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Addition of chlorogenic acid and caffeine during the processing of cooled boar semen

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[*Adição de ácido clorogênico e cafeína durante o processamento do sêmen suíno resfriado*]

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ABSTRACT

A study was conducted to evaluate the effect of chlorogenic acid (ChA) added pre-cooling and its combination with caffeine added during warming on cooled-stored boar semen parameters. Ten ejaculates were diluted in commercial extender with or without 4.5mg/ml ChA and stored at 15°C. After 0, 24 and 72 hours of storage, aliquots of these doses were taken and incubated at 37°C in the presence or absence of 8.0mM caffeine. Semen quality was evaluated after 10 and 120 minutes of incubation. The ChA increased (P <0.01) the sperm motility, viability, acrosomal integrity and the percentage of spermatozoa with high mitochondrial activity (PMHA), however, decreased (P <0.01) the malondialdehyde (MDA) concentration. Caffeine increased (P <0.05) the sperm motility, viability, PMHA and the MDA concentration and reduced (P <0.05) the acrosome integrity. When associated (ChA+caffeine), there was an increase (P <0.05) in sperm motility and viability, PMHA and acrosome integrity. The addition of ChA to the dilution medium improves the quality of the swine inseminating doses. The addition of caffeine during re-warming is only recommended when the semen is stored for prolonged periods (72h), and the inseminating dose should be used immediately after its addition.

Keywords: antioxidant, boar semen storage, metabolic activator, methylxanthine, polyphenol

RESUMO

O objetivo deste estudo foi avaliar os efeitos da adição de ácido clorogênico (ChA) antes do resfriamento e sua combinação com cafeína adicionada durante o reaquecimento sobre a qualidade do sêmen suíno resfriado. Dez ejaculados foram diluídos em diluidor comercial com adição ou não de 4,5mg/mL de ChA e armazenados a 15°C. Após zero, 24 e 72 horas de armazenamento, 10mL foram retirados e incubados a 37°C na presença ou ausência de 8,0mM de cafeína. A qualidade seminal foi avaliada após 10 e 120 minutos de incubação. O ChA aumentou (P<0,01) a motilidade, a viabilidade, a integridade acrosomal e a porcentagem de espermatozoides com alta atividade mitocondrial (PMHA), entretanto diminuiu (P<0,01) a concentração de malondialdeído (MDA). A cafeína aumentou (P<0,05) a motilidade, a viabilidade, a PMHA e a concentração de MDA e reduziu a integridade acrossomal. Quando associados (ChA+cafeína), houve aumento (P<0,05) na motilidade, na PMHA, na viabilidade e na integridade acrossomal. Conclui-se que a adição de ChA ao meio de diluição melhora a qualidade das doses inseminantes de suínos. A adição de cafeína durante o reaquecimento só é recomendada ao sêmen adicionado de ChA quando esse for armazenado por períodos prolongados (72h), devendo a dose inseminante ser utilizada imediatamente após sua adição.

Palavras-chave: antioxidante, sêmen suíno armazenado, ativador metabólico, metilxantina, polifenol

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INTRODUCTION

Currently, artificial insemination is widely practiced in swine production using fresh seminal doses or stored at 15°C for usual periods of 24 to 48h (Pinart *et al.*, 2013). Although the fertility of boar semen can be maintained under these conditions, cooling for longer periods results in decreased sperm quality. This occurs mainly due to excessive production of reactive oxygen species (ROS) by spermatozoa, which in turn cause oxidative damage in sperm cell membranes compromising viability and sperm function (Waterhouse *et al.*, 2004). The lipid peroxidation of cell membrane induced by ROS alters the structure and function of the sperm, generates DNA damage (Schulte *et al.*, 2010) and reduces intracellular ATP levels and sperm motility (Aitken *et al.*, 2012).

