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## Effect of Relaxin on Fertility Parameters of Frozen–Thawed Buffalo (*Bubalus bubalis*) Sperm

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### Contents

The aim of this work was to evaluate the effect of relaxin on fertility parameters of buffalo frozen/thawed sperm. Sperm were incubated in the absence of capacitating agents (negative control), with a known capacitating agent such as heparin (positive control) and with 50 and 100 ng/ml relaxin for 2 and 4 h. Sperm viability, motility, capacitation and the effect of relaxin on the fertilizing ability after heterologous IVF were evaluated. Although viability was not affected, relaxin increased ( $p < 0.05$ ) sperm motility compared to the negative and positive controls both after 2 h ( $60.0 \pm 2.0$ ,  $60.0 \pm 3.1$ ,  $68.3 \pm 1.7$  and  $69.4 \pm 2.7$ , respectively, in negative control, positive control, 50 and 100 ng/ml relaxin) and 4 h ( $55.0 \pm 2.5$ ,  $53.3 \pm 3.0$ ,  $62.2 \pm 3.0$  and  $65.0 \pm 3.2$ , respectively, in negative control, positive control, 50 and 100 ng/ml relaxin) incubation. When sperm were incubated with both 100 ng/ml relaxin and heparin, a decrease ( $p < 0.01$ ) of pattern A, that is low capacitation level, was observed compared to the negative control both after 2 h (54.4, 34.3 and 36.4%, respectively, in negative control, positive control and 100 ng/ml relaxin) and 4 h (51.9, 35.0 and 34.3%, respectively, in negative control, positive control and 100 ng/ml relaxin). Moreover, an increase ( $p < 0.01$ ) of pattern EA, that is high capacitation level, was recorded with 100 ng/ml relaxin and heparin compared to the negative control both after 2 h (44.1, 59.3 and 57.7%, respectively, in negative control, positive control and 100 ng/ml relaxin) and after 4 h (43.0, 54.4 and 56.0%, respectively, in negative control, positive control and 100 ng/ml relaxin). Finally, relaxin increased ( $p < 0.01$ ) cleavage rate compared to the negative control ( $57.1 \pm 4.4$ ,  $72.5 \pm 6.0$ ,  $71.4 \pm 5.5$  and  $73.6 \pm 2.9$ , respectively, in negative control, positive control, 50 and 100 ng/ml relaxin). In conclusion, relaxin has a beneficial effect on motility, capacitation and fertilizing ability of frozen–thawed buffalo sperm.

### Introduction

The global interest in buffalo breeding has been steadily increasing, due to the competitive role of this species as a dairy animal in developing countries. In the current scenario, the success of buffalo breeding depends on the genetic improvement and hence on the application of reproductive biotechnologies. Due to the limitations of multiple ovulation in this species (Misra 1997; Zicarelli 1997), the *in vitro* embryo production (IVEP) technology is the best tool to improve maternal contribution to genetic progress. Although the IVEP efficiency has greatly improved over the years, cleavage rates are still

low compared to other species (Neglia et al. 2003; Boccia et al. 2013). Many factors are known to affect IVF efficiency such as sperm quality, bull, environment, time of insemination and appropriate capacitation of frozen–thawed sperm.

In standard IVF procedure, frozen–thawed sperm are commonly used. However, it is known that freezing determines several sperm damages including loss of enzymes, proteins and antioxidants (Bilodeau et al. 2000; Marti et al. 2008). In addition, sperm washings that are routinely carried out before IVF also lead to leakage of essential components of seminal plasma that may account for reduced fertilization ability (Lessing et al. 1986). Relaxin, a regulatory peptide of the insulin superfamily, is an important component of seminal plasma that is negatively affected by cryopreservation (Lessing et al. 1985). Special attention has been recently given to the role of relaxin on male fertility. Relaxin has been identified in boar testes (Kohsaka et al. 2009) and in seminal plasma of several species (Ferlin et al. 2012). Kohsaka et al. 2003 reported that among domestic animals, bovine seminal plasma contains the highest concentration ( $41.9 \pm 25.2$  ng/ml) of relaxin followed by humans ( $17.4 \pm 2.9$  ng/ml), rams ( $13.1 \pm 1.7$  ng/ml), boars ( $2.4 \pm 0.5$  ng/ml) and goats ( $1.0 \pm 0.3$  ng/ml). Relaxin is produced by the male accessory glands, secreted into seminal plasma and saturated with sperm just after ejaculation (Sasaki et al. 2001). It was demonstrated that relaxin improves motility of human (Ferlin et al. 2012), porcine (Miah et al. 2008) and bovine sperm (Miah et al. 2007). It has also been reported that relaxin induces capacitation and acrosome reaction (AR) in fresh and frozen–thawed porcine or bovine sperm (Miah et al. 2006a, 2008, 2011). Furthermore, relaxin improves the *in vitro* fertilization rate of porcine oocytes (Han et al. 2006) and the penetration ability of human sperm (Park et al. 1988).

