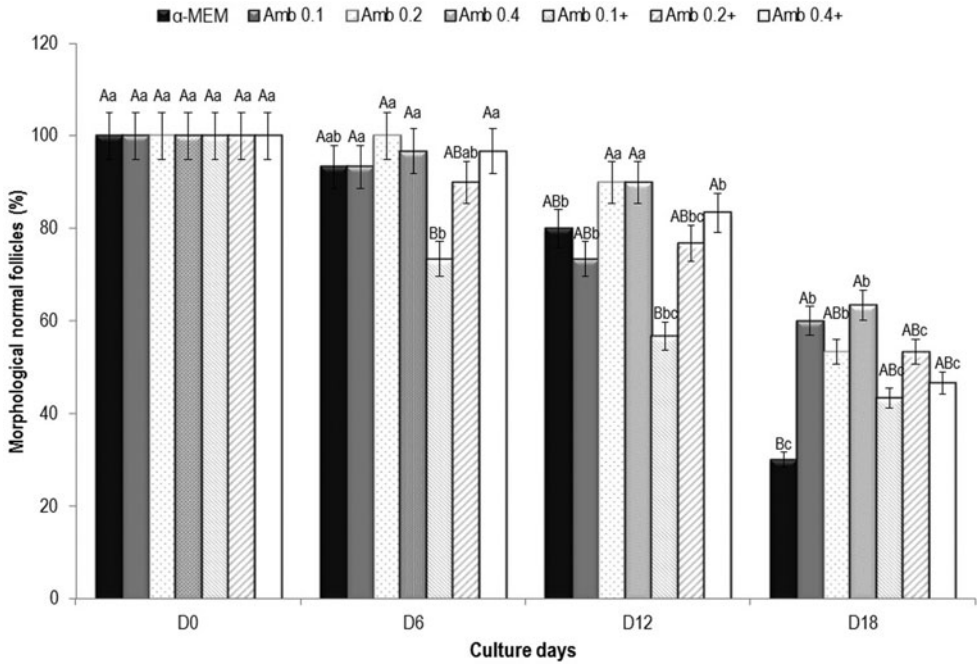

Figure 3 Percentages of morphologically normal follicles cultured in α-MEM$^+$ or different concentrations of *Amburana cearensis* extract in the absence (Amb 0.1; 0.2 and Amb 0.4) or presence of supplements (Amb 0.1$^+$; Amb 0.2$^+$ and Amb 0.4$^+$). ^{a,b,c}Different letters denote significant differences among culture periods in the same treatment ($P < 0.05$). ^{A,B}Different letters denote significant differences among treatments in the same period ($P < 0.05$).

± standard error mean (SEM) and differences were considered significant when $P < 0.05$.

Results

Follicular survival after *in vitro* culture

Morphologically normal follicles showed centrally located oocytes and normal granulosa cells, which were enclosed by an intact basement membrane (Fig. 2*A*). As early as day 6 of the culture, a small antral cavity (Fig. 2*B*) and atretic follicles (Fig. 2C) could be observed in all the treatments. Extruded follicles were observed only after culture in α-MEM$^+$ (Fig. 2*D*).

The percentage of follicular survival after 18 days of culture in the different concentrations of *A. cearensis* extract is shown in Fig. 3. The percentage of morphologically normal follicles decreased significantly from day 0 to day 6 only in the Amb 0.1$^+$ mg/ml treatment. Nevertheless, the percentage of normal follicles decreased significantly from day 6 to day 12 in all the treatments, except in Amb 0.2 and Amb 0.4 mg/ml without supplements. A significant decrease in the percentage of normal follicles in these two *A. cearensis* concentrations was observed only after 18 days of culture.

