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Inhibition of Hsp90 during *in vitro* maturation under thermoneutral or heat shock conditions compromises the developmental competence of bovine oocytes

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Summary

Heat shock protein 90 (Hsp90) is critical for cell homeostasis but its role on bovine oocyte maturation is not well known. We investigated the importance of Hsp90 for competence of bovine oocyte using 17-(allylamino)-17-demethoxygeldanamycin (17AAG), an inhibitor of Hsp90, during *in vitro* maturation (IVM). Three experiments evaluated the effect of 17AAG on developmental competence of oocytes matured *in vitro* under thermoneutral (38.5ºC) or heat shock (HS; 41.5ºC) temperatures. The first experiment found that the blastocyst rates were lower ($P < 0.05$) with 2 μM 17AAG compared with the untreated control (0 μM). The abundance of *HSF1* transcripts was higher in oocytes matured with 2 μM than with 0 and 1 μM 17AAG, whereas the abundance of *HSP90AA1* and *HSPA1A* transcripts was lower ($P < 0.05$) with 1 and 2 μM than with 0 μM. The second experiment found that 2 μM 17AAG for 12 or 24 h during IVM decreased ($P < 0.05$) the blastocysts rates. In the third experiment, the association of 2 μM 17AAG with HS for 12 h during IVM resulted in lower ($P < 0.05$) blastocysts rates than 17AAG, HS or untreated control. In conclusion, inhibition of Hsp90 during *in vitro* maturation compromises further embryo development; the association of Hsp90 inhibition with HS aggravates the deleterious effect of both on oocyte developmental competence.

Introduction

Heat stress has been a major concern in the cattle industry because of its deleterious effect on production and reproduction performance, resulting in economical losses (Key *et al.*, 2014). The effects of heat stress on reproduction include changes in hormone secretion (Wolfenson *et al.*, 2000; Torres-Júnior *et al.*, 2008), follicular microenvironment (Rispoli *et al.*, 2019) and developmental competence of oocytes and embryos (Silva *et al.*, 2013; Roth, 2015; Wolfenson and Roth, 2019), which result in lower conception rates during the summer (Wolfenson and Roth, 2019). *In vitro* studies have reported that heat shock (HS) reduces protein synthesis in oocytes (Edwards and Hansen, 1997), disturbs microfilaments, microtubules and architecture of the meiotic spindle (Roth and Hansen, 2005), and induces cell death by apoptosis (Roth and Hansen, 2004).

The cell response to HS involves multiple molecular and cellular mechanisms, in which HS proteins (Hsp) play a significant role (Morimoto, 1993). Heat shock proteins are chaperones highly conserved among species, being involved in cytoprotective functions with important roles on protein folding (Kregel, 2002). Hsp70 and Hsp90 are the two major Hsps and both cooperate in protein folding (Morán Luengo *et al.*, 2019). Two Hsp90 proteins can be found: Hsp90α and Hsp90β. Whereas the expression of *HSP90α* and *HSP90β* genes are constitutive, the expression of *HSP90α* can be also induced by heat shock (Barnier *et al.*, 1987; Schopf *et al.*, 2017). Similarly to *HSP70* gene, the expression of *HSP90* in response to acute heat shock is mediated by heat shock factor 1 (HSF1) transcription factor (Lang *et al.*, 2021), although expression of *HSP90* gene can also be activated by other transcription factors (Prodromou 2016). HSF1 activity can be repressed by an increase in Hsp90 and stimulated by Hsp90 inhibition (Zou *et al.*, 1998). Hsp90 plays several other roles on cell function in addition to protein folding,

for example in cell signalling (Lang *et al.*, 2022), chromatin remodelling and transcriptional regulation (Zhao and Houry, 2005; Yahara, 2019), apoptosis, cell cycle control and hormone signalling (Hoter *et al.*, 2018).

The role of Hsp90 during maturation of oocyte is not well known. In mice, Hsp90 inhibition interferes in oocyte meiosis progression due to an inadequate function of kinases involved in the control of germinal vesicle breakdown (GVBD) such as mitogen activated protein kinase (MAPK) (Metchat *et al.*, 2009). The MAPK cascade mediates the activation of maturation promoting factor (MPF), a complex formed by kinase p34cdc2/cyclin B, triggering GVBD and chromatin condensation by phosphorylation of proteins involved in nuclear membrane formation and microtubular reorganization (Gautier *et al.*, 1990; Trounson *et al.*, 2001), which enables the progression of the cell cycle. In *Xenopus* oocytes, the pro-oncogene *c*-MOS, responsible for triggering the MAPK cascade, also requires Hsp90 during maturation (Fisher *et al.*, 1999, 2000). Although changes in expression of Hsp90 protein can be found in bovine oocytes exposed to low temperature in contrast with high temperature (Pöhland *et al.*, 2020), there are no data showing that Hsp90 is required during oocyte maturation and whether it can influence the competence of bovine oocyte.

