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Effect of indole-3-acetic acid (plant auxin) on the preservation at 15 °C of boar semen for artificial insemination

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Summary — In order to extend the duration of boar sperm survival at 15 °C for artificial insemination, we tested the effect of indole-3-acetic acid (IAA), which appeared to be the main sperm protective substance present in the *Coco nucifera* endosperm (coconut water). Two IAA concentrations (10 and 100 ng/mL) in Beltsville extender (BTS) were studied for their in vitro effects. The motility, the percentage of motile spermatozoa and the acrosome morphology of sperm were recorded each day over 13 days of storage at 15 °C, after 5 min and 3 h of incubation at 39 °C. The IAA effect on sperm preservation was also studied in vivo at a concentration of 10 ng/mL in BTS by inseminating groups of females twice at 24 h intervals either at D0 (day of sperm collection) and D1 (D0/1) or at D5 and D6 (D5/6). At D0/1, the two groups of females (control and IAA) were inseminated with a total of 6.3×10^9 spermatozoa (3.15×10^9 at D0 and the same dose at D1) whereas at D5/6, one IAA group was inseminated with a total of 6.3×10^9 spermatozoa and another one with 12.6×10^9 spermatozoa. The animals in the D5/6 control group were inseminated each with a total of 12.6×10^9 spermatozoa. For each group of females ($n = 106-140$), fertility rate (% farrowing) and prolificacy rate (litter size) were recorded. No effect of IAA in vitro on the motility rate and on the percentage of motile spermatozoa was observed over a 13 day storage. However, IAA (10 ng/mL) had a significant positive effect on the percentage of living spermatozoa with intact acrosomes after 13 days (66 vs 54%, $P < 0.05$). The fertility and prolificacy rates after 5–6 days of sperm preservation in BTS extender alone did not differ significantly between D5/6 and D0/1 but the total number of inseminated spermatozoa was 12.6×10^9 at D5/6 instead of 6.3×10^9 at D0/1. When the spermatozoa were stored in the presence of 10 ng/mL IAA for 5–6 days at 15 °C, the fertility and prolificacy of the females inseminated with only 6.3×10^9 spermatozoa were identical to those of the females inseminated with an equal number of spermatozoa at D0/1 in the presence or absence of IAA.

pig / sperm / 3-indole-acetic acid / fertility / prolificacy / artificial insemination

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Résumé — Effet de l'auxine végétale, l'acide 3-indole-acétique, sur la conservation du sperme de verrat pour l'insémination artificielle. Afin de prolonger la durée de conservation du sperme de verrat à 15 °C pour l'insémination artificielle, nous avons étudié l'effet de l'acide 3-indole acétique (IAA) que nous avons identifié comme la principale substance protectrice du sperme présente dans l'endosperme de *Coco nucifera* (eau de coco). Deux concentrations d'IAA (10 et 100 ng/mL) dans le dilueur de Beltsville (BTS) ont été étudiées in vitro. La mobilité et le pourcentage de spermatozoïdes mobiles ainsi que l'intégrité de leurs acrosomes ont été étudiés sur une période de 13 jours de conservation à 15 °C après des incubations de 5 minutes et 3 heures à 39 °C. L'effet de l'IAA a aussi été étudié in vivo à la concentration de 10 ng/mL de BTS en inséminant des groupes de femelles deux fois soit, le jour de la collecte et le lendemain (j0/1) soit, aux cinquième et sixième jours après la collecte (j5/6). À j0/1, les femelles des deux groupes (témoin et IAA) ont été inséminées avec un total de $6,3 \times 10^9$ spermatozoïdes ($3,15 \times 10^9$ à j0 et j1) tandis qu'à j5/6 les femelles du groupe IAA ont été inséminées avec $6,3 \times 10^9$ spermatozoïdes ($3,15 \times 10^9$ à j5 et j6) et les femelles des deux autres groupes (témoin et IAA) par $12,6 \times 10^9$ spermatozoïdes ($6,3 \times 10^9$ à j5 et j6). La fertilité et la prolificité des animaux de chaque groupe ($n = 106-140$) ont été enregistrées. Aucun effet de l'IAA in vitro n'a été observé sur la mobilité massale et le pourcentage de spermatozoïdes mobiles sur une période de 13 jours. Cependant, l'IAA (10 ng/mL) a un effet favorable significatif sur le pourcentage de spermatozoïdes vivants dont l'acrosome est intact après 13 jours (66 % versus 54 % ; $p < 0,05$). Aucune baisse de la fertilité et de la prolificité n'est observée chez les truies inséminées avec $12,6 \times 10^9$ spermatozoïdes conservés pendant 5-6 jours par rapport à celles inséminées par $6,3 \times 10^9$ spermatozoïdes conservés 0-1 jour. La fertilité et la prolificité des truies inséminées par seulement $6,3 \times 10^9$ spermatozoïdes conservés pendant 5-6 jours en présence d'IAA (10 ng/mL) à 15 °C sont les mêmes que celles des animaux inséminés par le même nombre de spermatozoïdes conservés seulement pendant 0-1 jour avec ou sans IAA.