When the treatments were compared at day 18, there were more morphologically normal follicles in Amb 0.1 or Amb 0.4 mg/ml (without supplements) than in

https://doi.org/10.1017/S0967199415000179 Published online by Cambridge University Press

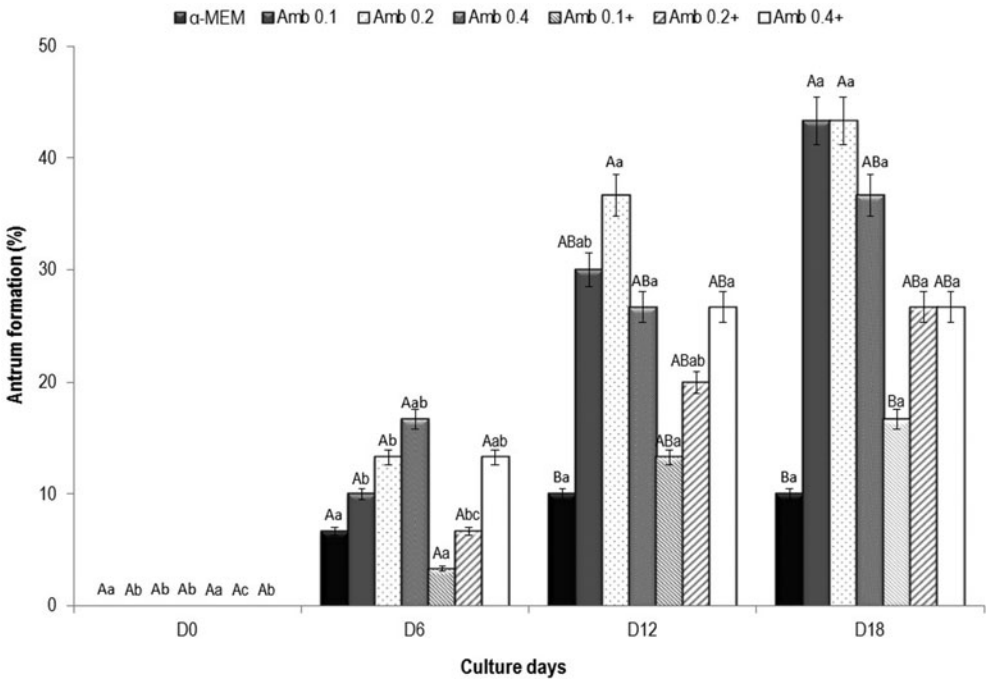

Figure 4 Percentages of antral cavity formation in follicles cultured in α-MEM$^+$ or different concentrations of *Amburana cearensis* extract in the absence (Amb 0.1; 0.2 and Amb 0.4) or presence of supplements (Amb 0.1$^+$; Amb 0.2$^+$ and Amb 0.4$^+$). ^{a,b,c}Different letters denote significant differences among culture periods in the same treatment ($P < 0.05$). ^{A,B}Different letters denote significant differences among treatments in the same period ($P < 0.05$).

control medium (α-MEM$^+$). In addition, there was no significant difference in the rate of follicular survival among the *A. cearensis* concentrations, with or without supplements ($P > 0.05$).

Antral cavity formation during *in vitro* culture

Compared with day 0, the rates of antral cavity formation increased significantly only from day 12 of culture onwards in Amb 0.2, Amb 0.4, Amb 0.2$^+$ or Amb 0.4$^+$ mg/ml treatments (Fig. 4). Moreover, follicles cultured in Amb 0.1 mg/ml increased significantly the percentage of antrum formation only at day 18.

After comparing the different treatments at day 18 of culture, the rate of antrum formation was significantly higher in Amb 0.1 and Amb 0.2 mg/ml (without supplements) than in α-MEM$^+$ (control medium) and Amb 0.1$^+$ mg/ml, and similar to the other treatments ($P > 0.05$).

Follicular diameter and growth rate *in vitro*

All *A. cearensis* extract media induced a progressive and significant increase in follicular diameter throughout the culture period (Fig. 5). After 12 days, all the treatments had similar follicular diameters ($P > 0.05$), which was significantly higher than that found after Amb 0.4$^+$ treatment. At the end of the culture, follicles cultured in non-supplemented 0.1, 0.2 or 0.4 mg/mL *A. cearensis* had larger diameters than those after Amb 0.2$^+$ mg/ml treatment and similar to the other treatments ($P > 0.05$).