To address these problems, addition of antioxidants in semen extenders have been used, ensuring better quality of the cooled semen (Martin-Hidalgo *et al.*, 2011; Mendez *et al.*, 2013). The chlorogenic acid (ChA) is a polyphenol known for its antioxidant properties *in vitro* (Rice-Evans *et al.*, 1996) that has been associated with suppressing the activation of transcription factors induced by ROS (Xu *et al.*, 2010). Recently, a study has proved the efficiency of ChA in improving the quality of the cooled boar semen (Pereira *et al.*, 2018). Thus, the addition of antioxidants could have a beneficial effect on increasing the useful storage time of boar semen.

There are other substances that can improve stored boar semen quality. For instance, metabolic activators, such as caffeine, have also been used in this sense. Caffeine is a methylxanthine capable of improving the motility of spermatozoa and mitochondrial activity (Nabavi *et al.*, 2013), resulting in an increase in fertilization rates (Numabe *et al.*, 2001), when added immediately prior of insemination. In swine, there is evidence that the addition of caffeine during the semen re-warming of cooled boar semen reduces the sperm viability, however, increases in the vigour of spermatozoa (Nunes, 2012). The reduction of sperm viability was attributed by the authors to the higher metabolic activity of the cells. In frozen-thawed boar semen, the use of caffeine in the thawing solution improved the progressive motility, straightness, and linearity of sperm movement (Yamaguchi e Funahashi, 2012; Yamaguchi *et al.*, 2013). It is known that caffeine positively affects sperm function by upregulating glycolysis via a buildup of cyclic adenosine monophosphate (cAMP), which generates adenosine triphosphate (ATP), used to power sperm movement (Lardy *et al.*, 1971). Rhemrev *et al.* (2001) suggested that the motility of the spermatozoa was related to their fertilizing capacity and the sperm motility was dependent on mitochondrial function.

However, one undesirable side effect linked with the utilization of metabolic activators is a concomitant increase of sperm ROS production, which would act against sperm function. Thus, an effective technique to utilize metabolic activators to improve boar semen conservation would include a system that allows us to avoid the undesirable increase in ROS. A possibility would be the simultaneous utilization of a metabolic activator, like caffeine, together with an antioxidant, like ChA. In this manner, the antioxidant effect of the ChA could counteract the caffeine-caused increase in ROS. Taking this into consideration, the main objective of this study was to determine if combined use of ChA, in the dilution media, and caffeine, during re-warming of inseminating doses, could be useful to improve the quality of boar semen stored at 15°C until 72 hours.

MATERIALS AND METHODS

All procedures were approved by the Ethics Committee on Animal Use of UFLA with protocol number 027/13. Ten ejaculates from three fertility proven pure breed boars (Pietrain, Duroc and Large White) were used. The animals were 1.0-1.5 years of age and were kept in individual pens (3.0m long, 2.0m wide and 1.30m high) received water *ad libitum* and 3.0kg of feed divided into two daily rations. Animals were housed at the Experimental Swine Center; Department of Animal Science, Federal University of Lavras (UFLA; Lavras; Minas Gerais, Brazil).

Ejaculates were obtained using the gloved hand method during routine collection on the farm. After collection, ejaculates were immediately sent to the Laboratory of Swine Breeding (Department of Physiology and Pharmacology,

UFLA). Initial subjective observations on motility and vigour were carried out on a drop of semen placed between a slide and coverslip previously heated to 37ºC under phase microscope (Nikon Eclipse E200-LED, Nikon, Tokyo, Japan) at 100×. For analysis of sperm morphology 200µL of fresh semen was addition in 1000µL of formal saline solution, and a drop was evaluated under a phase microscope (Olympus CX31, Olympus Corporation, Hamburg, Germany) at 1000×. Sperm concentration was evaluated in a Neubauer cell counter chamber after dilution of 10µL fresh semen in 1000µL of formal saline solution, and the number of spermatozoa/ml was determinate under a phase microscope at (Olympus CX31, Olympus Corporation, Hamburg, Germany) 400×. Only the rich fraction of motile ejaculates was used for processing of insemination doses.