porc / spermatozoïde / acide 3-indole-acétique / fertilité / prolificité / insémination artificielle

INTRODUCTION

Artificial insemination (AI) in the porcine species is usually performed with boar semen stored at 15 °C for up to a maximum of 3 days, after which time a significant decrease in fertilizing ability is observed (Paquignon, 1984). For an efficient organization of inseminations and optimal use of semen doses in breeding, it is essential that sperm can be stored over long periods of time with no or minimal loss in fertilizing ability (Paquignon et al, 1982a; Rillo et al, 1991). The best current extender for porcine AI is Beltsville thawing solution (BTS) (Paquignon et al, 1987; Blichfeld et al, 1988; Reed and Curnock, 1991) which permits economically acceptable boar sperm preservation for 2-3 days after collection (Paquignon et al, 1988). Taking into consideration work organization in pig farms, it would be of great interest to conserve fertile sperm over a 5-6 day period.

Preliminary studies have shown that endosperm from *Coco nucifera* (coconut water) can be used for the preservation of sperm from bucks (Toniolli, 1989), boars (Toniolli, 1990, 1991) and rams (Freitas and Ferreira-Nunès, 1994). Fractionation of coconut water has permitted isolation of a component, named JYP, which appeared to be, at least in part, responsible for the favourable effect of coconut water (Ferreira-Nunès, Combarnous and Leclercq, unpublished data). Later, indole-3-acetic acid (IAA) was identified as the main active molecule in JYP (Combarnous and Ferreira-Nunès, 1995).

In the present paper, we report the effect of the addition of IAA to BTS extender in the survival of boar sperm at 15 °C over a 0-13 day period of time. These effects were evaluated in vitro on sperm motility parameters and acrosome morphology integrity and in vivo on fertility and prolificacy rates in

sows inseminated after 0–1 or 5–6 days of sperm conservation.

MATERIALS AND METHODS

Forty-nine boars (Landrace and Large White full-bred and crossbred) in regular service (one ejaculate per week on average) were used in the present study. In all ejaculates, sperm motility rates were ≥ 3 and percentages of motile spermatozoa were $\geq 75\%$.

Immediately after collection, each ejaculate was split into two (in vivo assays) or three (in vitro assays) fractions diluted at a concentration of 35×10^6 spermatozoa per mL in BTS extender alone or in BTS containing 10 ng/mL IAA (5.7×10^{-9} M) in vivo and in vitro assays or 100 ng/mL IAA (5.7×10^{-8} M) in vitro assays. The BTS extender was purchased in dry form from Cobiporc (Saint-Gilles, France) and prepared as recommended by the manufacturer.

In in vitro studies, 3 mL fractions were incubated in 5 mL glass tubes for time periods of 0 to 13 days at 15 °C. Each day, except D8, D10 and D12, one tube from each group was removed and incubated for 3 h at 39 °C in a water bath. Motility parameters were evaluated microscopically at $\times 128$ magnification after 5 min and 3 h at 39 °C as described by Bork et al (1988). The observations were made at a concentration of 35×10^6 spermatozoa per mL in blind tests on at least three fields. The integrity of spermatozoa acrosome morphology was assessed on 300 to 400 spermatozoa at D13 using Congo red and Giemsa stain (Kovacs and Foote, 1992).