To date, there is no report on the effects of relaxin on fertility parameters of buffalo frozen sperm. Therefore, the aim of this work was to evaluate the effect of relaxin on motility, viability and capacitation of buffalo frozen/thawed sperm (Experiment 1). A further objective was to assess the influence of relaxin on the fertilizing ability of buffalo frozen–thawed sperm, after heterologous IVF (Experiment 2).

## Materials and Methods

Unless otherwise stated, reagents were purchased from Sigma-Aldrich (Milan, Italy).

### Experimental design

The concentrations of relaxin used in this trial were chosen after a preliminary dose–response trial that showed no differences in sperm viability, motility and capacitation with concentrations lower than 50 ng/ml.

In Experiment 1, frozen sperm from three Mediterranean buffalo bulls (6 straws corresponding to different ejaculates per each bull) were thawed at 37°C for 40 s and separated by Percoll. At the end of separation, the sperm pellet of each straw was equally divided into 4 aliquots that were resuspended in Tyrode albumin lactate pyruvate (TALP) medium in the absence of capacitating agents (negative control), with standard concentration (10 µg/ml) of heparin, a known capacitating agent (positive control) and two concentrations of relaxin (50 and 100 ng/ml) at  $20 \times 10^6$ /ml concentration. Incubation was then carried out for 2 h and 4 h in a controlled gas atmosphere of 5% CO<sub>2</sub> in humidified air. The choice of the incubation times was based on previous observations indicating that *in vitro* capacitation of frozen–thawed buffalo sperm occurs between 30 min (Kitiyanant et al. 2002) and 4 h (Boccia et al. 2013; Elkhawagah et al. 2014). In particular, in our system, an increased capacitation is recorded either after 2 h or 4 h according to the capacitating agents employed (Boccia et al. 2013; Elkhawagah et al. 2014). After thawing and following each incubation period, sperm viability, motility and capacitation (the latter evaluated both by dual staining and the immune localization of tyrosine-phosphorylated protein) were assessed as described in sections “Evaluation of sperm motility, Trypan-Blue Giemsa staining to evaluate viability and capacitation and Localization of tyrosine phosphorylated protein”.

In Experiment 2, the effect of relaxin on the fertilizing ability was assessed following heterologous IVF. Therefore, frozen–thawed sperm were used for IVF of *in vitro* matured abattoir-derived bovine oocytes (n = 917, over 6 replicates). In particular, the oocytes were fertilized in the absence of capacitating agents (negative control), with standard concentration (10 µg/ml) of heparin (positive control) and two concentrations of relaxin (50 and 100 ng/ml).

### Evaluation of sperm motility

Sperm motility was examined by phase contrast microscopy (Nikon Diaphot 300 inverted microscope equipped with phase contrast and fluorescence filters) at 40x magnification on a clean and dry glass slide maintained on thermoregulated stage at 37°C. Sperm motility of each semen sample was estimated by routine method used in the livestock industry where

the frozen semen is supplied for artificial insemination (Vale 1997).

### Trypan blue–Giemsa staining to evaluate viability and capacitation

Capacitation was assessed indirectly by estimating the percentage of acrosome-reacted (AR) sperm after 15 min of incubation with lysophosphatidylcholine (LPC), a fusogenic agent known to induce acrosome reaction only in capacitated sperm (Parrish et al. 1988). To evaluate the sperm viability and the acrosome reaction, the sperm were fixed and stained with Trypan blue–Giemsa (Boccia et al. 2013). Sperm were counted under differential interference light microscopy (Nikon Diaphot 300), and the number of acrosome-intact live (AILS), acrosome-intact dead (AIDS), acrosome-reacted live (ARLS) and acrosome-reacted dead (ARDS) sperm was recorded. For each replicate, at least 100 sperm were evaluated per slide. To compare viability and capacitation among groups, the percentage of total live sperm (TLS) was calculated out of the total sperm counted and that of ARLS was calculated out of the TLS.