All ejaculates fulfilled the minimum requirements for use in artificial insemination: >70% total motility, <20% proximal cytoplasmic droplets and total number of spermatozoa $>200\times10^6$ per ml (Silva *et al.*, 2011). After the sperm concentration was calculated, the semen volume that contained 1.5 billion sperm was diluted in BTS extender previously prepared and heated to 37°C with or without addition of ChA. From each ejaculate, four insemination doses were processed and distributed in a randomized block design (blocked by ejaculate) in a 2 x 2 factorial scheme (with or without ChA, with or without caffeine) in a split plot in time (incubation time of semen) with ten repetitions that were representative of the ejaculate.

For this, immediately before the processing of insemination doses, 180mg of ChA (Chlorogenic acid crystalline, C3878, Sigma-Aldrich Co., Ltd., St. Louis, MO, USA) was diluted in 5.0ml of BTS extender (Beltsville Thawing Solution®, Minitube, Porto Alegre, Brazil) at 37°C using an ultrasonic sonicator (QR 300W, Ultronique, Indaiatuba, Brazil). This solution was then added to the volume of semen that contained 1.5 billion sperm. After, BTS at 37°C were then added until the final volume of 40ml, so that each insemination dose contained 4.5mg of ChA per ml of semen (Pereira *et al.*, 2018). Two inseminate doses were processed with ChA and two without this substance. Then, the pH of doses was measured using pH-indicator strips (109535, Merck Millipore Corporation, Darmstadt, Germany). The pH of all samples remained between 7 and 8, which is considered ideal for sperm survival (Johnson *et al.*, 2000).

Ninety minutes after this process, all four doses were stored at 15°C in a refrigerator. Before storage, a 10ml aliquot of each insemination dose was heated in test tubes in a water bath at 37°C. One aliquot from a ChA-added dose and another aliquot from a negative control were supplemented with caffeine (8.0mM) in a concentration determined in previous study (Nunes, 2012). For this, 15.33mg of caffeine (pure caffeine, 533, Isofar, Rio de Janeiro, Brazil) was diluted in 1.0ml of BTS at 37°C, which was added into 9.0ml of the inseminate dose. The other two 10ml aliquots were added to 1.0ml of BTS without caffeine and they were used as controls for the caffeine effect. After 10 and 120min of incubation, all samples were evaluated to determine the percentage of mobile and viable cells, integrity of sperm acrosome, mitochondrial activity of the cells and concentration of glucose to determine the glucose uptake during this time. Furthermore, after 60min of incubation samples were collected to determine the concentration of malondialdehyde (MDA). The same incubation procedure was carried out after 24 and 72h of storage, with the exception of glucose intake that was evaluated only at 0 and 72h of storage.

To evaluate sperm motility, a drop of semen was placed between a slide and a cover slip previously heated to 37°C. Observations were made using a phase-contrast microscope (Nikon Eclipse E200-LED, Nikon, Tokyo, Japan) with 100x magnification. Two trained persons subjectively evaluated 10 fields, to determine the percentage of motile sperm. Analyses were done blind and in triplicate. The vigour was evaluated using a system scale from 0 to 5, where 0 represents the absence of movement and 5 is maximum intensity.

Sperm membrane integrity was measured by a smear of drop of semen mixed with a drop of eosin-nigrosin (Blom, 1950). The cells were evaluated under optical microscope (Olympus CX31, Olympus Corporation, Hamburg, Germany) at 400×. The values were expressed by the percentage of cells with intact membranes in relation to the total number of cells counted (200 cells). The same process was used to determine

the acrosomal integrity, however, a single dye of POPE (Fast Green - F7258 and Rose Bengal – 198250, Sigma-Aldrich Co. Ltd., St. Louis, USA) was used (Pope *et al.*, 1991). The mitochondrial oxidative activity was estimated by the DAB technique (Hrudka, 1987) in the same phase contrast microscopy at 1000× magnification. Only cells with fully stained midpieces that indicate high mitochondrial activity were analyzed. All analyses were performed in duplicate in a blinded manner by the same investigator.