For the in vivo study, a total of 634 crossbred sows were used among which only 54 (8.5%) were nulliparous and evenly distributed among the five experimental groups randomly distributed among 112 farms. Forty-nine ejaculates from 37 boars were used. The experiment was carried out between December 1992 and December 1994 over all seasons. Four technicians were in charge of artificial inseminations and the collection of farrowing data. Inseminations were performed twice at 24 h intervals at days 0 and 1 (D0/1) or at days 5 and 6 (D5/6) after sperm collection. The females in the D0/1 groups (control and IAA) were inseminated with 2×90 mL diluted sperm (total of 6.3×10^9 spermatozoa). The females in two D5/6 groups (control and IAA) were inseminated with 2×180 mL diluted sperm (total of 12.6×10^9 sper-

matozoa) or with 2×90 mL diluted sperm (total of 6.3×10^9 spermatozoa) in the third D5/6 group (IAA).

Data are expressed as means and standard deviations for each treatment. Differences between means have been evaluated by multifactorial analysis in the in vitro experiment (SAS program type II-SS). In the in vivo experiment, differences between means have been analyzed either by Student's *t*-test (litter sizes) or by chi-square test including Yates' correction (percentages of birth) (Pearce, 1965). The motility data were analyzed using the following function in which simple, double and triple interactions were taken into consideration:

$$Y_{ijklm} = \mu + \alpha_i + \beta_{ij} + \gamma_k + \sigma_l + \xi_m + (\alpha\gamma)_{ik} + (\alpha\delta)_{il} + (\alpha\xi)_{im} + (\gamma\delta)_{kl} + (\gamma\xi)_{km} + (\delta\xi)_{lm} + (\alpha\gamma\delta)_{ikl} + (\alpha\gamma\xi)_{ikm} + (\alpha\delta\xi)_{ilm} + (\beta\xi\delta)_{ijk} + (\beta\delta\xi)_{ijkm} + (\gamma\delta\xi)_{klm} + E_{ijklm}$$

where *Y* = motility, μ = overall mean, α_i = simple race effect, β_{ij} = within race ejaculate effect, γ_k = diluent effect, σ_l = day effect, ξ_m = incubation effect and *E* = residue. The day variable was treated as a repetition factor.

RESULTS

Figure 1 shows that the presence of IAA at 10 or 100 ng/mL was without any significant effect over a 13 day period at 15 °C on the percentage of motile spermatozoa (fig 1A) and their motility rate (fig 1B) after 5 min or 3 h of incubation at 39 °C. Although not significant, a slight effect of IAA at a concentration of 10 ng/mL can be observed on both parameters between D7 and D13. In the scope of storing sperm over a 5–6 day period for AI, it is noteworthy that there is almost no decrease in motility parameters between D0 and D5/6 whether IAA is present or not in BTS extender.

Thirty-four ejaculates, each from different boars, were used to study acrosome morphology after 13 days at 15 °C. Table 1 shows that the presence of IAA (10 ng/mL) in BTS during the 13 day storage leads to a significant increase in the proportion of living spermatozoa with intact acrosome (66 vs 54%; $P < 0.05$).