Follicles cultured in α-MEM$^+$, in *A. cearensis* without supplements or in Amb 0.1$^+$ (8.9 μm/day) or Amb 0.2$^+$ (8.3 μm/day) mg/ml showed similar rates of daily growth ($P > 0.05$), which were significantly higher than Amb 0.4$^+$ mg/ml (7.0 μm/day) treatment.

Discussion

To the best of our knowledge, this study constitutes the first report to demonstrate the beneficial effect of *A. cearensis* extract on *in vitro* culture of ovine isolated follicles. It is known that the levels of antioxidant defenses are lower during *in vitro* oocyte and embryo culture than *in vivo*. Therefore, the addition of an antioxidant to the medium may be important to reduce the damage caused by oxidative stress (Crocomo *et al.*, 2012; Rajabi-Toustani *et al.*, 2013). In the present study, after 18 days of culture, appropriate concentrations of medium composed of *A. cearensis* extract (0.1 or 0.4 mg/ml) showed higher percentages of morphologically normal follicles than for the control medium (α-MEM$^+$).

A recent study performed by our group used high performance liquid chromatography (HPLC) to determine the fingerprint chromatogram of the

https://doi.org/10.1017/S0967199415000179 Published online by Cambridge University Press

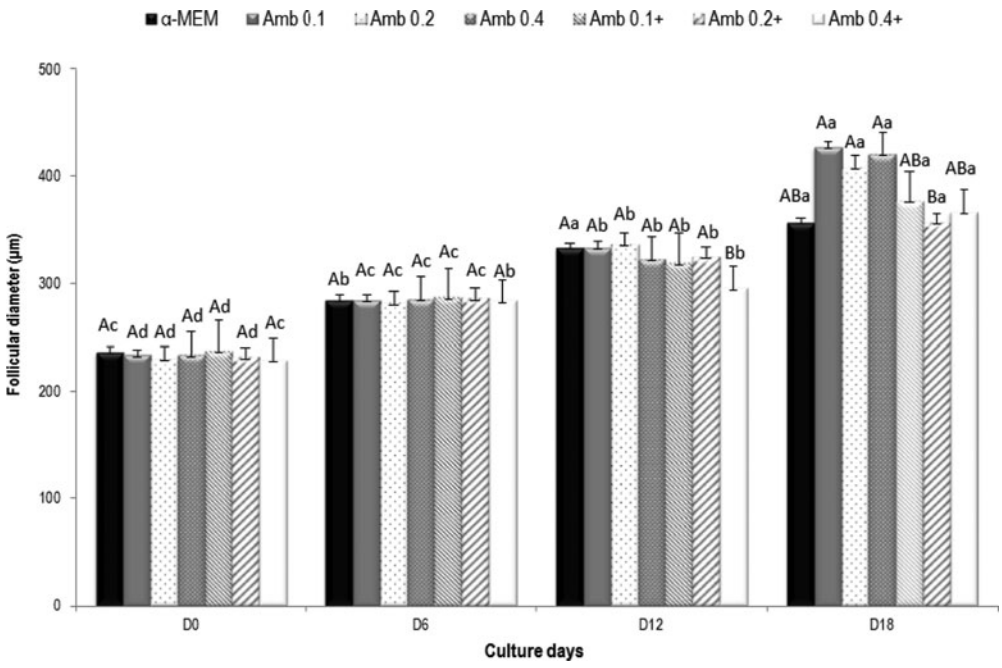

Figure 5 Diameter (μm) of follicles cultured for 18 days in α-MEM$^+$ or different concentrations of *Amburana cearensis* extract in the absence (Amb 0.1; 0.2 and Amb 0.4) or presence of supplements (Amb 0.1$^+$; Amb 0.2$^+$ and Amb 0.4$^+$). ^{a,b,c,d}Different letters denote significant differences among culture periods in the same treatment ($P < 0.05$). ^{A,B}Different letters denote significant differences among treatments in the same period ($P < 0.05$).