### Localization of tyrosine-phosphorylated protein

Localization of phosphotyrosine-containing protein was detected using an indirect immunofluorescence assay as described by Tardif et al. (2001). Aliquots of 20 million sperm were fixed in 2% (v/v) formaldehyde for 1 h at 4°C and centrifuged at 300 g for 10 min. The sperm pellets were incubated overnight at 4°C in modified phosphate-buffered saline (mPBS: 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 137 mM NaCl, 5.5 mM glucose and 1.0 mM pyruvate, pH 7.4) containing 2% (w/v) BSA. Twenty µl of sperm suspension was smeared onto a slide, air-dried and permeabilized with absolute ethanol for 5 min. The permeabilized sperm were incubated with primary antibody (Sigma, Cat no: T1325; diluted 1 : 10 in tris-buffered saline – TBS: 20 mM Tris-HCl, 0.8% NaCl, pH 7.6) for 1 h at room temperature. Excess antibodies were removed by plunging 4–5 times in TBS. The slides were incubated with secondary antibody, FITC-conjugated goat anti-rabbit IgG (Sigma, Cat no: F0382; diluted 1 : 10 in TBS) for 1 h in the dark at room temperature. Excess antibodies were removed by plunging the slides 4–5 times in TBS, and the slides were mounted with 90% (v/v) glycerol. Green fluorescence was observed by epifluorescent microscope (Nikon Diaphot 300) using FITC filter. A total of 100 sperm were screened per slide and classified according to one of the three fluorescence patterns described by Cormier and Bailey (2003):

Pattern A, that is uniform fluorescence over the entire acrosome (low capacitation level); Pattern E, that is a short line or triangle of fluorescence in the equatorial segment (medium capacitation level) and Pattern EA,

that is fluorescence at both equatorial and anterior acrosomal regions (high capacitation level; Fig. 2).

### *In vitro* fertilizing ability

Abattoir-derived bovine oocytes were matured and fertilized *in vitro* according to standard procedure (Rubessa et al. 2011). Briefly, cumulus–oocyte complexes (COCs) with uniform cytoplasm and multilayered cumulus cells were matured in TCM 199 supplemented with 15% bovine serum (BS), 0.5 µg/ml FSH, 5 µg/ml LH, 0.8 mM L-glutamine and 50 µg/ml gentamycin for 22 h at 39°C and 5% CO<sub>2</sub> in air. *In vitro* matured COCs were fertilized in TALP buffered with 25 mM sodium bicarbonate and supplemented with 0.2 mM penicillamine and 0.1 mM hypotaurine (IVF medium), in the absence of capacitating agents (negative control), with 10 µg/ml heparin (positive control) and with two concentration of relaxin 50 and 100 ng/ml. Frozen–thawed sperm were selected by centrifugation (25 min at 300 g) on a Percoll discontinuous gradient (45 and 80%). The pellet was reconstituted into 2 ml of IVF medium and centrifuged twice at 160 and then at 108 g for 10 min each. The pellet was diluted with IVF medium and added in the fertilization wells at the concentration of  $2 \times 10^6$  sperm/ml. Gametes were co-incubated for 20 h at 39°C, in 5% CO<sub>2</sub> in air, after which presumptive zygotes were vortexed for 2 min to remove cumulus cells in Hepes-TCM with 5% BS and incubated in a humidified mixture of 5% CO<sub>2</sub>, 7% O<sub>2</sub> and 88% N<sub>2</sub> in air at a temperature of 39°C. After 24 h of culture, the cleavage rate was assessed and confirmed by fixation of zygotes with absolute ethanol overnight and staining with DAPI for nuclei examination under epifluorescence microscope (Nikon Diaphot 300) after zona removal by digestion with 2 mg/ml protease (P5147). The penetration, normal fertilization and polyspermy rates were assessed.

### Statistical analysis

In Experiment 1, the differences among groups in the percentages of sperm showing different tyrosine-phosphorylated protein patterns, as well as in the percentages of TLS, and ARLS cells were analysed by chi-squared test. Sperm motility was analysed by ANOVA, and the differences among groups were tested by Tukey's test, using SPSS 20.0 statistical software (2012). In Experiment 2, the percentages of total, normospermic and polyspermic penetration were also analysed by ANOVA, using Tukey's test to show differences among groups (SPSS 20.0; IBM Corp, Armonk, NY, USA, 2012).