The 17-(allylamino)-17-demethoxygeldanamycin (17AAG) is a Hsp90 inhibitor with activities similar to geldanamycin, but with lower toxicity (Schulte and Neckers, 1998). It binds to the N-terminal ATP binding site of Hsp90 protein to prevent the binding to client proteins, blocking Hsp90 function. The result is the degradation of the Hsp90 client proteins through a ubiquitin-proteasome pathway (Johnson *et al.*, 2010). In mice oocytes, 1.78 µM 17AAG during the pre-maturation caused changes in meiotic maturation as a delay to GVBD (Metchat *et al.*, 2009). In pig, treatment of oocytes during 44 h of maturation with 2 µM 17AAG reduced meiotic progression and affected the expression of genes in preimplantation stage embryos (Son *et al.*, 2010, 2011). The lack or the reduction of Hsp90 function induced by 17AAG can be useful for demonstrating the importance of this chaperone for the acquisition of competence in bovine oocyte during maturation under thermoneutral or heat stress conditions.

In this study we aimed to evaluate the effect of Hsp90 inhibition on bovine oocyte developmental competence by testing 1 and 2 µM 17AAG concentrations for 12 or 24 h of *in vitro* maturation (IVM). We also evaluated the associative effects between Hsp90 inhibition and HS on oocyte competence by exposing oocytes simultaneously to HS and 17AAG during IVM.

Materials and methods

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Experimental design

Three experiments were carried out to evaluate the effect of Hsp90 inhibition by 17AAG on the developmental competence of bovine oocytes matured *in vitro* under thermoneutral (38.5ºC) or HS (41.5ºC) temperatures. The first experiment evaluated the effect of 17AAG concentration and time of exposure on oocytes matured *in vitro* at 38.5ºC in two trials. Immature oocytes were randomly allocated in a 3 × 2 factorial design (concentration vs. exposure time): 0 (untreated), 1 or 2 µM of 17AAG for 12 h or 24 h IVM in tissue culture medium at 38.5ºC under 5% CO_2 and saturated humidity. Oocytes exposed to 17AAG for 12 h were washed and allocated in maturation medium without the inhibitor for an additional 12 h to complete 24 h of IVM. Trial 1 assessed oocyte nuclear maturation (three replicates), cleavage and blastocyst rates (nine replicates). Trial 2 assessed the transcript abundance of target genes in oocytes exposed to 0, 1 or 2 µM of 17AAG for 12 or 24 h during IVM (three replicates). The second experiment was carried out to confirm the main results achieved in Experiment 1. It evaluated the effects of 2 µM 17AAG for 12 or 24 h during IVM on embryo development, blastocysts cell number and apoptosis. For that, immature oocytes were randomly allocated in three groups: no 17AAG (untreated; control), 2 µM 17AAG for 12 h (17AAG-12h) or 2 µM 17AAG for 24 h (17AAG-24h) of IVM at 38.5ºC. Cleavage and blastocysts rates, blastocyst total cell number and apoptotic index were assessed (six replicates). The third experiment evaluated the associative effects between 17AAG and HS during IVM on oocyte competence. For that, immature oocytes were randomly allocated into four groups: no heat shock or 17AAG (untreated; control); 2 µM 17AAG for the first 12 h of IVM (17AAG); heat shock (41.5ºC) for the first 12 h of IVM (HS); and 2 µM 17AAG plus HS for the first 12 h of IVM (17AAG+HS). Cleavage and blastocysts rates were assessed (seven replicates).

Oocytes recovery and in vitro maturation (IVM)

Bovine ovaries from crossbred cows were obtained from a local slaughterhouse and transported to the laboratory in physiological saline solution, 0.9% NaCl (w/v) at 35–37°C with 0.1 g L^{-1} streptomycin. Cumulus–oocyte complexes (COCs) were aspirated from 3–8 mm follicles and those with more than three compact cumulus cell layers and homogeneous cytoplasm were selected for IVM. *In vitro* maturation was performed in tissue culture medium (TCM-199; Gibco Life Technologies, Inc., Grand Island, NY, USA) supplemented with 20 µg/ml follicle-stimulating hormone (FSH; Pluset, Calier, Barcelona, Spain) and 10% fetal bovine serum (FBS) for 24 h at 38.5°C under 5% CO_2 in the air and saturated moisture or according to the experimental groups.

Heat shock during IVM

Heat shock was performed for 12 h at 41.5°C under 7% CO_2 followed by 12 h at 38.8°C under 5% CO_2 in the air and saturated moisture in TCM-199 supplemented with 20 µg/ml FSH and 10% FBS, completing 24 h of IVM. The percentage of CO_2 was adjusted to 7% to ensure that the concentrations of dissolved CO_2 and pH in the medium were similar between treatments (Roth and Hansen 2005).

Assessment of nuclear maturation

After 24 h of IVM, oocytes were denuded from cumulus cells by vortexing the COCs for 5 min in 1 ml of HEPES-TALP. Denuded oocytes were fixed for 30 min in 4% paraformaldehyde and incubated for 10 min in Hoechst 33342 (10 µg/ml) at room temperature in the dark. Afterwards, oocytes were washed three times in phosphate-buffered saline (PBS) with 0.1% polyvinyl alcohol (PVA) solution and transferred to a glass slide covered with a coverslip. The extrusion of the first polar body was checked and oocytes with an extruded first polar body and a metaphase II plate were defined as matured. Maturation rate was assessed under a ×400 magnification epifluorescence microscopy (excitation of 330 ± 50 nm and emission of 460 ± 50 nm). Three replicates per group were performed.