ethanolic extract of *A. cearensis* (Gouveia *et al.*, 2015). This extract was the same used in the current work and the following compounds were identified and quantified: gallic acid, protocatechuic acid, epicatechin, *p*-coumaric acid and kaempferol. Gallic acid (GA) and protocatechuic acid (PCA), compounds, found in large amounts in the *A. cearensis* extract, have antioxidant action and scavenging ROS. The GA, an endogenous plant phenol (Singh *et al.*, 2004), showed anti-lipid peroxidative and antioxidant effects in pancreatic tissue of Wistar rats after induced toxicity by streptozotocin (Punithavathi *et al.*, 2011). PCA, one of the major benzoic acid derivatives from vegetables and fruits (Guan *et al.*, 2009), extends lifespan and increases osmotic and heat stress resistance in *Caenorhabditis elegans*, by elevate antioxidant enzyme activities such as catalase and superoxide dismutase (Kim *et al.*, 2014).

In addition, epicatechin and kaempferol are phenols belonging to the flavonoids class. Their prominent and most useful properties are scavenge ROS and metal chelating activities (Lopez-Revuelta *et al.*, 2006), being considered more efficient antioxidants than vitamin C (ascorbic acid), vitamin E (α-tocoferol) and β-carotene (Gao *et al.*, 2001). Recent studies have demonstrated that epicatechin protects human vascular endothelial cells against oxidative stress (Ruijters *et al.*, 2013) and that kaempferol has antimicrobial and antioxidant properties (Tatsimo *et al.*, 2012).

With respect to *p*-coumaric acid (CA), an intermediate product of the phenylpropanoid pathway in plants, it was able to directly scavenge ROS and protect low-density lipoprotein from oxidation (Zang *et al.*, 2000). Therefore, in our study, it is possible that these natural antioxidants, especially GA and PCA, may act in isolation or in association to decrease oxidative damage during follicular cell culture in sheep, contributing to the maintenance of higher morphologically normal follicle rates.

Besides its antioxidant activity, PCA stimulated neural stem cells proliferation *in vitro* (Guan *et al.*, 2009). In the current study, it is possible that PCA may have supported follicular development *in vitro* without the necessity of medium supplementation, as, after 18 days, the rates of antrum formation were higher in follicles cultured in 0.1 or 0.2 mg/ml of *A. cearensis* extract without supplements, compared with α-MEM$^+$. This finding is important because the ability to form an antrum may be considered as a marker of follicular functionality (Rossetto *et al.*, 2012), as the mechanisms by which small cavities of fluid develop inside the follicle to form the antral cavity are related to the secretion of osmotically active molecules into small spaces between the granulosa cells (Rodgers & Irving-Rodgers, 2010). However, in previous reports, the rate of antrum formation was higher than that observed in our study (Saraiva *et al.*, 2010; Barboni *et al.*, 2011). This situation could be explained because our culture

https://doi.org/10.1017/S0967199415000179 Published online by Cambridge University Press

media were FSH-free, which is a different formulation from the culture media used by the above-cited authors. After antrum formation, the follicle becomes dependent on follicle-simulating hormone (FSH) (Erickson & Shimasaki, 2001) and the absence of this hormone in the basic medium may have contributed to lower rates of antrum formation in the present study. Similar results were obtained by Luz *et al.* (2013), who observed a low and constant rate of antrum formation during 18 days of culture of sheep secondary follicles in a FSH-free medium.