## Results

### Effect of relaxin on sperm motility

Sperm motility at thawing was 80% and decreased ( $p < 0.05$ ) after incubation. However, as shown in Fig. 1, relaxin (50 and 100 ng/ml) increased ( $p < 0.05$ )

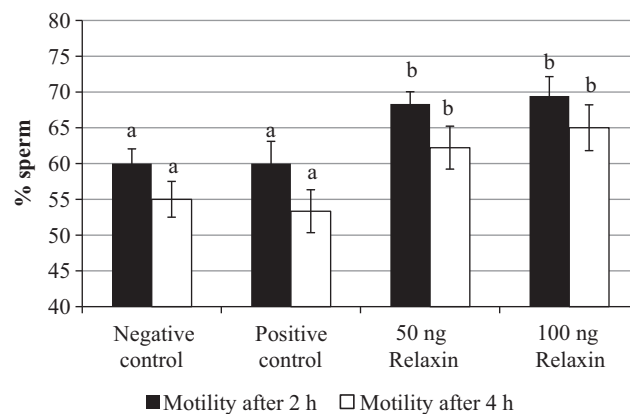


Fig. 1. Effect of relaxin on the sperm motility (Mean  $\pm$  SE). <sup>a,b</sup>Bars with different letters are significantly different;  $p < 0.05$

sperm motility compared to the negative and positive controls both after 2- and 4-h incubation. On the contrary, no differences were recorded between positive and negative controls.

### Effect of relaxin on viability and capacitation assessed by Trypan blue–Giemsa staining

Immediately after thawing (time 0), the percentage of TLS was 93.0% and the percentage of ARLS was 13.6%. The percentages of AIDS and ARDS were, respectively, 4.5 and 2.5%. The percentages of TLS and ARLS under different incubation conditions are reported in Table 1. After Percoll selection, sperm viability increased up to 98% and remained very high after both 2- and 4-h incubation, with no differences among groups (Table 1). The percentages of AIDS and ARDS were negligible and similar among groups (on average 0.6 and 0.8%, respectively).

The percentage of ARLS was higher ( $p < 0.05$ ) in the positive control than in the negative control at both incubation times. When sperm were incubated with either 50 or 100 ng/ml relaxin, an increase ( $p < 0.01$ ) of this parameter was recorded at both 2- and 4-h incubation compared to the negative control, reaching values similar to those recorded in the positive control. The percentage of ARLS increased ( $p < 0.01$ ) at the higher incubation time in all groups.

### Effect of relaxin on the localization of tyrosine-phosphorylated proteins

At thawing (time 0), the majority of sperm exhibited patterns A (64.8%) and EA (31.7%), with a negligible proportion displaying pattern E (0.2%) and a small proportion exhibiting no fluorescence (3.3%). Within 2-h incubation, no differences were observed among groups in non-fluorescent sperm (on average 1.3%) and in pattern E sperm (on average 3.7%). After 4-h incubation, the percentages of non-fluorescent sperm (on average 2.8%) and of pattern E sperm (on average 5.6%) were also similar among groups.

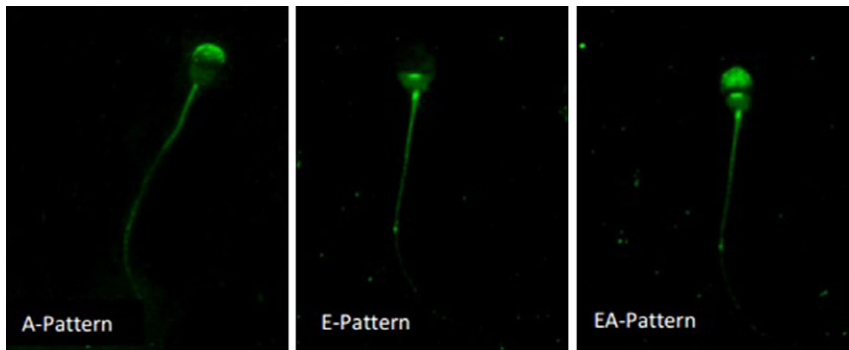


Fig. 2. Different categories of buffalo frozen/thawed sperm immunolocalized tyrosine-phosphorylated protein. A-Pattern: uniform fluorescence over the entire acrosome; E-Pattern: a short line or triangle of fluorescence in the equatorial segment and EA-Pattern: fluorescence at both equatorial and anterior acrosomal regions