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In vitro *fertilization (IVF)*

In vitro fertilization was performed with frozen–thawed semen after sperm separation carried out on a discontinuous density gradient with 400 µl 90% and 45% Percoll. Semen in the Percoll gradient was centrifuged at 5000 *g* for 7 min, supernatant was discarded and the pellet was resuspended with IVF medium (Fert-TALP) and then centrifuged at 915 *g* for 5 min. The final pellet was resuspended with 100 µl of IVF medium. *In vitro* fertilization was performed using 2×10^6 spermatozoa/ml for 20 h in 100-µl drops of IVF medium supplemented with 20 µg/ml of heparin and 6 mg/ml of fatty acid free bovine serum albumin fraction V, covered with mineral oil, in a humidified atmosphere of 5% CO_2 and 38.5ºC in air.

In vitro *culture of embryos*

Presumptive zygotes were completely denuded in a PBS solution with 0.1% hyaluronidase and then cultured in wells with 500 µl of modified CR2aa medium supplemented with 2.5% fetal calf serum overlaid with 300 µl mineral oil and cultured for 8 days in incubator at 38.5 ºC under 5% CO_2, 5% O_2, 90% N_2 and saturated humidity. Cleavage rate was assessed 72 h post-fertilization and blastocyst rate was assessed on day 7 (D7) and day 8 (D8).

Assessment of total cell number and apoptosis

Total cell number and apoptosis in blastocysts on day 8 were analyzed using terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end-labelling (TUNEL) assay using a commercial kit (Dead End Fluorimetric TUNEL System, Promega, *Madison*, *WI*, USA), according to the manufacturer's instructions. Briefly, embryos were washed three times in PBS-PVA solution, fixed in 4% paraformaldehyde for 25 min and then permeabilized with 0.2% Triton X-100 (Promega). Positive control embryos had been treated previously with DNase (Promega). Samples were then incubated in 50-µl drops with a reagent mixture containing the enzyme solution (terminal deoxynucleotide transferase enzyme) and 90% staining solution (fluorescein dUTP conjugate) for 1 h at 37°C in a dark humid chamber. Negative control embryos were incubated only in the staining solution without enzyme. Embryos were stained with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) plus 4′6-diamidino-2-phenylindole (DAPI) and mounted on slides for evaluation by fluorescence microscopy. The total number of cells and number of apoptotic cells per embryo were counted, and the apoptotic cell index was calculated as the proportion of apoptotic cells per total number of cells.

Relative quantification of mRNA in oocytes

Denuded *in vitro* matured oocytes were washed three times in PBS plus 0.1% polyvinyl alcohol and then frozen rapidly in liquid nitrogen, and stored at −80°C. Three pools of 10 oocytes per group were used for total RNA extraction with an RNeasy Micro Kit (Quiagen, Valencia, CA, USA) according to the manufacturer's instructions. Samples were treated with DNase I (27 units for 15 min at room temperature) to prevent DNA contamination. RNA was eluted in 12 µl RNase-free water. RNA samples were reverse transcribed using the SuperScript III First-Strand Synthesis Supermix (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions using 6 µl RNA (equivalent to five oocytes per reverse transcription reaction), oligo$(dT)_{20}$ primers, dNTP mix, Superscript III RT, RNaseOUT™, $MgCl_2$, RT buffer in a final volume of 20 µl. Samples were first incubated at 65ºC for 5 min and then for 50ºC for 50 min. The reaction was terminated at 85ºC for 5 min and then chilled on ice. After that, RNase H was added to the samples, which were incubated at 37ºC for 20 min.

Relative quantification was performed in triplicate using real-time polymerase chain reaction (ABI Prism 7300 Sequence Detection Systems, Foster City, CA, USA). Reactions were prepared using a mixture of SYBR Green PCR Master Mix (Applied Biosystems), 0.1 µM primers, nuclease-free water and reverse transcribed cDNA. The cDNA template was denatured at 95°C for 15 min, followed by 45 cycles of 94°C for 15 s, annealing at a specific temperature for each primer for 15 s (Table 1) and extension at 60°C for 30 s. Negative template controls, comprising the PCR mixture without nucleic acids, were also run with each group of samples. Primer efficiency was calculated using LinRegPCR software (Ramakers *et al.*, 2003) for each reaction. All technical replicates were analyzed. After each PCR run, a melting curve analysis was performed to confirm that a single specific product was generated.