The presence of supplements (BSA, insulin, transferrin, selenium, glutamine, hypoxanthine and ascorbic acid) in the *A. cearensis* media did not improve follicular survival and antrum formation compared with the control medium (α-MEM$^+$). Previous studies have shown that addition of supplements into the culture medium could maintain ovarian follicle viability (Silva *et al.*, 2004; Peng *et al.*, 2010), due its antioxidant capacity (selenium and transferrin) or to be effective energy substrate (glutamine and BSA) for follicular cells (Songsasen *et al.*, 2007; Abedelahi *et al.*, 2010; Rodrigues *et al.*, 2010). The concentrations of the supplements used in our study are the same previously used in the culture of isolated preantral follicles in α-MEM$^+$ (Saraiva *et al.*, 2010). Therefore, it is likely that these concentrations could be unsuitable for the *A. cearensis* extract medium. It is known that some antioxidant substances may act as pro-oxidants when used at high concentrations (Andrade *et al.*, 2010). In fact, an excess of antioxidant compounds can increase the redox state, which may have deleterious effects on embryos (Guérin *et al.*, 2001). In this way, some studies have shown that high concentrations of ascorbic acid, an important antioxidant added to the culture medium, can inhibit important physiological processes in the ovary, resulting in follicular degeneration (Murray *et al.*, 2001), besides promoting oxidative damage to cellular DNA (Li & Schellhorn, 2007). Thus, as the balance between antioxidants and pro-oxidants must be preserved in the cells, future studies should be done to investigate the suitable concentrations of the supplements added to the medium containing *A. cearensis* extract.

It is important to note that the costs for purchasing medium containing *A. cearensis* extract may make *in vitro* culture studies cheaper and more available. These costs were limited to acquisition of 95% ethanol and 0.9% saline solution for extract dilution. Adversely, MEM is an expensive commercial medium that would cost about 40 times more than plant extract. Therefore, our findings encourage future studies of follicle culture in *A. cearensis* because this medicinal plant is cheaper than MEM.

In conclusion, this study showed that an appropriate concentration of *A. cearensis* extract, without supplementation, maintains follicular survival and promotes the development of ovine isolated secondary follicles *in vitro*. The concentration of 0.1 mg/ml must be highlighted due to the satisfactory results in all evaluated parameters after 18 days of culture. This extract can be a cheap and efficient alternative culture medium for preantral follicle development. Therefore, a greater knowledge of *A. cearensis* extract's chemical composition is important because it would make this protocol efficient and reproducible as that of MEM and also allows that this protocol becomes a commercial product. In future studies, we also suggest that more techniques should be performed to permit a deeper examination of the cells, identifying even slight damage in the follicle ultrastructure caused by *in vitro* culture.

Acknowledgements

This work was supported by the National Council for Scientific and Technological Development (CNPq; Process 562548/2010–6). R. Sousa Barberino receives a scholarship from the CNPq/Univasf. M.H.T. Matos is supported by a grant from CNPq.

Declaration of interest

None of the authors have any conflict of interest to declare.

References

Abedelahi, A., Salehnia, M., Allameh, A.A. & Davoodi, D. (2010). Sodium selenite improves the *in vitro* follicular development by reducing the reactive oxygen species level and increasing the total antioxidant capacity and glutathione peroxide activity. *Hum. Reprod.* **25**, 977–85.

Albuquerque, U. P., Medeiros, P.M., Almeida, A.L.S., Monteiro, J.M., Neto, E.M.F.L., Melo, J.G. & Santos, J.P. (2007). Medicinal plants of the Caatinga (semi-arid) vegetation of NE Brazil: A quantitative approach. *J. Ethnopharmacol.* **114**, 325–54.

Andrade, E.R., Melo-Sterza, F.A., Seneda, M.M. & Alfieri, A.A. (2010). Consequências da produção das espécies reativas de oxigênio na reprodução e principais mecanismos antioxidantes. *Rev. Bras. Reprod. Anim.* **34**, 79–85.

Andrade, E.R., Van Den Hurk, R., Lisboa, L.A., Hertel, M.F., Melo-Sterza, F.A., Moreno, K., Bracarense, A.P.F.R.L., Landim-Alvarenga, F.C., Seneda, M.M. & Alfieri, A.A. (2012). Effects of ascorbic acid on *in vitro* culture of bovine preantral follicles. *Zygote* **20**, 379–88.