Overall, the more relevant changes in tyrosine-phosphorylated protein patterns regarded pattern A and EA (Fig. 2). The incubation affected the tyrosine-phosphorylated protein patterns: after 2-h incubation, a decrease ( $p < 0.01$ ) in pattern A accompanied by an increase ( $p < 0.01$ ) of pattern EA was recorded compared to time 0, with no further changes observed after 4 h. Furthermore, when sperm were incubated both for 2 and 4 h with either 100 ng/ml relaxin or heparin (positive control), a decrease ( $p < 0.01$ ) of pattern A together with an increase ( $p < 0.01$ ) of pattern EA was found compared to the negative control (Table 2).

Furthermore, the concentration of 100 ng/ml of relaxin gave similar results to the positive control (Table 2). The lower relaxin concentration tested (50 ng/ml) also improved capacitation, but it was less effective than the other treatments.

### Effect of relaxin on sperm fertilizing ability

As reported in Table 3, both concentrations of relaxin increased ( $p < 0.01$ ) cleavage, total penetration and normospermic penetration rates compared to the negative control. The polyspermic rate was similar in all groups.

### Discussion

The results of the present study demonstrated that relaxin improves motility, capacitation and fertilizing ability of buffalo frozen–thawed sperm. Relaxin is a peptide belonging to the family of insulin-like hormones that acts as a pleiotropic endocrine and paracrine factor (Lee et al. 2005). As relaxin is one of the seminal plasma components negatively affected by cryopreservation (Lessing et al. 1985), we hypothesized that it may be added into the IVF medium to maintain the normal physiological functions of cryopreserved sperm. Both the concentrations tested had similar effects on the majority of the fertility parameters, but the tyrosine-phosphorylated protein patterns improved with the higher concentration, that is 100 ng/ml.

In the present study, relaxin did not affect the viability of frozen/thawed buffalo sperm at both incubation times. Yet, it is worth pointing out that the percentage

Table 1. Sperm viability (TLS) and acrosome-reacted sperm (ARLS) at 2 and 4 h post-incubation in different groups

Groups	n.	TLS		ARLS	
		Times post-incubation (h)			
		2 n (%)	4 n (%)	2 n (%)	4 n (%)
Negative control	1800	1775 (98.6)	1779 (98.8)	692 (38.4) <sup>AaX</sup>	822 (45.7) <sup>AaY</sup>
Positive control	1800	1769 (98.3)	1775 (98.6)	767 (42.6) <sup>bX</sup>	889 (49.4) <sup>bY</sup>
50 ng relaxin	1800	1770 (98.3)	1781 (98.9)	779 (44.4) <sup>BbX</sup>	897 (49.8) <sup>bY</sup>
100 ng relaxin	1800	1772 (98.4)	1774 (98.6)	797 (44.3) <sup>BbX</sup>	929 (51.6) <sup>BbY</sup>

<sup>A,B</sup>Values with different superscripts within columns are different;  $p < 0.01$ .

<sup>a,b</sup>Values with different superscripts within columns are different;  $p < 0.05$ .

<sup>X,Y</sup>Values with different superscripts within rows are different;  $p < 0.01$ .

of viable sperm was very high in all groups also after 4-h incubation, indicating a good quality of the sperm used in this trial. These results are in agreement with those of previous studies on fresh and frozen/thawed bovine sperm (Miah et al. 2007), as well as on boar fresh sperm (Miah et al. 2006b).

However, relaxin at the concentrations of 50 and 100 ng/ml significantly improved the motility of frozen/thawed buffalo sperm both after 2- and 4-h incubation. The positive effect of relaxin on motility has been recorded in different mammalian species including human (Park et al. 1988; Ferlin et al. 2012), porcine (Kohsaka et al. 2001; Han et al. 2006; Miah et al. 2006a, 2008) and bovine (Miah et al. 2007). It was suggested that seminal relaxin has a physiological influence on sperm motility and fertility via specific cell-surface receptors for relaxin on sperm (Kohsaka et al. 2003).