The relative quantification was performed for the following genes: murine sarcoma viral proto-oncogene homologue (*c*-MOS), mitogen activated protein kinases 1 (*MAPK1*), heat shock factor 1 (*HSF1*), heat shock protein family A, member 1A (*HSPA1A*, also known as *HSP70*) and heat shock protein 90 alpha class A member 1 (*HSP90AA1*, also known as *HSP90*). The genes tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), actin beta (*ACTB*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used as endogenous references. The volume of cDNA-converted RNA added to each PCR was 0.3 µl (equivalent to 0.075 oocyte) for *YWHAZ*, *ACTB* and *GAPDH* genes, 0.6 µl (equivalent to 0.15 oocyte) for *c*-MOS, 0.8 µl (equivalent to 0.2 oocyte) for *HSPA1A* and *HSP90AA1*, 1.2 µl (equivalent to 0.3 oocyte) for *MAPK1*, and 1.8 µl (equivalent to 0.45 oocyte) for the *HSF1* gene. The primer efficiency was 1.93, 1.8, 1.83, 1.86, 1.84, 1.82, 1.82 and 1.70 for *YWHAZ*, *ACTB*, *GAPDH*, *c*-MOS, *MAPK1*, *HSF1*, *HSP90AA1* and *HSPA1A*, respectively.

Relative quantification was performed using the comparative Ct quantification method, adapted from Schmittgen and Livak (2008), relative to the sample with the highest delta C_T value (calibrator sample) and was based on primer efficiency. The calibrator was the sample with the highest delta C_t value in the 0 µM 17AAG group.

Statistical analysis

Cleavage and blastocysts rates were analyzed using the general linear model (Proc GLM) and means compared by Student–Newman–Keuls (SNK) test. Apoptosis index and nuclear maturation proportion were analyzed by logistic regression (Proc Logistic), and total cell number, apoptotic cell number and relative abundance of transcripts by the linear mixed model (Proc Mixed). The statistical package used was SAS statistical software (version 9.1). Values are shown as mean ± standard error of the mean (SEM). Differences were considered significant at the 95% confidence level ($P < 0.05$).

Results

Experiment 1, trial 1: Effect of 17AAG on oocyte nuclear maturation and further embryo development

There was no effect ($P > 0.05$) of concentration (76.4 ± 2.2%, 77.3 ± 2.8% and 76.4 ± 4.4%, for 0, 1 and 2 µM 17AAG, respectively)

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Table 1. Primers for real-time PCR and mean efficiency from real-time PCR to each gene used in relative expression

Gene	Primer sequence 5→3´	Annealing temperature	Product size (bp)	GenBank access number
YWHAZ	F GCAAAAGACGGAAGGTGCTG R CCTCAGCCAAGTAGCGGTAG	59°C	197 bp	NM_174814.2
ACTB	F GAGGCTCTCTTCCAGCCTTC R GGGCAGTGATCTCTTTCTGC	53°C	184 bp	NM_173979.3
GAPDH	F GGCGTGAACCACGAGAAGTATAA R CCCTCCACGATGCCAAAGT	53°C	119 bp	NM_001034034.2
c-MOS	F CACCTTGCACCAGGTCATCT R GATGTCGGCCTTGGGTGTAA	59°C	325 bp	XM_002692654.4
MAPK1	F CCTGCTCTCTCTTCCACACA R TCATCGCTCGGGTCGTAGTA	59°C	177 bp	NM_175793.2
HSF1	F ACTCCAACCTGGACAACCTG R TGAGTCTGGGCTGCTTTTCT	59°C	209 bp	XM_010811828.3
HSP90AA1	F GAGGATCCCCAGACACATGC R GACGTGTCGTCGTCTCCTTC	62°C	143 bp	NM_001012670.2
HSPA1A	F AACAAGATCACCATCACCAACG R TCCTTCTCCGCCAAGGTGTTG	59°C	275 bp	NM_203322.3

Table 2. Effect of 17-(allylamino)-17-demethoxygeldanamycin (17AAG) concentration and time during *in vitro* maturation of bovine oocyte on cleavage rate on day 3 and blastocyst rates on day 7 (D7) and day 8 (D8) post-fertilization

17AAG concentration	*n*	Cleavage (%)	Blastocysts D7 (%)	Blastocysts D8 (%)
0 μM	591	70.8 ± 2.1	29.2 ± 2.5[a]	34.0 ± 3.3[a]
1 μM	627	66.5 ± 2.7	25.6 ± 2,7[a,b]	27.9 ± 3.1[a,b]
2 μM	618	63.2 ± 2.7	18.6 ± 2.2[b]	20.4 ± 2.2[b]
Time of exposure				
12 h	842	66.0 ± 1.9	26.6 ± 2.2	29.4 ± 2.9
24 h	994	67.7 ± 2.3	22.3 ± 2.0	25.5 ± 2.2

[a,b]Superscript letters indicates significant differences ($P < 0.05$) among concentrations. There was no significant difference ($P > 0.05$) between time of exposure. Values are shown as mean ± standard error of the mean (SEM); *n* = number of oocytes.

and time of exposure (78.8 ± 2.6% and 74.6 ± 2.4%, for 12 h and 24 h, respectively) on the proportion of oocytes in metaphase II. Similarly, there was no effect of concentration and time on the cleavage rate; however, the blastocyst rates on D7 and D8 were lower ($P < 0.05$) with 2 μM 17AAG than with 0 μM (Table 2). There was no difference ($P > 0.05$) between 0 μM and 1 μM and between 1 μM and 2 μM (Table 2). There was no interaction between 17AAG concentration and time of exposure ($P > 0.05$) for any developmental parameters assessed.