To carry out the biochemical analysis, 1.0ml semen aliquots were removed at 10, 60 and 120min of incubation. Samples were centrifuged in 2.0mL centrifuge tubes at 3360g for 10min. Supernatants were then transferred to another centrifuge tube and frozen at −80°C until analysis.

The glucose and malondialdehyde (MDA) concentration in the seminal plasma were measured by enzyme colorimetric methods (Analyze Glucose-PP, Gold Analisa Diagnostica, Belo Horizonte, Brazil and QuantiChromTM TBARS, Assay Kit DTBA-100, Bioassay Systems, Hayward, CA, USA, respectively), following the manufacturer's instructions. The glucose intake by sperm was determined by difference between the glucose concentration in the extracellular medium at 10 and at 120min of incubation. The MDA concentration was measured at 60min of incutabion.

In relation to statistical analysis, the Shapiro Wilk test was used to evaluate the normality of residuals. Data from motility and sperm viability, and mitochondrial activity were subjected to analysis of variance and means within each main factor (ChA and caffeine) and time of incubation were compared by the F test, considering α= 0.05. For vigour, a nonparametric statistical analysis was used and the averages were compared using the Friedman test. All statistical analysis was performed with the Action 2.3 statistical program.

RESULTS

The ChA improved (P <0.01) the sperm motility after 120min of incubation of the semen before and at 24 hours of storage, regardless of the addition of caffeine (Table 1). With 72 hours of storage, ChA improved (P <0.01) the sperm motility at both 10 and 120 minutes of incubation. Caffeine increased (P <0.05) the sperm motility only in semen stored for 72h at 10min of incubation, both in the presence or absence of ChA. Throughout the incubation for 120 minutes, there was decrease (P <0.05) of semen motility, with or without addition of caffeine and ChA. Although there was improvement of the motility in some times of evaluation, both caffeine and ChA failed to maintain sperm motility during 120min of incubation.

Table 1. Sperm motility of boar semen containing or not chlorogenic acid (CH) and caffeine (CA), evaluated in different times incubation at 37ºC after different times of storage at 15ºC. n= 10

Incubation time	Chlorogenic acid	Caffeine		SEM	P =		
		without	with		CA	CH	CH x CA
- Before storage (diluted semen) -							
10 minutes	without	81.0 *	82.5 *	1.03	0.23	<0.01	0.24
	with	82.0 *	84.5 *				
120 minutes	without	72.5 A	71.0 A				
	with	79.0 B	81.0 B				
- 24 hours of storage -							
10 minutes	without	81.0 *	82.5 *	1.03	0.23	<0.01	0.24
	with	82.0 *	84.5 *				
120 minutes	without	72.5 A	71.0 A				
	with	79.0 B	81.0 B				
- 72 hours of storage -							
10 minutes	without	57.5 Aa*	61.5 Ab*	1.00	0.34	<0.01	0.10
	with	67.5 Ba*	70.6 Bb*				
120 minutes	without	47.5 A	46.5 A				
	with	64.0 B	61.5 B				

* Incubation period differ by F test (P <0.05)

[ab] Within each incubation time, means followed by different upper letters in the column and lower case letters in the line differ by the F-test (P <0.05)

The combination of ChA and caffeine was able to maintain (P<0.01) the percentage of mitochondria with high mitochondrial activity (PMHA) during 120min of incubation both in diluted fresh semen and in stored semen for 72h (Table 2). Alone, ChA was able (P<0.05) to maintain this characteristic during incubation time only in the stored semen for 72h. ChA increased (P<0.01) the PMHA throughout the 120min of incubation in all semen samples. Caffeine increased (P <0.01) the PMHA, with exception in the diluted fresh semen and semen stored for 72h containing ChA at 10min of incubation and in the semen stored for 24h, at 120min of incubation.

