RESEARCH ARTICLE

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Effects of pentoxifylline, platelet activating factor and prostaglandin F 2-alpha on Giant Panda (*Ailuropoda melanoleuca*) post-thawed sperm *in vitro* fertilizing capability

Abstract   Is it possible that Giant Panda (*Ailuropoda melanoleuca*) post-thawed sperm fertilization is improved with pentoxifylline (PF), platelet activated factor (PAF) and prostaglandin F 2-alpha ($PGF_{2\alpha}$)? In our study Giant Panda post-thawed sperm was incubated in Ham's F-10 medium with different concentration of PF, PAF and $PGF_{2\alpha}$ under 37°C. The effects of PF, PAF and $PGF_{2\alpha}$ on Giant Panda post-thawed sperm fertility were evaluated through sperm motility, survival time, sperm membrane integrity, acrosome state and heterospecific egg penetration. The results were that PF, PAF and $PGF_{2\alpha}$ all can affect Giant Panda post-thawed sperm *in vitro* fertilizing capability. In the experiment: 1 mg·mL^{-1} PF was most suitable for improving Giant Panda post-thawed sperm *in vitro* fertilizing capability. In the 1 mg·mL^{-1} PF group, sperm survival time was (15.33 ± 4.73) h, the heterospecific egg penetration was 51.44% after incubating for 4 hours, the heterospecific egg penetration was 7.49% after incubating for 6 hours. The results of the 1 mg·mL^{-1} PF group were significantly higher than those of the control group ($P < 0.01$). The results were also higher than those of the other treatment groups; Treated Giant Panda post-thawed sperm with 50 ng·mL^{-1} PAF had a better effect than 100 ng·mL^{-1} PAF, but the sperm fertilizing capability was damaged when incubation time exceeded 2 hours; 50 ng·mL^{-1}

$PGF_{2\alpha}$ had no significant effect on Giant Panda post-thawed sperm, but when the $PGF_{2\alpha}$ treated concentration was increased, sperm *in vitro* fertilizing capability decreased because of the damaged motility and declined acrosomal reaction rate. The conclusions suggest that it is possible to improve post-thawed Giant Panda sperm fertility with 1 mg·mL^{-1} PF.

1   Introduction

The Giant Panda is an endangered species and unique to China. Due to its low fertility, the continued shrinking and isolation of its habitat and consequent decrease in its genetic diversity. The number of Giant Pandas in the wild has decreased rapidly over the past 50 years and the species is at the verge of extinction. A frozen sperm bank of Giant Panda has been set up at Chengdu Research Base of Giant Panda Breeding in China but conception rate was low by artificial insemination with cryopreservation sperm. It's very significant to improve post-thawed spermatozoa fertilizing capacity by adding exogenous substance. We could not find previous reports about pentoxifylline (PF), platelet activated factor (PAF) and Prostaglandin F 2-alpha ($PGF_{2\alpha}$) in improving sperm fertilizing capability of Giant Panda except in domestic animals. Some studies about PF, PAF and $PGF_{2\alpha}$ improving sperm function have been reported. Fertilization is a complicated biological process. Sperm which have motility can finish fertilization after acrosomal reaction and capacitation. To improve Giant Panda post-thawed sperm survival time, motility and post-thawed sperm fertilizing capability *in vitro*, we firstly investigated the effects of PF, PAF and $PGF_{2\alpha}$ on Giant Panda post-thawed sperm *in vitro* fertilizing capability.

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In the past years, PF, a methylxanthine derivate, was regarded as a sperm motility-promoting supplement. It's obvious that PF could improve sperm motility and prolong sperm lifespan. The effects were obvious, especially in post-thawed and low-motility sperm. More and more researchers would choose PF to improve sperm fertilizing capability (Yovich et al., 1990). PF could improve sperm fertilizing capability by promoting sperm straight movement (STR) (Yovich et al., 1990; Tesarik et al., 1992). Wu et al. (1997) studied the effects of PF on human ejaculate-sperm motility and acrosomal reaction. The results suggested that PF promoted sperm movement. Particularly, PF promoted a rectilinear motion in sperm movement. The discovery is practical in artificial insemination (AI) and sperm treatment *in vitro*. Yue et al. (2000) studied the effects of PF on human sperm fertilizing capability, that after treating human sperm with PF, zona pellucida-free oocytes from golden shrewmouse were penetrated by treated sperm. The results showed that PF evidently could improve sperm motility. Clinically, PF improved the infecund female pregnancy rate (Tesarik et al., 1992).