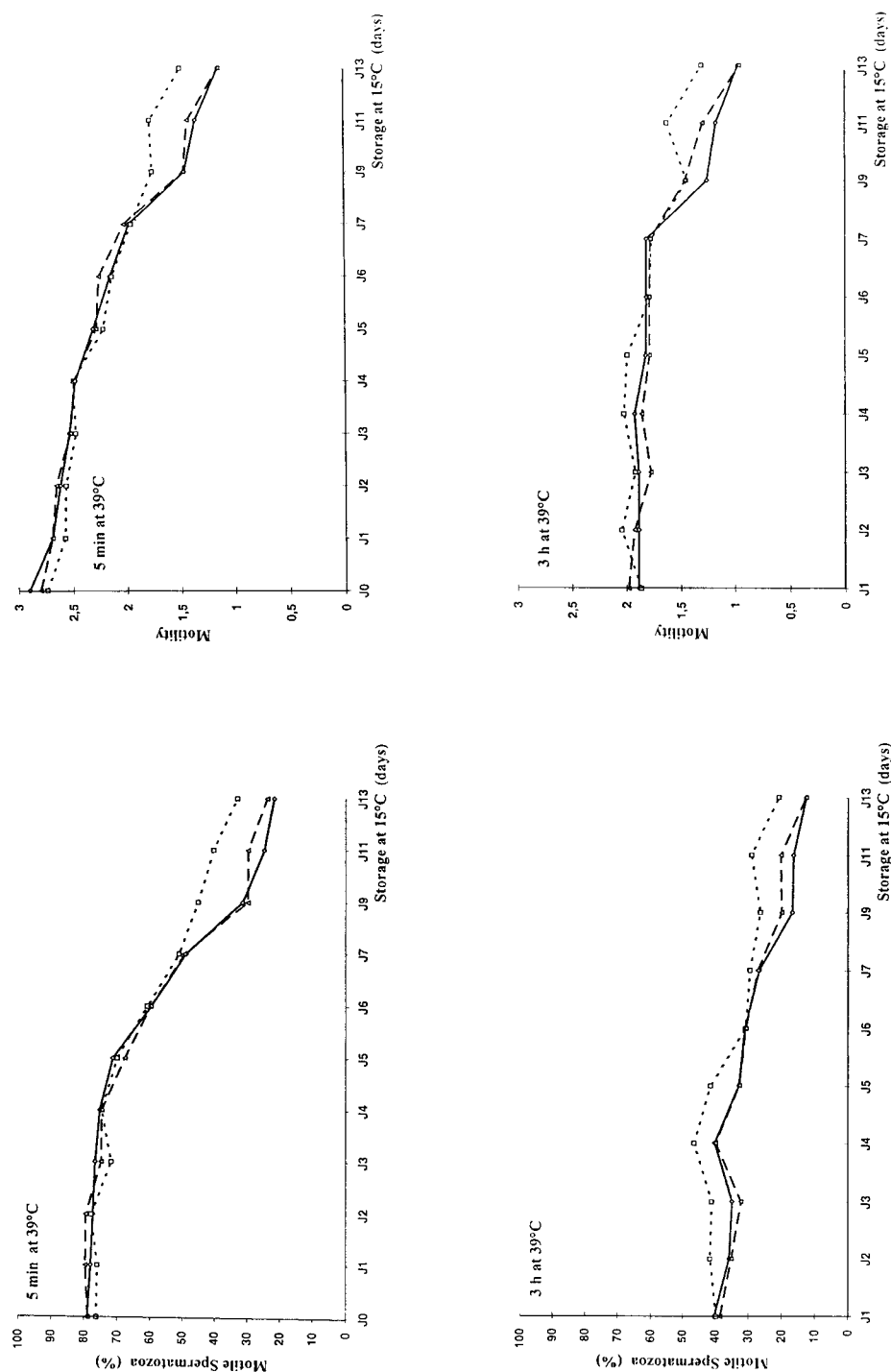


Fig 1. Effect of indole-3-acetic acid (IAA) over a 13 day period on the percentage of motile spermatozoa (left panels) and on motility (right panels) after 5 min (top) or 3 h (bottom) of incubation at 39 °C. Results represent the means for 49 ejaculates. Standard deviations are not shown for the sake of clarity since there is no significative difference between treatments at any day. (◇ — ◇) control (IAA = 0); (□ ---- □) IAA 10 ng/mL; (Δ Δ) IAA 100 ng/mL.

Table I. Acrosome morphology of sperm stored for 13 days in BTS extender in the absence or presence of IAA (10 ng/mL).

<i>Spermatozoa</i>	<i>Acrosomes</i>	<i>BTS alone</i>	<i>IAA in BTS</i>
Living	Intact (%)	54 ± 9	66 ± 9
	Altered (%)	7 ± 5	4 ± 2
	Total (%)	39 ± 11	30 ± 11
Dead			

The data are expressed in percent spermatozoa belonging to each class.

The results of the *in vivo* experiment are shown in table II. There was no significant difference ($P > 0.05$) in fertility and prolificacy between the two groups (A and B) in which the females were inseminated with 6.3×10^9 spermatozoa collected the same or previous day (D0/1), whether IAA is present

(B) or not (A). Similarly, no difference was observed between groups D (IAA in BTS) and E (BTS alone) in which the females were inseminated with 12.6×10^9 spermatozoa stored for 5–6 days at 15 °C. Unexpectedly, the fertility and prolificacy rates with BTS alone at D5/6 (group E) were not

Table II. Fertility and prolificacy rates of sows inseminated with sperm stored for up to 6 days in BTS extender in the absence or presence of IAA (10 ng/mL).