Experiment 1, trial 2: Effect of 17AAG during IVM on relative transcripts abundance of in vitro matured oocytes

The relative abundance of *HSF1* transcripts was higher ($P < 0.05$) in oocytes exposed to 2 μM 17AAG than in oocytes exposed to 0 or 1 μM. In contrast, the abundances of *HSP90AA1* and *HSPA1A* transcripts were lower ($P < 0.05$) with 1 and 2 μM than with 0 μM. There was no difference ($P > 0.05$) among 17AAG concentrations for the amount of *c-MOS* and *MAPK1* transcripts (Fig. 1). The longest time of exposure to 17AAG (24 h) increased ($P < 0.05$) the abundance of *c-MOS* transcripts. There was no effect of time ($P > 0.05$) on the amount of *MAPK1*, *HSF1*, *HSP90AA1* and *HSPA1A* transcripts (Fig. 2). There was no interaction between 17AAG concentration and time of exposure ($P > 0.05$).

Experiment 2: Effect of 2 μM 17AAG for 12 or 24 h during IVM on further embryo development and apoptosis index

This experiment was carried out to confirm the findings of Experiment 1 concerning the effect of 2 μM 17AAG during IVM on preimplantation embryo development. The cleavage rate was lower ($P < 0.05$) in the 17AAG-24h group than in the untreated control group but it was similar ($P > 0.05$) to 17AAG-12h group. The blastocysts rates on D7 and D8 were lower ($P < 0.01$) in the 17AAG-12h and 17AAG-24h groups than in the control group (Fig. 3). There was no ($P > 0.05$) difference in blastocyst total cell number. The highest ($P < 0.05$) number of apoptotic cells and apoptotic index was found in blastocysts for the 17AAG-12h group (Table 3).

Experiment 3: Effect of the association between 17AAG and heat shock during IVM on further embryo development

This experiment evaluated the combination of 2 μM 17AAG with HS for 12 h during IVM to detect an associative effect between the inhibition of Hsp90 activity and HS on further embryo development. No effect ($P > 0.05$) of 17AAG, HS and 17AAG+HS on cleavage rate was detected; however, the blastocyst rates on D7 and D8 were lower ($P < 0.05$) in the 17AAG and HS groups than in the control group (Fig. 4). The lowest ($P < 0.05$) blastocyst rate was found in the 17AAG+HS group (Fig. 4).

Discussion

Heat shock proteins have critical roles for the regulation of cellular homeostasis, being required to overcome stressful cellular condition, as that imposed by heat stress. Hsp90 is one of the chaperones necessary to keep cellular homeostasis under physiological and stress conditions in different species of eukaryotes (Schopf *et al.*, 2017). In this study we used an inhibitor of Hsp90 (17AAG) to demonstrate that Hsp90 activity is required for the competence

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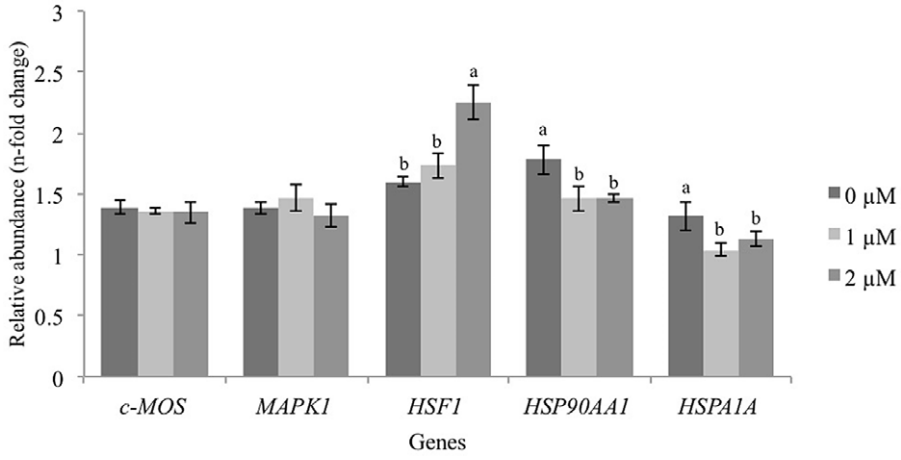

Figure 1. Effect of 17-(allylamino)-17-demethoxygeldanamycin (17AAG) concentration during *in vitro* maturation of bovine oocytes on the relative abundance of target transcripts. [a,b]Letters above the bars within the same gene indicate differences ($P < 0.05$) among concentrations. Means are shown as mean ± standard error of the mean (SEM). Data are from three replicates.