In 1987, Kumar found that PAF was synthesized in rabbit sperm. Some experiments demonstrated that PAF enhanced human sperm motility (Ricker et al., 1989). Researchers became interested in the relationship between male reproduction and PAF. Later, PAF was found in the semen of many species of animals (Diaz and Szeto, 1999; William and Roudebush, 2000; Mook et al., 1998; John et al., 1990). PAF, also known as acetyl-glycero-phospholipid (1-O-alkyl-2-acetyl-Sn- glycero-3-phosphocholine) (Fayed, 1996), is a component of sperm membrane lipid. PAF dosage in semen was significantly relative to sperm motility (Rudebush and Diehl, 2001; William et al., 2003). As a kind of phospholipid, PAF induced the cytoplasmic membrane to shape vesicles to act as an endogenous inducer of human sperm acrosomal reaction (Krausz et al., 1994), and improved mouse (Kazuo et al., 1992) and rabbit (Roudebush et al., 1993) oocytes *in vitro* fertilizing rate. People ulteriorly studied the effects of PAF on sperm motility, acrosomal reaction, capacitation and fertilizing capability (Luconi et al., 1995; Muguruma and Johnston, 1997; Huo and Yang, 2000; Wu et al., 2001; Kordan and Strzezek, 2002). The results showed that PAF can improve sperm motility, especially with low motility sperm. Sperm used in AI or *in vitro* fertilization could improve the fertilization rate after receiving treatment with PAF (Minhas, 1993; Angle et al., 1993; Wild and Roudebush, 2001).

$PGF_{2\alpha}$ is found in seminal plasma and it acts to adjust the smooth muscle shrinkage of cervix, uterus and oviduct which promote ovum and sperm one-way movement (Wang, 1993). It's possible that low conception in AI is caused by a decrease in the concentration of $PGF_{2\alpha}$ after semen dilution. Some experiments have shown that $PGF_{2\alpha}$ supplements can improve conception rate in AI.

2 Methods

2.1 Semen collection

Frozen sperm was obtained from the Giant Panda sperm bank of Chengdu (Chengdu Research Base of Giant Panda Breeding). Semen was collected by eletroejaculation from five Giant Pandas (6–20 years of age; weight: 100.5–123.0 kg) after the Giant Panda were anesthetized with Telazol® (Fort Dodge, Iowa, USA). The Giant Pandas were kept at the Chengdu Research Base of Giant Panda Breeding.

2.2 Experiment design

The canaliculi filled with Giant Panda semen were placed into a 37°C water bath for 45 s immediately after being removed from liquid nitrogen. Post-thawed sperm were washed with Ham's F-1 mediums at different concentrations adjusted to between $(2–7) \times 10^6$ mL^{-1} among the experimental groups listed in Table 1. The sperm were then incubated under 37°C. Sperm motility, survival time, acrosomal reaction rate, membrane integrity rate and heterogenetic penetrated-egg rate were tested after incubation at time intervals of 0, 1, 2, 4, 6 and 8 hours with 5 replications per measurement.

2.3 Sperm motility and survival time

Ten μL of semen was dropped onto a 37°C microcell-slide and covered with a glass-lid. The sperm on microcell-slide were observed and assessed for motility at 37°C and magnified $200 \times$ with an Olympus BX-51 microscope. The semen was kept at 37°C until all movements stopped, and this time was recorded as the sperm survival time.

2.4 Hypo-osmotic swelling test (HOST) for sperm membrane integrity

HOST has been applied to measure sperm membrane integrity (Kumi, 1993; Correa and Zavos, 1994; Vazquez et al., 1997; Nie and Wenzel, 2001; Mitsugu and Junjiro, 2003). Ten μL of semen at different incubation time in all experimental groups was added into a 100 μL low-osmotic medium (osmotic pressure: 150 mOsm; ion concentration: 0.15 mol·L^{-1}(0.735 g citrate sodium and 1.351 g fructose were dissolved in 100 mL pure water)). After being kept in the low-osmotic medium for 30 minutes at 37°C under humidified atmosphere containing 5% CO_2 and 95% air. A drop of sperm in low-osmotic suspension was spread onto a slide and 200 sperm were observed under a phase contrast microscope. Coil tail sperm (Plate 1 (a)) has intact plasma membrane. Finally, plasma membrane integrity rate of sperm was measured.