Barboni, B., Russo, V., Cecconi, S., Curini, V., Colosimo, A., Garofalo, M.L., Capacchietti, G., Di Giacinto, O. & Mattioli, M. (2011). *In vitro* grown sheep preantral follicles yield oocytes with normal nuclear-epigenetic maturation. *PLoS One* **6**, 27550.

https://doi.org/10.1017/S0967199415000179 Published online by Cambridge University Press

Cartaxo, S.L., Souza, M.M.A. & Albuquerque, U.P. (2010). Medicinal plants with bioprospecting potential used in semi-arid northeastern Brazil. *J. Ethnopharmacol* **131**, 326–42.

Chaves, R.N., Martins, F.S., Saraiva, M.V.A., Celestino, J.J.H., Lopes, C.A.P., Correia, J.C., I.B. Verde, Matos, M.H.T., Báo, S.N., Name, K.P.O., Campello, C.C., Silva, J.R.V. & Figueiredo, J.R. (2008). Chilling ovarian fragments during transportation improves viability and growth of goat preantral follicles cultured *in vitro*. *Reprod. Fertil. Dev.* **20**, 640–7.

Chaves, R.N., Duarte, A.B.G., Rodrigues, G.Q., Celestino, J.J.H., Silva, G.M., Lopes, C.A.P., Almeida, A.P., Donato, M.A.M., Peixoto, C.A., Moura, A.A.A., Lobo, C.H., Locatelli, Y., Mermillod, P., Campelo, C.C. & Figueiredo, J.R. (2012) The effects of insulin and follicle-simulating hormone (FSH) during *in vitro* development of ovarian goat preantral follicles and the relative mRNA expression for insulin and FSH receptors and cytochrome P450 aromatase in cultured follicles. *Bio. Reprod.* **87**, 69.

Crocomo, L.F., Marques Filho, F.C. & Landim-Alvarenga, F.C. (2012). Bicudo, S.D. Produção de embriões *in vitro*: estresse oxidativo e antioxidantes. *Vet. e Zootec.* **19**, 470–9.

Erickson, G.F. & Shimasaki, S. (2001). The physiology of folliculogenesis: the role of novel growth factors. *Fertil. Steril.* **76**, 943–9.

Esmaielzadeh, F., Hosseini, S.M., Hajian, M., Nasiri, Z., Chamani, M., Amin Afshar, M. & Nasr-Esfahani, M.H. (2013). Role of follicle stimulating hormone in the survival, activation and further growth of *in vitro* cultured sheep primordial follicles. *Iran. J. Appl. Anim. Sci.* **3**, 785–90.

Gao, Z., Huang, K. & Xu, H. (2001). Protective effects os flavonoids in the roots of *Scutellaria baicalensis* Georgii against hydrogen peroxide-induced oxidative stress in HS-SYSY cells. *Pharmacol. Res.* **2**, 173–8.

Gouveia, B.B., Barros, V.R.P., Gonçalves, R.J.S., Barberino, R.S., Menezes, V.G., Lins, T.L.B, Macedo, T.J.S., Santos, J.M.S., Rolim, L.A., Rolim Neto, P.J., Almeida, J.R.G.S. & Matos, M.H.T. (2015). Effect of ovarian tissue transportation in Amburana cearensis extract on the morphology and apoptosis of goat preantral follicles. *Anim. Reprod.* **12**, 316–23.

Guan, S., Ge, D., Liu, T. Q., Ma, X. & Cui, Z.F. (2009). Protocatechuic acid promotes cell proliferation and reduces basal apoptosis in cultured neural stem cells. *Toxicol. In Vitro* **23**, 201–8.

Guérin, P., El Mouatassim, S. & Ménézo, Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum. Reprod. Update* **7**, 175–89.

Gupta, P.S.P., Rameshb, H.S., Nandi, S. & Ravindra, J.P. (2007). Recovery of large preantral follicles from buffalo ovary: effect of season and corpus luteum. *Anim Reprod Sci* **101**, 145–52.

Kim, Y.S., Seo, H.W., Lee, M.H., Kim, D.K., Jeon, H. & Cha, D.S. (2014). Protocatechuic acid extends lifespan and increases stress resistance in *Caenorhabditis elegans*. *Arch. Pharm. Res.* **37**, 245–52.