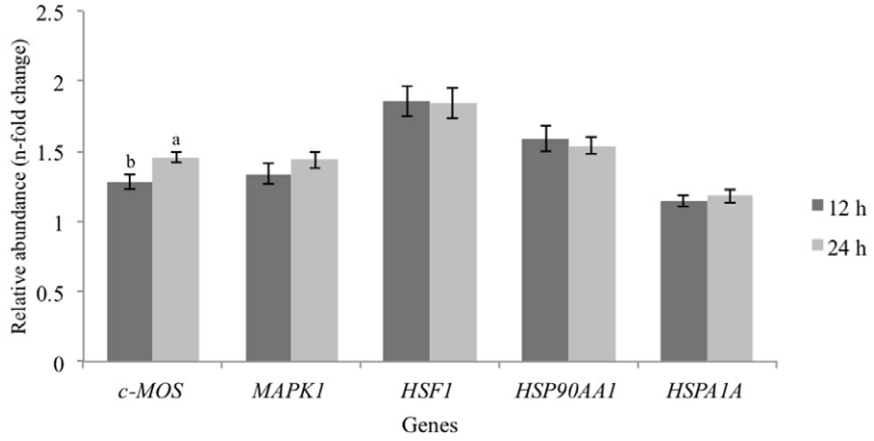

Figure 2. Effect of time of exposure of bovine oocytes to 17-(allylamino)-17-demethoxygeldanamycin (17AAG) during *in vitro* maturation on the relative abundance of target transcripts. [a,b]Letters above the bars within the same gene indicate differences ($P < 0.05$) between time of exposure. Means are shown as mean ± standard error of the mean (SEM). Data are from three replicates.

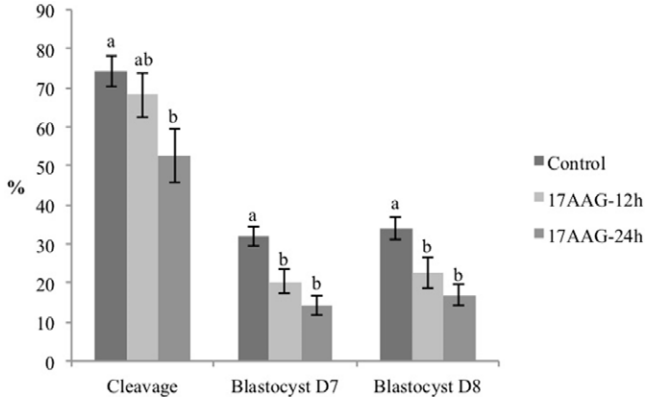

Figure 3. Effect of 2 µM 17-(allylamino)-17-demethoxygeldanamycin (17AAG) for 12 h or 24 h during *in vitro* maturation of bovine oocyte on cleavage rate on day 3 and blastocyst rates on day 7 (D7) and day 8 (D8) post-fertilization. [a,b]Letters above bars within the same developmental stage indicate differences ($P < 0.05$) among groups. Means are shown as mean ± standard error of the mean (SEM). Data are from six replicates (control $n = 240$ oocytes; 17AAG-12h $n = 240$ oocytes; 17AAG-24h $n = 243$ oocytes).

of bovine oocyte matured under physiological and stressful conditions.

First, we investigated the effect of two concentrations of 17AAG for 12 and 24h during *in vitro* maturation on nuclear maturation and on the ability of *in vitro*-fertilized oocytes to progress towards the blastocyst stage. We did not find the effect of 17AAG on the proportion of oocytes at metaphase II, in contrast with a previous study with mice oocytes that reported a delayed germinal vesicle breakdown when 1.78 µM 17AAG was used in the pre-maturation period (Metchat *et al.*, 2009). In pigs, also, 2 µM 17AAG for 24 h delayed germinal vesicle breakdown and for 44 h delayed the extrusion of the first polar body (Liu *et al.*, 2018) and decreased the rate of metaphase II oocytes (Son *et al.*, 2011). The reason we did not find any effect of 17AAG on meiosis progression may be because the inhibitory action on Hsp90 activity by geldanamycin may take hours to occur, as seen during cell mitosis (de Cárcer, 2004). Although no effect on nuclear maturation was found, the inhibition of Hsp90 by 2 µM 17AAG compromised the ability of oocyte in generating blastocysts after IVF.

Consistent with the findings for nuclear maturation, we did not find any effect of 17AAG on the relative amounts of *c*-MOS and *MAPK1* transcripts in matured oocytes. The MAPK pathway plays a critical role on meiosis progression, as c-Mos protein is an important component of cell cycle regulation (Verlhac *et al.*, 1996). A study with cancer cells reported that Hsp90 inhibitors can down-regulate components of the MAPK pathway (Haupt *et al.*, 2012). The lack of effect of 17AAG concentration on the amount of *MAPK1* transcripts in our study with oocytes may be because of the short time required for IVM. Indeed, an altered amount of *c*-MOS transcripts was found only when oocytes were exposed to the Hsp90 inhibitor for 24 h, suggesting that long exposition to the inhibitor may affect the transcription of genes that encode