Table 1 Dosage of PF, PAF and PGF$_{2\alpha}$ in different experimental groups

experimental group	basic medium	end concentration of treatment supplement
control (Co)	Ham's F-10 (Gibco BRL)	–
A$_1$	Ham's F-10 (Gibco BRL)	1 mg·mL^{-1} PF (Sigma, MO, USA)
A$_2$	Ham's F-10 (Gibco BRL)	2 mg·mL^{-1} PF
B$_1$	Ham's F-10 (Gibco BRL)	50 ng·mL^{-1} PAF (Sigma, MO, USA)
B$_2$	Ham's F-10 (Gibco BRL)	100 ng·mL^{-1} PAF
C$_1$	Ham's F-10 (Gibco BRL)	50 ng·mL^{-1} PGF$_{2\alpha}$ (Ningbo Hormone, China)
C$_2$	Ham's F-10 (Gibco BRL)	100 ng·mL^{-1}L PGF$_{2\alpha}$
C$_3$	Ham's F-10 (Gibco BRL)	200 ng·mL^{-1} PGF$_{2\alpha}$

2.5 Trypan blue and Gimsa stain for acrosomal reaction (AR).

First, 10 μL semen from all experimental groups at different incubation time was taken out and added into 10 μL 1% Trypan blue staining solution per sample, and then thoroughly mix. This was incubated under 37°C for 15 minutes and dropped onto the slides. After the slides were dried and rinsed, the sperm on slides were fixed with Orth's solution for 45 minutes. The process of rinsing and drying was repeated, and then the slides were dipped into 80% ethanol for 10 seconds. Then, the sperm on the slide were stained with Gimsa staining solution for 90 minutes. The sperm were observed under a microscope (Olympus BX-51) after rinsing, drying and mounting, and then sorted according to Plate 1 (b–e). Finally, acrosomal reaction rate in all experimental groups were calculated.

2.6 Heterogenetic penetrated-egg (HPE) rate assay for fertilizing capacity

Mature Kunming mice were fed and drunk freely under natural illumination. Every mouse was injected with 10 IU PMSG (Ningbo hormone, China) via the intra-abdomen method at 4 pm. 48–52 hours later, every mouse was injected with 10 IU HCG (Ningbo hormone, China). After 15–17 hours of injection with HCG, the abdominal cavities of the mice were opened for observation. COCs were collected from the ampulla of uterine tube. COCs were twice washed with a basic medium of M199 (Hyclone, USA) + 2.2 mg·mL^{-1} NaHCO$_3$ (Sigma, USA) + 25 μg·mL^{-1} sodium pyruvate (Sigma, MO, USA) + 4.8 mg·mL^{-1} HEPES (GibcoBRL, USA) + 75 μg·mL^{-1} penicillinum (Sigma, MO, USA) + 50 μg·mL^{-1} streptomycin (Sigma, MO, USA) and then transferred into the oocyte maturing culture medium of Basic medium + 15% FBS (Hyclone, USA) + 10 IU·mL^{-1} FSH(Sigma, MO, USA) + 20 μg·mL^{-1} LH (Sigma, MO, USA). After being cultured at 37°C under humidified atmosphere containing 5% CO$_2$ and 95% air for 6 h, COCs were transferred into the basic medium including 0.1% hyaluronidase (Sigma, MO, USA) to remove granular cells around oocytes. Finally, the oocytes were washed with the mature culturing medium three times and transferred into a hypersaline solution (Herrick and Swanson, 2003), and then refrigerated at 4°C.