Table 2. Percentage of mitochondria with high activity of boar semen containing or not chlorogenic acid (CH) and caffeine (CA), evaluated in different times incubation at 37ºC after different times of storage at 15ºC. n= 10

Incubation time	Chlorogenic acid	Caffeine		SEM	P =		
		without	with		CA	CH	CH x CA
		- Before storage (diluted semen) -					
10 minutes	without	38.6 Aa*	40.7 b*	0.40	<0.01	<0.01	0.09
	with	40.2 B*	41.2				
120 minutes	without	36.5 Aa	38.5 Ab				
	with	38.5 Ba	40.8 Bb				
		- 24 hours of storage -					
10 minutes	without	38.4 Aa	40.9 Ab*	0.49	<0.01	<0.01	0.88
	with	41.2 Ba*	43.2 Bb*				
120 minutes	without	37.6 A	38.7 A				
	with	39.6 B	41.0 B				
		- 72 hours of storage -					
10 minutes	without	35.4 Aa*	37.4 Ab*	0.47	<0.01	<0.01	0.12
	with	37.8 B	39.1 B				
120 minutes	without	33.1 Aa	35.5 Ab				
	with	36.6 Ba	38.6 Bb				

* Incubation period differ by F test (P <0.05)

[ab] Within each incubation time, means followed by different upper letters in the column and lower case letters in the line differ by the F-test (P <0.05)

Both ChA and caffeine did not influenced (P>0.05) the MDA concentration in the diluted semen (Table 3). However, caffeine increased (P<0.01) the MDA concentration of samples stored for 72h, with exception when ChA was present in the semen. ChA decreased (P<0.01) the MDA concentration of stored semen.

Table 3. Malondialdehyde concentration in the boar semen containing or not chlorogenic acid (CH) and caffeine (CA), evaluated after 60min of incubation at 37ºC before and after storage at 15ºC. n= 10

Chlorogenic acid	Caffeine		SEM	P =		
	without	with		CA	CH	CH x CA
	- Before storage (diluted semen) -					
Without	2.27	2.05	0.10	0.68	0.88	0.55
With	2.10	2.15				
	- 72 hours of storage -					
Without	5.17Aa	5.93Ab	0.15	0.01	<0.01	0.66
With	4.33B	4.88B				

[a.b] Within storage time, means followed by different upper letters in the column and lower case letters in the line differ by the F-test (P <0.05).

The presence of ChA in the extender improved (P <0.01) the sperm viability only on the stored semen (24 or 72h), with more expressive results in the semen stored for 72h, evaluated at 120min of incubation (Table 4). Caffeine improved the sperm viability only at 120min of incubation of semen without ChA stored for 24h or at 10min of incubation of semen stored for 72h with or without ChA. The addition of ChA and caffeine did not able to maintain the sperm viability throughout 120min of incubation.

ChA improved (P <0.01) the integrity of sperm acrosome in the diluted fresh semen only when caffeine was added (Table 5). In the semen stored for 72h, ChA improved the integrity of sperm acrosome in all evaluated samples. Caffeine reduced (P <0.05) the integrity of sperm acrosome in some samples without ChA, however, increased when ChA was present. ChA decreased (P <0.05) the integrity of sperm acrosome throughout 120min of incubation in all semen samples. Caffeine decreased this characteristic throughout the incubation time of stored semen and the combination ChA and caffeine decreased only in the semen stored for 72h.