To acquire fertilizing ability, sperm need to undergo the process of capacitation that *in vivo* takes place in the female genital tract, while *in vitro* is induced by adding capacitating agents in the medium. The present study demonstrated an effect of relaxin on capacitation of buffalo frozen/thawed sperm, estimated by both the percentage of ARLS following LPC treatment and the

Table 2. The percentages of buffalo sperm with tyrosine-phosphorylated protein patterns A and EA, under different incubation conditions

Groups	n.	Pattern A		Pattern EA	
		Times post-incubation (h)			
		2 n (%)	4 n (%)	2 n (%)	4 n (%)
Negative control	1800	980 (54.4) <sup>A</sup>	934 (51.9) <sup>A</sup>	794 (44.1) <sup>A</sup>	774 (43.0) <sup>A</sup>
Positive control	1800	618 (34.3) <sup>B</sup>	630 (35.0) <sup>B</sup>	1068 (59.3) <sup>BX</sup>	980 (54.4) <sup>BY</sup>
50 ng relaxin	1800	864 (48.0) <sup>CX</sup>	774 (43.0) <sup>CY</sup>	822 (45.7) <sup>Ax</sup>	884 (49.1) <sup>Cy</sup>
100 ng relaxin	1800	656 (36.4) <sup>B</sup>	618 (34.3) <sup>B</sup>	1038 (57.7) <sup>B</sup>	1008 (56.0) <sup>B</sup>

<sup>A,B</sup>Values with different superscripts within columns are different;  $p < 0.01$ .

<sup>X, Y</sup>Values with different superscripts within rows are different;  $p < 0.01$ .

<sup>x, y</sup>Values with different superscripts within rows are different;  $p < 0.05$ .

Table 3. Percentages of cleavage, total penetration, normospermic and polyspermic penetration in different groups. Values are expressed as means  $\pm$  SEM

Groups	COC N.	Cleavage %	Total penetration %	Normospermy %	Polyspermy %
Negative control	227	57.1 $\pm$ 4.4 <sup>A</sup>	71.9 $\pm$ 3.9 <sup>A</sup>	66.5 $\pm$ 4.4 <sup>A</sup>	5.4 $\pm$ 2.1
Positive control	237	72.5 $\pm$ 6.0 <sup>B</sup>	82.0 $\pm$ 4.0 <sup>B</sup>	79.8 $\pm$ 4.1 <sup>B</sup>	2.2 $\pm$ 0.8
50 ng relaxin	218	71.4 $\pm$ 5.5 <sup>B</sup>	82.7 $\pm$ 5.6 <sup>B</sup>	80.5 $\pm$ 4.6 <sup>B</sup>	2.2 $\pm$ 1.0
100 ng relaxin	235	73.6 $\pm$ 2.9 <sup>B</sup>	84.8 $\pm$ 3.2 <sup>B</sup>	82.8 $\pm$ 2.8 <sup>B</sup>	2.4 $\pm$ 1.2

<sup>A,B</sup>Values with different superscripts within columns are different;  $p < 0.01$ .

localization of tyrosine-phosphorylated proteins. Relaxin at the two concentrations tested (50 and 100 ng/ml) significantly increased the percentage of LPC-induced acrosome-reacted sperm after both 2- and 4-h of incubation, similar to the positive control. A positive effect of relaxin on AR was also previously reported in fresh and frozen/thawed bovine (Miah et al. 2007), as well as in fresh and cryopreserved boar sperm (Miah et al. 2006a,b). A significant enhancement of ARLS at increasing incubation times was recorded in all experimental groups. This may be attributed to the spontaneous increase in the capacitation and acrosome reaction at longer incubation times, as previously demonstrated in cattle (Cormier and Bailey 2003), boar (Choi et al. 2008) and buffalo (Elkhawagah et al. 2014).

Sperm capacitation has been correlated with an increase in protein tyrosine phosphorylation in various mammalian species including bovine (Galantino-Homer et al. 1997) and buffalo (Kadirvel et al. 2011). The protein tyrosine phosphorylation assay showed that cryocapacitation occurs immediately after thawing, as indicated by more than 30% sperm displaying tyrosine-phosphorylated protein pattern EA, corresponding to a high capacitation level. This may be due to the cryopreservation-induced modifications of sperm plasma membrane as previously reported (Elkhawagah et al. 2014). Interestingly, the results of the present study demonstrated that relaxin significantly decreases the percentage of sperm exhibiting pattern A, while increasing that of sperm showing pattern EA after 2 and 4 h of incubation, similar to a known capacitating

agent, such as heparin. This pattern indicates an advancement of the capacitation process as previously reported (Gualtieri et al. 2005). These results are in agreement with those reported by Miah et al. (2011) who found an increase in the protein tyrosine phosphorylation of specific proteins correlated to capacitation in the presence of relaxin in fresh and frozen/thawed bovine sperm.