50 μL drops of Ham's F-10 including 5% FCS (GibcoBRL, USA) were covered with pre-sterilized paraffin oil and then put into a CO$_2$ incubator to balance for 2 hours. Eggs in the hypersaline solution were transferred into the drop for *in vitro* fertilizing (IVF). 15–20 eggs were transferred into a drop and 10 μL of semen from the different experimental groups at different times were added into the drop. In the IVF drop, sperm concentration was adjusted to $(4–8) \times 10^6$ mL^{-1}. The drops with egg and sperm were then incubated at 37°C under humidified atmosphere containing 5% CO$_2$ and 95% air for 6 hours. Eggs were taken out from the drop and transferred into 10% formalin for fixation. Finally, eggs were observed with a differential interference contrast (DIC) microscope (Olympus, IX-70). Some sperm adhered to the zona pellucida (ZP) of oocytes (Plate 1 (f)). Other sperm whose head came into the perivitelline space (Plate 1 (g)) and half penetrated into the ZP or more were considered as successful penetrations. Penetrated-egg rate of sperm in all experimental groups were observed.

2.7 Statistic analysis

The percentage (mean ± SEM) of motility, acrosomal reaction rate, sperm membrane integrity rate and sperm heterogenetic penetrated-egg rate in different experimental groups at the same time were analyzed by using F test. Values were considered significant when $P < 0.05$.

3 Results

3.1 Effects of PF, PAF and PGF$_{2\alpha}$ on post-thawed sperm motility and survival time

Sperm motility and survival time in the different experimental groups are presented in Table 2. The results showed that the effects of different concentrations and different substances on sperm motility and survival time were different. Comparison of sperm motilities in all the experimental groups at 0 hour showed that the differences were not significant. When sperm were treated for 1–2 h, the sperm motilities in all treatment groups except for groups C$_2$ and C$_3$ were significantly higher than that of the controlled group ($P < 0.05$). The sperm
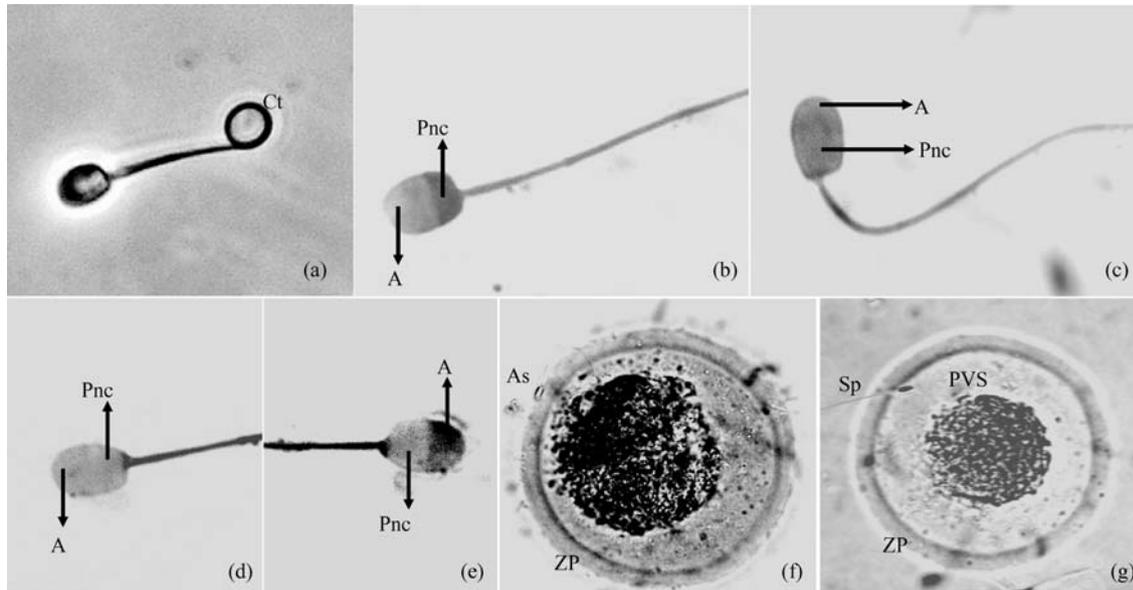
Plate 1 Sperm under different treatments