Table 4. Percentages of sperm viability in the boar semen containing or not chlorogenic acid (CH) and caffeine (CA), evaluated in different times incubation at 37ºC after different times of storage at 15ºC. n= 10

Incubation time	Chlorogenic acid	Caffeine		SEM	P =		
		without	with		CA	CH	CH x CA
		- Before storage (diluted semen) -					
10 minutes	without	92.8	92.7	0.74	0.14	0.22	0.28
	with	93.6	94.3				
120 minutes	without	93.2	91.4				
	with	93.3	92.3				
		- 24 hours of storage -					
10 minutes	without	89.4 A*	88.6 A*	0.70	0.41	<0.01	0.01
	with	93.1 B*	91.2 B*				
120 minutes	without	81.4 Aa	83.7 Ab				
	with	90.0 B	89.1 B				
		- 72 hours of storage -					
10 minutes	without	57.5 Aa*	61.5 Ab*	1.00	0.34	<0.01	<0.01
	with	67.0 Ba*	70.5 Bb*				
120 minutes	without	47.5 A	46.5 A				
	with	64.0 B	61.5 B				

* Incubation period differ by F test (P <0.05)
[ab] Within each incubation time, means followed by different upper letters in the column and lower case letters in the line differ by the F-test (P <0.05)

Table 5. Percentages of sperm acrosome integrity of boar semen containing or not chlorogenic acid (CH) and caffeine (CA), evaluated in different times incubation at 37ºC after different times of storage at 15ºC. n= 10

Incubation time	Chlorogenic acid	Caffeine		SEM	P =		
		without	with		CA	CH	CH x CA
		- Before storage (diluted semen) -					
10 minutes	without	97.1 a	96.0 Ab	0.32	0.79	<0.01	<0.01
	with	97.8 *	98.3 B				
120 minutes	without	96.3	95.4 A				
	with	96.8 b	98.0 Ba				
		- 24 hours of storage -					
10 minutes	without	93.8	95.3 *	0.62	0.07	<0.01	0.04
	with	95.0 *	95.8				
120 minutes	without	92.7	91.0 A				
	with	92.5 a	94.3 Bb				
		- 72 hours of storage -					
10 minutes	without	89.8 A*	90.4 A*	0.41	0.15	<0.01	0.18
	with	92.3 B*	91.8 B*				
120 minutes	without	88.5 Aa	85.8 Ab				
	with	90.2 B	90.6 B				

* Incubation time differ by F test (P <0.05)
[a,b] Within each incubation time, means followed by different upper letters in the column and lower case letters in the line differ by the F-test (P <0.05)

The vigour was not influenced (P>0.05) by the presence of both ChA and caffeine (Table 6). Despite this, there was a decrease (P<0.01) of movement intensity throughout the incubation period when caffeine was added to the semen. No effect of ChA and caffeine was observed in the glucose intake (Table 7).

Table 6. Vigour in scale from 0 (absence of movements) to 5 (high intensity of movements) of boar semen containing or not chlorogenic acid (CH) and caffeine (CA), evaluated in different times incubation at 37ºC after different times of storage at 15ºC. n= 10

Incubation time	Chlorogenic acid	Caffeine		P =
		without	with	
		- Before storage (diluted semen) -		
10 minutes	without	4.0	4.7*	<0.01
	with	3.9	4.6*	
120 minutes	without	3.0	2.8	
	with	3.0	2.8	
		- 24 hours of storage -		
10 minutes	without	3.0	4.2*	<0.01
	with	3.0	4.1*	
120 minutes	without	2.6	2.0	
	with	2.8	2.3	
		- 72 hours of storage -		
10 minutes	without	2.6	3.6*	<0.01
	with	2.8	3.5*	
120 minutes	without	2.0	1.9	
	with	2.1	1.9	

* Incubation time differ by the Friedman test (P <0.01)

Table 7. Glucose intake in the boar semen containing or not chlorogenic acid (CH) and caffeine (CA), evaluated after 60min of incubation at 37ºC before and after storage at 15ºC. n= 10

Chlorogenic acid	Caffeine		P=
	without	with	
	- Before storage (diluted semen) -		
without	20.2	15.2	0.14
with	17.0	17.7	
	- 72 hours of storage -		
without	14.5	12.0	0.25
with	13.2	10.2	

No significance by Frideman test (P >0.05).