<i>Day *</i>	<i>Conditions</i>			<i>Results</i>		
	<i>Group (n)</i>	<i>IAA (ng/mL) in BTS</i>	<i>Spz (10⁹)</i>	<i>Fertility (% farrowing)</i>	<i>Prolificacy ** (litter size)</i>	
					<i>Total</i>	<i>Alive</i>
D0/1	A (128)	0	6.3	80.5	11.3 ± 2.9	10.7 ± 2.9
	B (128)	10	6.3	76.6	11.5 ± 3.6	11.1 ± 3.1
D5/6	C (140)	10	6.3	81.4	11.2 ± 3.5	10.6 ± 3.2
	D (132)	10	12.6	80.3	11.4 ± 3.2	10.8 ± 2.9
	E (106)	0	12.6	77.4	11.3 ± 3.1	10.7 ± 2.8

* Storage duration: D0/1 = 0–1 day, D5/6 = 5–6 days; ** mean ± SD. Spz: spermatozoa.

significantly lower than at D0/1 (group A). Nevertheless, the number of sperm used at D5/6 was twice as much as at D0/1. When the sows were inseminated with sperm stored for 5–6 days in the presence of 10 ng IAA per mL of BTS (group C), there was no decrease in fertility and prolificacy even though only 6.3×10^9 spermatozoa were used. Because poor results were expected with 6.3×10^9 spermatozoa stored for 5–6 days in BTS alone (Paquignon, 1984), such a control group could not be included in the present study.

DISCUSSION

Numerous studies have been carried out to increase the survival duration of boar sperm in liquid form (Bariteau et al, 1977; Slaweta et al, 1981; Paquignon et al, 1987, 1988; Cheng, 1988; Galli and Bosisio, 1988; Rillo et al, 1991; Sone et al, 1992; Galli et al, 1993). The BTS extender is the most largely used extender for boar sperm survival at 15 °C as it allows economically acceptable fertility and prolificacy rates for time periods up to 3 days (Bariteau et al, 1977; Strzezek et al, 1979; Paquignon et al, 1982b, 1987; Galli and Bosisio, 1988; Revell and Glosop, 1989; Sone et al, 1992). Since previous work had shown a beneficial effect of coconut water on sperm survival in different species (Toniolli, 1989, 1990, 1991), we tested here the effects of low concentrations of IAA which was identified as the favourable component from coconut water previously called JYP (Ferreira-Nunès, Combarous and Leclercq, unpublished data). The effects of IAA during conservation of sperm were studied on motility and morphological parameters *in vitro* and on fertility and prolificacy parameters *in vivo*.

Our observation that the percentage of living spermatozoa with undamaged acrosomes after 13 day storage at 15 °C was significantly higher in the presence of IAA

(10 ng/mL) compared to control strongly supports an effect of IAA on sperm over this period of time. Nevertheless, there was no evidence that any difference was already present over the initial 5 day period.

Many previous reports have indicated that there is a decrease in boar sperm fertility and prolificacy parameters between 3 and 5 days of conservation in liquid form in various extenders such as BTS, Modena, Kiev, BL1, Androhep and Zorlesco (Johnson et al, 1982, 1988; Weitze, 1990; Machàty et al, 1992; Waberski et al, 1992). Most extenders can be used up to 2 days without any loss in sperm fertilizing capacity (Bariteau et al, 1977; Paquignon et al, 1980) but they can also be used for up to 4 days provided that inseminations are performed with double doses of sperm. Until now, the best results were reported by Machàty et al (1992) who inseminated Large White sows and gilts with 5×10^9 sperm stored for 4 days in BTS extender. They obtained a fertility rate of 74.5% and a prolificacy rate of 9.5 live piglets per litter. For sows alone, the figures were 78.1% for fertility and 10.5 for prolificacy, respectively.

The females inseminated with only 6.3×10^9 spermatozoa stored for 5–6 days in BTS containing 10 ng/mL IAA (group C) exhibited fertility and prolificacy rates identical to those inseminated with the same number of spermatozoa stored for only 0–1 day in the absence or presence of IAA (groups A and B) as well as to those inseminated with a double number of spermatozoa (12.6×10^9) stored for 5–6 days without or with IAA (groups D and E). Thus, our data clearly demonstrate that it is feasible to conserve boar sperm in liquid form for 5–6 days with good results in AI without increasing the number of spermatozoa when IAA is present. This provides additional flexibility and efficiency in breeding technology at AI centers and pig farms since it allows better availability of sperm and its transport to more distant locations.