Leal, L.K.A.M., Nobre-Júnior, H.V., Cunha, G.M.A., Moraes, M.O., Pessoa, C., Oliveira, R.A., Silveira, E.R., Canuto, K.M. & Viana, G.S.B. (2005). Amburoside A, a glucoside from *Amburana cearensis* protects mesencephalic cells against 6-hydroxydopamine-induced neurotoxicity. *Neurosci. Lett.* **388**, 86–90.

Leal, L.K.A.M., Nobre, H.V., Cunha, G.M.A., Moraes, M.O., Pessoa, C., Oliveira, R.A., Silveira, E.R., Canuto, K.M. & Viana, G.S.B. (2010). A comparative chemical and pharmacological study of standardized extracts and vanillic acid from wild and cultivated *Amburana cearensis* A.C. Smith. *Phytomedicine* **18**, 230–3.

Li, Y. & Schellhorn, H.E. (2007). New developments and novel therapeutic perspectives for vitamin. *C. J. Nutr.* **137**, 2171–84.

Lopez-Revuelta, A., Sanches-Gallego, J.I., Hernandez-Hernandez, A., Sanchez-Yague, J. & Llanillo, M. (2006). Membrane cholesterol contents influence the protective effects of quercetin and rutin in erythrocytes damaged by oxidative stress. *Chemo-Biol. Interact.* **61**, 79–91.

Luz, V.B, Araújo, V.R., Duarte, A.B.G., Silva, G.M., Chaves, R.N., Brito, I.R., Serafim, M.K.B., Campello, C.C., Feltrin, C., Bertolini, M., Almeida, A.P., Santos, R.R. & Figueiredo, J.R. (2013) Kit ligand and insulin-like growth factor I affect the *in vitro* development of ovine preantral follicles. *Small Rumin. Res.* **115**, 99–102.

Magalhães, D.M., Fernandes, D.D., Mororó, M.B., Silva, C.M., Rodrigues, G.Q., Bruno, J.B., Matos, M.H., Campello, C.C. & Figueiredo, J.R. (2011). Effect of the medium replacement interval on the viability, growth and *in vitro* maturation of isolated caprine and ovine pre-antral follicles. *Reprod. Domest. Anim.* **46**, 134–40.

Martín-Aragón, S., Bened, J.M. & Villar, A.M. (1998). Effects of the antioxidant (6,7-dihydroxycoumarin) esculetin on the glutathione system and lipid peroxidation in mice. *Gerontology* **44**, 21–5.

Murray, A.A., Molinek, M.D., Baker, S.J., Kojima, F.N., Smith, M.F., Hillier, S.G. & Spears, N. (2001). Role of ascorbic acid in promoting follicle integrity and survival in intact mouse ovarian follicles *in vitro*. *Reproduction* **121**, 89–96.

Neichi, T., Koshihara, Y. & Murota, S. (1983). Inhibitory effect of esculetin on 5-lipoxygenase and leukotriene biosynthesis. *Biochim. Biophys. Acta* **753**, 130–2.

Peng, X., Yang, M., Wang, L., Tong, C. & Guo, Z. (2010). *In vitro* culture of sheep lamb ovarian cortical tissue in a sequential culture medium. *J. Assist. Reprod. Genet.* **27**, 247–57.

Punithavathi, V.R., Prince, P.S.M., Kumar, R. & Selvakumari, S. (2011). Antihyperglycaemic, antilipid peroxidative and antioxidant effects of gallic acid on streptozotocin induced diabetic Wistar rats. *Eur. J. Pharmacol.* **650**, 465–71.

Rajabi-Toustani, R., Motamedi-Mojdehi, R., Mehr, M. & Motamedi-Mojdehi, R. (2013). Effect of *Papaver rhoeas* L. extract on *in vitro* maturation of sheep oocytes. *Small Rumin. Res.* **114**, 146–51.

Rodgers, R.J. & Irving-Rodgers, H.F. (2010). Formation of the ovarian follicular antrum and follicular fluid. *Biol. Reprod.* **82**, 1021–9.