DISCUSSION

Our results clearly indicate that the addition of ChA during storage and refrigeration can be useful to improve the semen quality of storage boar semen at 15°C. Furthermore, our results also indicate that the presence of ChA has a beneficial effect through potentiating the positive effects of caffeine on boar semen quality with a clear mitigation of the side effects linked to caffeine addition. The results described are very important when taking into account the fact that one of the biggest current challenges for reproductive technology is the maintenance of boar semen quality stored in refrigerated conditions for prolonged periods. In addition, the

evaluation at different incubation times is important to simulate sperm resistance after re-warming. Under practical conditions, the use of semen at different times after re-warming may contribute to the reduction of fertility rate. Thus, alternatives that can prolong sperm resistance time after re-warming are important.

Pereira *et al.* (2018), evaluating different doses of ChA (0.0, 1.5, 3.0, 4.0 and 6.0mg/ml) concluded that the concentration of 3.2mg/ml of this substance is the best for semen stored for 24h. However, for semen stored for a longer period, 6.0mg/ml or more should be used. In the present study, the concentration of 4.5mg/mL was considered an intermediary dose, giving beneficial effects for both semen stored for 24 and 72h. By other hand, Nunes (2012), evaluating different doses of caffeine (0, 2, 4, 6 and 8mM) added in the cooled semen concluded that the higher dose improves the vigour only in the semen stored by 48h, but negatively affects in lower storage times, probably due to increased metabolic activity of spermatozoa. In this case, the association between a metabolic activator and a membrane protecting substance could bring benefits to the use of the cooled sperm semen.

Reduction in sperm quality during storage is commonly correlated with oxidative damage to cell structures due to metabolism (Schulte *et al.*, 2010), since preservation temperatures between 15 and 18°C do not completely stop sperm metabolism. The losses to the seminal quality are more evident after 48h of cooling, reducing the reproductive performance of the herd (Pinart *et al.*, 2013). The oxidative attack on the sperm membranes is able to modify the fluidity of the plasma membrane and interfere with enzymatic activity and in the function of ion channels (Marmunti *et al.*, 2012), thus reducing sperm quality. On the other hand, sperm metabolism is important to guarantee the semen quality (Yeste *et al.*, 2008). Thus, substances that accelerate sperm metabolism combined with substances with antioxidant properties could have positive effects on the quality of inseminating doses.

In the present study, we can only speculate about the mechanisms by which ChA exerts its positive effects. Other studies have reported the scavenger ability of polyphenols on sperm function (Martin-Hidalgo *et al.*, 2011; Wittayarat *et al.*, 2013). Thus, the scavenger activity of ChA could be an important mechanism leading to a reduction of the oxidative damage of sperm cell structures. Furthermore, the low MDA levels found in semen stored for 72h in the presence of ChA indicate that the effect was able to decrease the lipid peroxidation alterations caused by ROS (Gomez-Fernandez *et al.*, 2013; Fraser *et al.*, 2014). Since the integrity of cell structures is crucial to maintaining sperm functionality, the reduction of the oxidative stress induced by the addition of antioxidants would improve the quality of seminal doses (Maia *et al.*, 2009; Wittayarat *et al.*, 2013). In the non-stored semen, the effects of ChA were not evident, probably due to the presence of natural antioxidants in the seminal plasma, which are able to protect sperm cells from oxidative damage during short periods of storage (Kowalowka *et al.*, 2008). However, since storage is linked to a great dilution of seminal plasma factors, the addition of ChA could counteract the loss of the antioxidant effect because of the dilution of seminal plasma.

The maintenance of sperm function is also dependent on the metabolic status of the sperm since cellular activity occurs from the consumption of both extra and intracellular energy substrates. In this sense, energy production through mitochondrial pathways is of the utmost importance in eukaryotic cells. Centering on boar sperm, it is noteworthy that the total energy yielded by mitochondrial activity is a very low percentage (a maximum of 5%) of total energy production (Marin *et al.*, 2003). Despite this, the minimal mitochondria-based energy production is critical for the maintenance of motility and sperm capacitation in swine (Ramio-Lluch *et al.*, 2014). Thus, sperm mitochondrial activity becomes an important evaluation parameter regardless of their intrinsic importance in the production of energy in boar sperm. With respect to the seminal metabolic rate, the addition of caffeine increased sperm mitochondrial activity only after 10min of incubation of semen stored for 72h. This effect of caffeine had been associated with the increase of intracellular concentrations of cAMP (Lopez e Alvarino, 2000; Carrington *et al.*, 2011). This is important, since several sperm metabolic parameters, such as motility, are closely dependent on intracellular cAMP levels (Qu *et al.*, 2007).