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Table 3. Effect of 2 μM 17-(allylamino)-17-demethoxygeldanamycin (17AAG) for 12 h or 24 h during *in vitro* maturation of bovine oocyte on the blastocyst's total cell number and apoptosis

Treatment	*n*	Total cell number	Apoptotic cell number	Apoptotic index (%)
Control	20	113.1 ± 6.6	10.1 ± 0.9[b]	8.7 ± 0.6[b]
17AAG-12 h	18	105.2 ± 6.5	13.7 ± 1.4[a]	12.8 ± 1.2[a]
17AAG-24 h	17	102.0 ± 6.1	10.0 ± 0.9[b]	9.7 ± 0.7[b]

[a,b]Superscript letters indicates significant differences ($P < 0.05$) among treatments. Values are shown as mean ± standard error of the mean (SEM); *n* = number of blastocysts.

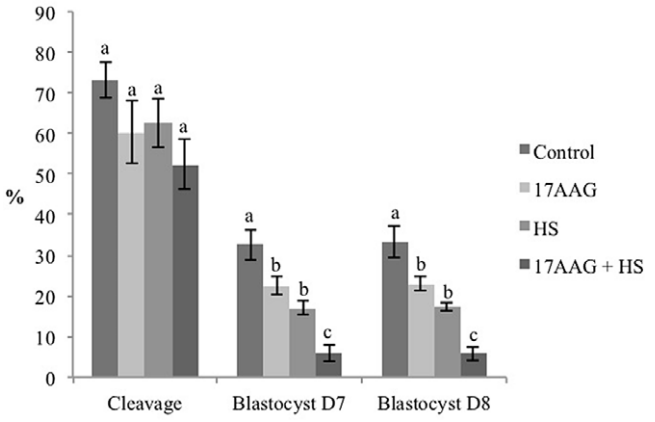

Figure 4. Effect of the association between 17-(allylamino)-17-demethoxygeldanamycin (17AAG) and heat shock (HS) for 12 h during *in vitro* maturation of bovine oocyte on cleavage rate on day 3 and blastocyst rates on day 7 (D7) and day 8 (D8) post-fertilization. [a,b,c]Letters above bars within the same developmental stage indicate differences ($P < 0.05$) among groups. Means are shown as mean ± standard error of the mean (SEM). Data are from seven replicates (control *n* = 315 oocytes, 17AAG *n* = 298 oocytes, HS *n* = 281 oocytes, 17AAG + HS *n* = 312 oocytes).

proteins that play a role in meiosis, but perhaps in a time frame not sufficient to disturb nuclear maturation in bovine oocytes.

The amount of *HSF1* transcripts in bovine oocytes was increased after *in vitro* maturation with 2 μM 17AAG. Although the transcription activity of oocyte genome is supposedly silenced after the breakdown of germinal vesicle (Bettegowda and Smith, 2007; Wu *et al.*, 2021), this finding suggests that an increase in the amount of determined mRNA still can occur. Indeed, a global mRNA expression analysis in bovine oocytes revealed that the abundance of 209 transcripts was increased, whereas 612 transcripts had a decreased abundance after maturation (Fair *et al.*, 2007). Another study performed RNA-seq in the single bovine oocyte and found that the amount of some polyadenylated transcripts increased from germinal vesicle to metaphase II stages (Reyes *et al.*, 2015). In addition, the surrounding corona radiata and cumulus cells can provide mRNA to the oocyte via transzonal projections (Macaulay *et al.*, 2014; Marchais *et al.*, 2022). Therefore, the response of oocytes and the surrounding cells to the changes in the maturation environment could involve an increase in the availability of some necessary transcripts for oocyte viability. We also found that the amounts of *HSP90AA1* and *HSPA1A* transcripts were lower in oocytes exposed to 17AAG. The low amount of chaperones transcripts stored in the cytoplasm may imply a lower ability of the oocyte or the derived embryo to deal with stressors, compromising the oocyte developmental competence. In human cells cultured *in vitro*, the inhibition of Hsp90 activates HSF1 protein, which, in turn, increases the expression of heat shock proteins (Kim *et al.*, 1999). It seems that, in our study, the increase in *HSF1* transcripts caused by the inhibition of Hsp90 activity may not be linked to a greater availability of activated HSF1 protein, which is required for the transcription of *HSP* genes, once the amounts of the *HSP90AA1* and *HSPA1A* transcripts were lower in oocytes exposed to 17AAG. One possible explanation is that the activation of HSF1 protein by 17AAG could not occur efficiently in bovine oocytes, as seen in *Xenopus* oocytes, in which activation of HSF1 by the Hsp90 inhibitor was only observed when the oocytes were under HS (Conde *et al.*, 2009). Conversely, the cytoplasm stores of the *HSP90AA1* and *HSPA1A* transcripts may be reduced due to a combination of a consumption of mRNAs for Hsp90 translation with an inefficient replacement of transcripts.