Note: (a) sperm membrane integrity and coiling tail (Ct) sperm by hypoosmotic swelling test (HOST) $\times 200$; (b) dead sperm during acrosomal reaction: postnuclear cap (Pnc) shows dark cyan, acrosome (A) cannot be stained or show dark red $\times 400$; (c) dead sperm without acrosomal reaction: postnuclear cap (Pnc) shows dark cyan, acrosome (A) shows amaranth $\times 400$; (d) living sperm during acrosomal reaction: postnuclear cap (Pnc) is not stained or shows weak cyan, acrosome (A) is not stained or shows amaranth $\times 400$; (e) living sperm without acrosomal reaction: postnuclear cap (Pnc) is not stained or shows weak cyan, and acrosome (A) shows amaranth $\times 400$; (f) adhesive sperm (As) on zona pellucida (ZP) $\times 100$; (g) sperm penetrate (Sp) and the zona pellucida (ZP) into perivitelline space (PVS) $\times 100$.

motilities in treatment groups A_1, B_1 and C_1 were over 50% higher than that in the control. Group A_1 had the highest motility with an increase in percentage at 58.33%. After sperm were incubated for 4 hours, the sperm motilities in groups A_1 and C_1 were 41.67% and 35.0% respectively, obviously higher than that in control group ($P < 0.01$). In addition, significant deviation of sperm motility was found at the different levels in the groups with the same treatment substance, but the difference in sperm motility wasn't significant between groups A_1 and C_1. In groups B_1 and B_2, sperm motility decreased quickly, but after sperm was treated for 4 hours, the differences in sperm motility were not significant compared to the control group.

Judging from sperm survival time and motility in all treatment groups, group A_1 was the best of all treatment groups for improving sperm quality. The sperm survival and the motility index in group A_1 reached 15.33 hours and 3.77 hours respectively. The results were significantly higher than those in the control and other treatment groups.

3.2 Effects of PF, PAF and $PGF_{2\alpha}$ on post-thawed sperm acrosomal reaction (AR) rate

Acrosomal reaction rate in the different experimental groups are shown in Fig. 2. The results suggested that PF, PAF and $PGF_{2\alpha}$ were able to influence sperm acrosomal reaction, but the differences of sperm acrosomal

Table 2 Effect of different treatment on sperm motility and survival time

experimental group	motility/%						survival time/hour
	0 h	1 h	2 h	4 h	6 h	8 h	
Co	$73.33^a \pm 12.58$	$58.33^a \pm 10.41$	$31.67^a \pm 10.41$	$16.67^a \pm \pm 2.89$	$8.33^{ac} \pm 2.89$	2.67 ± 2.52^a	$12.67^{ab} \pm 2.52$
A_1	$78.33^a \pm 7.64$	$71.67^a \pm 5.77$	$58.33^b \pm 10.41$	$41.67^b \pm 7.64$	$21.67^b \pm 7.64$	10.0 ± 5.0^b	$15.33^a \pm 4.73$
A_2	$76.67^a \pm 10.41$	$56.67^b \pm 10.41$	$45.0^c \pm 8.66$	$30.0^c \pm 5.0$	$13.33^a \pm 2.89$	6.67 ± 2.89^{ab}	$11.67^{abc} \pm 2.89$
B_1	$76.67^a \pm 7.64$	$73.33^c \pm 5.77$	$56.67^{bc} \pm 5.77$	$13.33^{ad} \pm 5.77$	$6.67^{ac} \pm 2.89$	1.67 ± 2.89^a	$13.33^{ab} \pm 2.89$
B_2	$73.33^a \pm 12.58$	$65.0^a \pm 13.23$	$46.67^{bc} \pm 5.77$	$16.67^a \pm 2.89$	$5.0^c \pm 0.0$	–	$7.0^{cd} \pm 0.0$
C_1	$75.0^a \pm 10.0$	$66.67^a \pm 5.77$	$55.0^{bc} \pm 5.0$	$35.0^{bc} \pm 5.0$	$15.0^{ab} \pm 5.0$	5.0 ± 2.14^{ab}	$10.0^{bc} \pm 3.0$
C_2	$78.33^a \pm 7.64$	$60.0^a \pm 10.0$	$36.67^{ac} \pm 5.77$	$20.0^{ac} \pm 10.0$	$10.0^{ac} \pm 5.0$	–	$9.0^{bc} \pm 3.46$
C_3	$80.0^a \pm 10.0$	$50.0^b \pm 10.0$	$11.67^d \pm 7.64$	$3.33^d \pm 5.77$	–	–	$3.67^d \pm 1.15$