When we started this experiment, the literature was very pessimistic about the ability of BTS extender alone to fully sustain sperm fertilizing ability for 5–6 days. For this reason, we could not include a control group inseminated with only 6.3×10^9 sperm stored for 5–6 days at 15 °C in BTS alone. Consequently, in the present study, we have not positively demonstrated that IAA is responsible for the good sperm fertilizing capacity observed in group C with only 6.3×10^9 sperm after 5–6 days at 15 °C since there was no corresponding control group in BTS alone. Nevertheless, data from the literature indicate that the BTS extender provides good results for only up to 4 days using 6×10^9 sperm for IA (Johnson et al, 1988; Machàty et al, 1992). A large-scale experiment has been set up to tackle this issue and should provide us with a clear evaluation of IAA effect on boar sperm preservation in liquid form during the year 1996. In parallel, recent data (Blesbois et al, 1996) have also shown a favourable effect of IAA on sperm preservation in the fowl.

It has been known for a long time that the indole-3-acetic acid (IAA) molecule is the main plant hormone named auxin. However, it is only recently that the molecular mechanisms of auxin action in plants have begun to be understood (Barbier-Brygoo, 1995). Auxin binds to a soluble auxin-binding protein and the so-formed complex consecutively associates with a trans-membrane receptor which is thought to trigger, directly or indirectly, the various cell responses.

Interaction between the animal epidermal growth factor (EGF) and IAA has been shown to occur and the EGF-IAA complex is more potent than IAA alone on coleoptile growth in various plants (Moon et al, 1994). The reverse situation in which IAA might bind to EGF or another molecule and would potentiate its action in animals thus appears plausible.

It has recently been shown that IAA can accelerate lipid peroxidation catalyzed by haem peroxidase and hydrogen peroxide (Candeias et al, 1995) and such a reaction is expected to have adverse effects on sperm fertilizing ability (Windsor et al, 1993). However, at pH 7.5 this reaction is only observed in the presence of horseradish peroxidase (10 µg/mL) with concentrations of IAA and H_2O_2 of 2.5×10^{-4} M and 1×10^{-3} M, respectively. In our experimental conditions (no added peroxidase, no H_2O_2 and 5.7×10^{-9} M IAA), it is very unlikely that IAA promotes sperm lipid peroxidation.

IAA has also been shown to bind to a glutathion-S-transferase (GST) and to reduce its activity towards its substrates in a noncompetitive manner in plants (Bilang and Sturm, 1995). GST activity has been demonstrated at several levels in the male genital tract: epididymal cells (Robaire and Viger, 1995), in pachytene spermatocytes, spermatids and cytoplasmic droplets (Yoganathan et al, 1989; Papp et al, 1994; Veri et al, 1994) and the μ -class of GST has been detected in mouse and rat spermatozoa (Aravinda et al, 1995; Fulcher et al, 1995). In the rat, GST was detected immunocytochemically on sperm acrosome (Aravinda et al, 1995). Therefore, a possible direct effect of IAA on sperm GST should be taken into consideration to explain its favourable effect on acrosome protection.

In addition, IAA has been demonstrated to accelerate the protein disulfide-isomerase (PDI) activity present in soybean plasma membranes (Morre et al, 1995). PDI activity has also been found at the external surface of platelet plasma membranes (Essex et al, 1995) and hepatocytes (Terada et al, 1995) in mammals and it is known to permit correct refolding of proteins through chaperone-like activity (Song and Wang, 1995). As it is also present in the acrosome of spermatozoa (Ohtani et al, 1993), it might participate in the modifications of acrosome proteins during capacitation. As the main effect of IAA in

boar semen was observed on the integrity of acrosome morphology, it is tempting to hypothesize that it acts by stimulating acrosome PDI.

Is IAA the sole molecule responsible for the protective effect of coconut water in boar sperm preservation? The composition of the liquid endosperm of coconut is very complex and includes various carbohydrates, amino acids and ions, etc. The IAA concentrations used in the present study (10–100 ng/mL) flank the known concentration of active auxin (40 ng/mL) in liquid endosperm of coconut at stages IV–V of development (Dua and Chandra, 1993). Therefore, although other molecules might be involved in the overall effect of coconut water on boar sperm survival, it seems that IAA is responsible for a part in it.

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