The second experiment confirmed the effects of 2 μM 17AAG during IVM on embryo development as the oocyte were fertilizable. In this experiment we observed the negative effect of the long exposure of oocytes to 17AAG on cleavage rate, similar to that found with pig oocytes exposed to 17AAG for 44 h during IVM (Son *et al.*, 2011). The exposure of oocytes to 17AAG also decreased their ability to develop towards the blastocyst stage when compared with the untreated control group, but this effect occurred regardless of the duration of exposure (i.e. 12 or 24 h). Although no effect of 17AAG was found on total cell number, the highest apoptotic index was observed in blastocysts derived from oocytes exposed to the Hsp90 inhibitor for 12 h during IVM. The inhibition of Hsp90 activity using Hsp90 inhibitors can induce apoptosis in cancer cells (MORI *et al.*, 2015; Massimini *et al.*, 2017). The same effect on apoptosis was reported in pig embryos exposed to 17AAG (Son *et al.*, 2011). Indeed, as Hsps can regulate cell apoptosis by interfering with caspases activation (Lanneau *et al.*, 2008), an inhibition of Hsp may alter this type of regulation. It is interesting that in our study only oocytes were exposed to 17AAG, not the embryos, which suggests a continuing or residual effect of Hsp90 inhibitor over fertilization that may influence apoptosis further in the embryo.

The data from the two experiments indicate that the inhibition of Hsp90 activity during early IVM may cause damage that results in a poor embryo development, highlighting the importance of Hsp90 activity for the developmental competence of bovine oocytes. This importance of Hsp90 activity was also shown in mice (Metchat *et al.*, 2009) and pigs (Son *et al.*, 2011; Liu *et al.*, 2018) oocytes. Interestingly, the findings for Hsp90 inhibition are comparable with that observed in some studies with bovine oocytes exposed to *in vivo* or *in vitro* heat stress, i.e. changes in the abundance of target transcripts in oocytes, lower blastocyst rates and higher apoptotic index in the embryos (Payton *et al.*, 2004; Roth and Hansen 2004; Camargo *et al.*, 2007, 2019; Gendelman and Roth 2012; Rodrigues *et al.*, 2016; Ascari *et al.*, 2017).

Based in our data that showed the importance of Hsp90 for oocyte competence, we questioned whether the inhibition of Hsp90 during the HS would increase the deleterious effects of high temperature on oocyte developmental competence. To answer this question, we evaluated the effects of the combination of 17AAG with HS for 12 h during IVM on embryo development. The effects of 17AAG, HS or 17AAG+HS during *in vitro* maturation on cell division were not detected in the early stages; however, development rate until blastocyst stage was disturbed by 17AAG, as already shown in the previous experiment of the present study, and by HS, as reported in other studies (Roth and Hansen, 2004; Camargo *et al.*, 2019). The lowest blastocyst rate was found when 17AAG and HS were combined during IVM. Blastocyst rate

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at D8 in the 17AAG+HS group was 3.9- and 2.9-fold lower than in 17AAG and HS groups, respectively. Under stress conditions both Hsp90 and Hsp70 are required to maintain a functional proteome with properly folded proteins (Vabulas *et al.*, 2010). It can be supposed that, in the 17AAG+HS group, the denatured protein is not properly refolded due the inhibition of Hsp90, resulting in an inadequate response to heat shock during IVM, which may culminate in the lowest developmental competence when compared with HS or 17AAG alone. In this sense, our data suggest that active Hsp90 can be required to attenuate the damage of HS in oocytes. However, it is needed to investigate whether Hsp90 has also a relevant role during *in vivo* heat stress, as differences between the cow body and intrafollicular temperatures may exist through a countercurrent heat exchange mechanism (Hunter and Einer-Jensen, 2005). In fact, the temperature in the bovine follicular fluid of pre-ovulatory follicle is cooler than the rectal temperature (Grøndahl *et al.*, 1996; López-Gatius and Hunter, 2017). Therefore, the control of follicular fluid temperature may help to protect the oocyte from the effects of high body temperature; nevertheless, the efficiency of this protection may vary among cattle breeds. It has been shown that, during a tropical summer, immature oocytes from Gir cows, a tropical-adapted breed, are more competent and have lower amounts of *HSP70* transcripts than oocytes from Holstein cows, a thermosensitive breed (Camargo *et al.*, 2007). The authors speculated that these differences could be the result of breed adaptation to the tropics, which could involve the control of follicular fluid temperature. Considering probable differences between breeds, the role of Hsp90 during *in vivo* heat stress may be more relevant for the competence of oocytes from thermosensitive breeds.

In summary, the inhibition of Hsp90 activity during *in vitro* maturation compromises further embryo development, demonstrating that this chaperone can play a role in developmental competence of bovine oocytes. Inhibition of Hsp90 activity and HS in oocytes causes similar effects in further embryo development, whereas the combination of both aggravates the consequences, suggesting that Hsp90 may play a role in cellular protection, also during maturation under high temperature. This novel information may contribute towards the development of alternatives aimed to modulate the influence of heat stress on oocyte developmental competence.

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Conflict of interest. The authors declare none.

Ethical standards. All applicable guidelines for the care and use of farm animals were followed and approved by the Ethics Committee on Animal Use (CEUA), protocol 02/2015.

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