Note: In the same column, the numbers with the same letters in the superscripts are not statistically significant ($P > 0.05$); the numbers with the different letters in the superscripts are statistically significant ($P < 0.05$). The same as below.
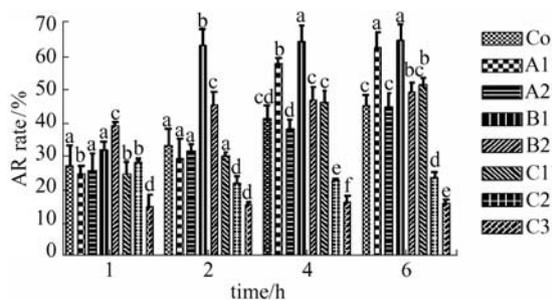
Fig. 2 Effect of different treatments on sperm acrosomal reaction (AR)
Note: In the different groups at the same treatment time, the histograms with the same letters in the superscripts are not statistically significant ($P>0.05$); the histograms with the different letters in the superscripts are statistically significant ($P<0.05$).

Fig. 3 Effect of different treatments on sperm membrane integrity
Note: In the different groups at the same treatment time, the histograms with the same letters in the superscripts are not statistically significant ($P>0.05$); the histograms with the different letters in the superscripts are statistically significant ($P<0.05$); Capital letters show highly statistically significant values ($P<0.01$).

reaction were relative to their concentration and treatment time. After sperm were treated for 2 hours, the rate of acrosomal reaction in group B_1 was significantly higher than that in the control group and other treatment groups ($P<0.05$); The acrosomal reaction rate in groups A_1 and C_1 peaked after the sperm were cultured for 4–6 hours, and the peak of acrosomal reaction rate in group A_1 reached 62.6%, which was significantly higher than that of the control group ($P<0.05$), but no significant differences were found between group C_1 and the control group. In our experiments, the rates of acrosomal reaction in groups C_2 and C_3, with higher concentrations of PGF$_{2\alpha}$, were significantly lower than those of the control and other treatment groups ($P<0.05$).

3.3 Effects of PF, PAF and PGF$_{2\alpha}$ on frozen-thawed sperm membrane integrity rate

Sperm membrane integrity rates in different groups are shown in Fig. 3. The different treatment substances and their dosages have different effects on sperm membrane integrity. In all experimental groups, between the 0th and 4th hour of treatment, as the length of the treatment increased, the intact rate of sperm membrane decreased.

In groups B_2, C_2 and C_3, the sperm membrane damage was more serious, and the intact rates of sperm membrane in groups B_2 and C_2 were significantly lower than those in the control and other treatment groups ($P<0.05$). In group C_3, the intact rates of sperm membrane were significantly lower than those in the control and other treatment groups ($P<0.01$). In the range between 1–2 hours, the sperm membrane integrity rates in the high concentration groups of PF, PAF and PGF$_{2\alpha}$ were lower than those in the low concentration groups. The higher intact rates of sperm membrane were found in groups A_1, B_1 and C_1, but only the treatment C_1 group had a significant difference ($P<0.05$) compared to the control group. In groups A_1 and B_1, as treatment time increased, sperm membrane retained better protection. After the sperm were treated for 4 hours, the sperm membrane intact rates in groups A_1
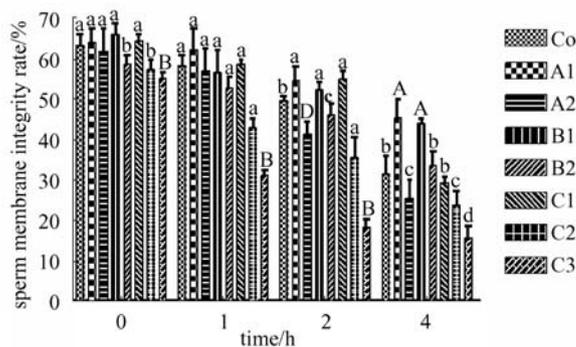
and B_1 were 45.61%, 44.17% respectively, and these results were significantly higher than those in the control and other treatment groups ($P<0.01$).