Although the molecular mechanisms of capacitation are not completely elucidated, it is known that cholesterol efflux is a key early triggering event, leading to an increase in membrane fluidity and hence of HCO<sub>3</sub><sup>-</sup> and Ca<sup>2+</sup> influx. This in turn increases intracellular cAMP and protein kinase-A (PKA) activity, resulting in an upregulation of protein tyrosine phosphorylation (Visconti et al. 2002). It has been reported that relaxin increases the cholesterol efflux in both fresh and frozen/thawed bovine sperm (Miah et al. 2011), as well as in porcine sperm (Miah et al. 2008). It was speculated that relaxin binds with the sperm receptor and leads to a change in membrane architecture that gives rise to the cholesterol efflux (Miah et al. 2011). It was also demonstrated that relaxin increases Ca<sup>2+</sup> influx, likely relaxing the sperm membrane, and cAMP in bovine sperm (Miah et al. 2011). The increase of the intracellular concentrations of cAMP and Ca<sup>2+</sup> is the most important cell signalling event which promotes hyper-activated motility and AR in mammalian sperm (Visconti et al. 1995; Baldi et al. 2000), through increasing protein tyrosine phosphorylation, known to be regulated by a cAMP-dependent pathway (Visconti et al.

1995; Kalab et al. 1998; Galantino-Homer et al. 2004). Furthermore, *in vitro* studies showed a role for relaxin in promoting nitric oxide (NO) biosynthesis by increasing the expression of nitric oxide (NO) synthase (Bani et al. 1998; Failli et al. 2001). It is known that physiological levels of nitric oxide are important for various sperm functions including capacitation (Choudhary et al. 2010; Kothari et al. 2010). An increased capacitation rate was previously recorded when buffalo sperm were incubated with sodium nitroprusside, a well-known generator of nitric oxide *in vitro* (Boccia et al. 2007). Therefore, it is not possible to rule out that the beneficial effect of relaxin on capacitation is due to its role of modulating reactive oxygen species.

The importance of these results is confirmed by the findings on fertilizing ability following heterologous IVF. It is well known that sperm acquire fertilizing ability during capacitation, and hence, the assessment of oocyte penetration, as well as cleavage rates, is of the utmost importance. Indeed, when IVF was carried out in the presence of relaxin, the overall penetration rate and the normal fertilization and cleavage rates were significantly improved compared to the non-capacitating medium, at the same extent of heparin, here used as a positive control. In addition, no effect on polyspermy was observed. In swine, relaxin was demonstrated to increase normal fertilization rate, while decreasing polyspermy (Han et al. 2006), and to improve cleavage and blastocyst rates (Kim et al. 2010; Feungang et al. 2011). A positive effect of relaxin on penetrating ability of human sperm has also been found (Park et al. 1988). The higher rates of penetration, cleavage and normal fertilization obtained after heterologous IVF in the present study confirm that a capacitating agent is indeed required to improve *in vitro* capacitation in buffalo

frozen/thawed sperm, despite the high level of cryopreservation-induced capacitation, to optimize IVF efficiency. However, as relaxin was added to the IVF medium, it is not possible to rule out that its beneficial effects on penetration and cleavage rates are also due to a positive action on the oocytes.

In conclusion, treatment of buffalo frozen-thawed sperm with relaxin results in increased motility, capacitation and acrosome reaction. Furthermore, supplementation of IVF medium with relaxin improves the *in vitro* fertilizing ability of buffalo frozen-thawed sperm.

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### Conflict of interest

The authors declare no conflict of interests. None of the authors have financial and/or personal relationships with people or organizations that could inappropriately influence their work. The corresponding author has full access to all the data in the study and as the final responsibility for the decision to submit the manuscript for publication.

### Author contributions

Dr Elkhawagah, Longobardi and Salzano were involved in the practical part of the study. Dr Gasparri and Neglia were involved in the analysis and interpretation of the data and Dr Campanile revised this article critically.

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