Rodrigues, G.Q., Silva, C.M.G., Faustino, L.R., Bruno, J.B., Magalhães, D.M., Campello, C.C. & Figueiredo, J.R. (2010). Bovine serum albumin improves *in vitro* development of caprine preantral follicles. *Anim. Reprod.* **7**, 382–8.

Rossetto, R., Saraiva, M.V.A., Santos, R.R., Silva, C.M., Faustino, L.R., Chaves, R.N., Brito, I.R., Rodrigues, G.Q.,

Lima, I.M., Donato, M.A., Peixoto, C.A. & Figueiredo, J.R. (2012). Effect of medium composition on the *in vitro* culture of bovine pre-antral follicles: morphology and viability do not guarantee functionality. *Zygote* **21**, 125–8.

Ruijters, E.J.B, Weseler, A.R., Kicken, C., Haenen, G.R.M.M. & Bast, A. (2013). The flavanol (–)-epicatechin and its metabolites protect against oxidative stress in primary endothelial cells via a direct antioxidant effect. *Eur. J. Pharmacol.* **715**, 147–53.

Santos, J., Menezes, V.M., Barberino, R.S., Macedo, T.J.S., Lins, T.L.B., Gouveia, B.B., Barros, V.R.P., Santos, L.P., Gonçalves, R.J.S. & Matos, M.H.T. (2014). Immunohistochemical localization of fibroblast growth factor-2 in the sheep ovary and its effects on pre-antral follicle apoptosis and development *in vitro*. *Reprod. Domest. Anim.* **49**, 222–8.

Saraiva, M.V.A., Rossetto, R., Brito, I.R., Celestino, J.J.H., Silva, C.M.G., Faustino, L.R., Almeida, A.P., Bruno, J.B., Magalhães, D.M., Matos, M.H.T., Campello, C.C. & Figueiredo, J.R. (2010). Dynamic medium produces caprine embryo from preantral follicles grown *in vitro*. *Reprod. Sci.* **17**, 35–43.

Singh, J., Rai, G.K., Upadhyay, A.K., Kumar, R. & Singh, K.P. (2004). Antioxidant phytochemicals in tomato (*Lycopersicon esculentum*). *Ind. J. Agric. Sci.* **74**, 3–5.

Silva, J.R.V., van den Hurk, R., Costa, S.H.F., Andrade, E.R., Nunes, A.P.A., Ferreira, F.V.A., Lôbo, R.N.B. & Figueiredo, J.R. (2004). Survival and growth of goat primordial follicles after *in vitro* culture of ovarian cortical slices in media containing coconut water. *Anim. Reprod. Sci.* **81**, 273–86.

Songsasen, N., Spindler, R. E. & Wildt, D. E. (2007). Requirement for, and patterns of, pyruvate and glutamine metabolism in the domestic dog oocyte *in vitro*. *Mol. Reprod. Dev.* **74**, 870–7.

Tatsimo, S.J.N., Tamokou, J.D., Havyarimana, L., Csupor, D., Forgo, P., Hohmann, J., Kuiate, J.R. & Tane, P. (2012). Antimicrobial and antioxidant activity of kaempferol rhamnoside derivatives from *Bryophyllum pinnatum*. *BMC Res. Notes* **5**, 158–63.

Tilly, J.L. & Tilly, KI (1995). Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. *Endocrinology* **136**, 242–52.

Wang, J. & Shahidi, F. (2013). Acidolysis of *p*-coumaric acid with omega-3 oils and antioxidant activity of phenolipid products in *in vitro* and biological model systems. *J. Agric. Food Chem.* **62**, 454–61.

Zang, L.Y., Cosma, G., Gardner, H., Shi, X., Castranova, V. & Vallyathan, V. (2000) Effect of antioxidant protection by *p*-coumaric acid on low-density lipoprotein cholesterol oxidation. *Am. J. Physiol. Cell Physiol.* **279**, 954–60.

https://doi.org/10.1017/S0967199415000179 Published online by Cambridge University Press