3.4 Effects of PF, PAF and PGF$_{2\alpha}$ on post-thawed sperm heterogenetic penetrated-egg rate

Sperm penetrated-egg rates in the different experimental groups are shown in Table 3. The sperm penetrated-egg rate was relative to the PF, PAF and PGF$_{2\alpha}$ concentrations and the length of time of incubation with PF, PAF or PGF$_{2\alpha}$ at 37°C. After being treated for 1–2 hours, the sperm in the control and treatment groups, except group C_3, had the ability to penetrate eggs *in vitro*. Sperm penetrated-egg rate in group B_1 was the highest in all of the experimental groups and it reached 48.55% and was significantly higher than that in the control and other treatment groups ($P<0.01$). After 4 hours of treatment, sperm penetrated-egg rates in groups B_1, B_2, C_2 and C_3 were zero, but those in groups A_1 and C_1 reached 51.44% and 40.66% respectively. The rates in these two groups were significantly higher than those of the control and other treatment groups ($P<0.01$). At the same time, sperm penetrated-egg rate in group A_1 was significantly higher than in group C_1 ($P<0.05$). In all treatment groups, judging from sperm penetrated-egg rate *in vitro*, the best treatment method was in group A_1. After sperm were treated for 6 hours, the sperm penetrated-egg rate in group A_1 was 7.49% while the rate for the other groups fell to zero.

4 Discussion

4.1 Effects of PF on post-thawed sperm fertility

In the experiments with PF treatments, PF concentration, treatment time and stimulating effects on sperm varied

Table 3 Effect of different treatments on sperm penetrated-egg rate *in vitro*

experimental group	sperm heterogenetic penetrated-egg rate/%			
	1 hour	2 hour	4 hour	6 hour
Co	$13.97^a \pm 2.51$	$32.53^a \pm 3.92$	$10.65^a \pm 2.12$	0
A_1	$16.58^{ab} \pm 2.86$	$24.54^b \pm 3.50$	$51.44^A \pm 4.97$	7.49 ± 1.45
A_2	$13.70^a \pm 2.80$	$14.18^D \pm 2.14$	$5.34^a \pm 4.64$	–
B_1	$34.51^A \pm 3.86$	$48.55^A \pm 5.31$	–	–
B_2	$24.29^c \pm 1.84$	$18.86^{bD} \pm 2.98$	–	–
C_1	$18.85^b \pm 2.96$	$24.44^b \pm 3.07$	$40.66^B \pm 1.95$	–
C_2	$9.19^d \pm 1.18$	$5.27^c \pm 0.28$	–	–
C_3	$9.14^d \pm 0.84$	–	–	–

Note: Capital letters show highly statistically significant values ($P<0.01$) as compared with other values in the same column.

between different researchers. Numabe et al. (2001) observed that the rate of cattle sperm fertilization *in vitro* was 16% higher than that in the control group ($P < 0.01$) after cattle sperm were treated with 5 mmol·L^{-1} PF, and the developmental rate of blastula *in vitro* was significantly increased (Numabe et al., 2001). Gradil and Ball (2000) treated horse post-thawed sperm with 3.5–7.0 mmol·L^{-1} PF, and the results revealed that sperm motility can be significantly improved ($P < 0.01$) after adding PF into post-thawed semen. In the experiments for improving human sperm motility, some researchers considered that 3.0, 5.0 and 10.0 mmol·L^{-1}, or higher concentration of PF, could gain the better stimulative effect (Gradi and Ball, 2000). However, some researchers considered that lower concentrations of PF (0.3 mmol·L^{-1}) could also gain satisfactory results (Rees et al., 1990). A 30-minute treatment is often applied to incubate PF and human sperm together *in vitro* (Yovich et al., 1990; Tesarik et al., 1992), and some studies reported that 60–75-minute treatment could also attain a favorable effect (Kay et al., 1993; Pang et al., 1993). There was very little influence on sperm survival time when the incubation time prolonged to 3 hours (Kay et al., 1993).