On the other hand, increased intracellular concentrations of cAMP are also associated with elevated overall cell metabolic rates. In the present study, caffeine was able to increase the number of mitochondria with maximum activity even in doses that were not supplemented with ChA. There is evidence that the phosphorylation of certain mitochondrial enzymes mediated by cAMP play a regulatory role in oxidative phosphorylation (Acin-Perez *et al.*, 2009). Therefore, increases in cAMP could increase mitochondrial activity by stimulating oxidative phosphorylation. In contrast, while the energy generated by mitochondria is essential for sperm function, this organelle is the major source of endogenous ROS production in the cell (Ramio-Lluch *et al.*, 2014). It is known that ROS can impair sperm metabolism during storage or incubation. In fact, a significant increase in the MDA concentration was detected in the present study after addition of caffeine in the semen stored for 72h. This increase may be linked to the reduction in the PMHA and, consequently, the reduction in the vigour after 120min of incubation. The increase of MDA concentration may be associated with an increased metabolic rate, generated by caffeine's capability to increase the rate of cellular oxidative phosphorylation (Carrington *et al.*, 2011). Despite this evidence of increased sperm metabolic rate, caffeine does not alter glucose uptake by sperm, possibly due to consumption of other energy sources, as fructose, from spermatozoa.

In the present study, the negative effects of caffeine are mitigated with the use of ChA. This fact indicates that the protective effect against sperm membrane damage can be associated with the antioxidant activity of ChA. While caffeine metabolically stimulates mitochondrial activity (Carrington *et al.*, 2011), ChA protects the organelle from oxidative damage (Li *et al.*, 2012), promoting good conditions for its performance. (Peña *et al.* (2003)) also observed the beneficial effects of adding an antioxidant (vitamin E) to the mitochondrial membrane potential of thawed boar semen, showing that mitochondria are more sensitive to oxidative damage, and antioxidants have a protective effect to this structure.

In summary, the present study showed that ChA has positive effects in sperm motility (mainly after 120 minutes of incubation), percentage of mitochondria with maximum activity, MDA concentration, sperm viability (at 24 and 72h of storage) and acrosome integrity (72h). Caffeine increased the sperm motility at 10 minutes of incubation of stored semen for 72h, percentage of mitochondria with maximal activity (0, 24 and 72h) and sperm viability (24 and 72h). On other hand, caffeine increased the MDA concentration and reduced the acrosome integrity (both with 72h of storage) and sperm vigour throughout the 120 minute incubation (0, 24 and 72h). When these substances were associated, there was an increase in the sperm motility, sperm viability, percentage of mitochondria with maximum activity (all with 72h of storage) and in the acrosome integrity (0 and 24h of storage). The reduction of sperm vigour throughout the incubation time caused by caffeine adding was not alleviated by the addition of ChA. These results suggest that the addition of ChA in the dilution medium is beneficial for the quality of the swine inseminating doses and that the use of caffeine before re-warming is only recommended for the semen stored for prolonged periods (72h). Although the vigour has been reduced during incubation time when these substances were used, studies *in vivo* and *in vitro* must be conducted to evaluate if this reduction could compromise the fertilization capacity of semen.

CONCLUSIONS

The addition of ChA to the dilution medium improves the quality of the swine inseminating doses. The addition of caffeine during re-warming is only recommended when the semen is stored for prolonged periods (72h), and the inseminating dose should be used immediately after its addition.

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