In our tests, 1 mg·mL^{-1} (3.5 mmol·L^{-1}) and 2 mg·mL^{-1} PF Giant Panda post-thawed sperm *in vitro*, were incubated and the results suggested that sperm motility in two groups (groups A_1 and B_1) declined more slowly than that in the control group and the sperm motility in the PF groups A_1 and A_2 was higher than that in the control group. For the 1 mg·mL^{-1} PF treatment (group A_1), the sperm survival time was longer than that in the control group, and the sperm acrosomal reaction, intact rate of sperm membrane and sperm penetrated-egg rate were significantly higher than those in the control group ($P < 0.01$). Post-thawed sperm was able to enter the ovum even after being treated *in vitro* for 6 hours with PF. The results indicated that the effect of PF on sperm was dosage-dependent. To improve the sperm fertilizing capability, the treatment efficiency of 1 mg·mL^{-1} was better than 2 mg·mL^{-1}. Furthermore, a higher concentration of PF would result in some damage on sperm motility and a notable decrease in sperm fertilizability ($P < 0.01$).

Up till now, the method of treating post-thawed Giant Panda sperm for improving sperm fertilizing capability with PF has not been reported. The aim of our study was to investigate whether PF has the ability to elevate Giant Panda post-thawed sperm fertilizability *in vitro*. The results indicated that PF could elevate sperm motility and fertilizability *in vitro*, and prolong the duration in which the sperm remains able to fertilize. That is to say, it is possible to elevate the fecundation rate of Giant Panda in artificial insemination by improving sperm motility with PF before the procedure, but further researches are necessary to select and confirm the most effective dose of PF.

4.2 Effects of PAF on post-thawed sperm fertility

The result of adding exogenous PAF into Giant Panda post-thawed semen suggested that the sperm motility in groups with low concentrations of PAF (50 ng·mL^{-1}) was higher than that in the control group (0 mg·mL^{-1} PAF). After sperm were treated for 2 hours with PAF, sperm penetrated-egg rate reached 48.55%, which was significantly higher than that in the control group ($P < 0.01$), and sperm acrosomal reaction rate achieved 63.24% which was also significantly higher than that in the control group ($P < 0.05$). Similar results had been previously observed by He (2000) on rats in which 50 ng·mL^{-1} of PAF was used to treat rat sperm for 90 minutes which led to a 66.3% sperm capacitation. Kordan and Strzezek (2002) observed that exogenous PAF at 1×10^{-8}–1×10^{-6} mol·L^{-1} concentrations can cause significant improvement in the movement ability of swine fresh and post-thawed sperm, with an optimal concentration of 1×10^{-7} mol·L^{-1}. Higher concentrations of PAF may result in disruption of sperm membrane.

In our study, the degree of sperm membrane damage in extremely high concentrations of PAF (100 ng·mL^{-1}) was more severe than that in extremely low concentrations ($P < 0.05$), as supported by the observation that groups with higher levels of PAF yielded significantly higher acrosomal reaction rates ($P < 0.05$). Accordingly, sperm penetrated-egg rate *in vitro* in groups with high

concentrations of PAF was lower than that in groups with low concentrations. Thus, there was an effect of PF dosage on sperm. The results suggested that PAF has an effect on elevating the rate of sperm acrosomal reaction and motility, but the incubation time of sperm with PAF has to be carefully controlled. The sperm survival time in PAF groups was not different from that in the control group ($P < 0.05$) since PAF could not prolong the sperm survival time. After sperm were treated for 2 hours with PAF, the sperm motility and penetrated-egg rate significantly decreased ($P < 0.01$) as prolonged exposure to PAF during incubation could disrupt sperm function.

4.3   Effects of PGF$_{2\alpha}$ on post-thawed sperm fertility

Certain concentrations of PGF$_{2\alpha}$ can be found in the semen of some species of animals. The average concentrations of PGF$_{2\alpha}$ were 58.9 pg·mL^{-1} in boar semen (Henrique et al., 2003), 800 ng·mL^{-1} in bull semen (Ledwozw et al., 1986), 35.5 ng·mL^{-1} in ram semen (Oliw, 1989) and 2.1–18.3 μg·mL^{-1} in human semen (Cosentino et al., 1984). Fayed (1996) studied the influence of PGF$_{2\alpha}$ on bull sperm motility. They found that bull sperm motility was suppressed at high levels (1200 ng·mL^{-1}) of PGF$_{2\alpha}$, and that high concentrations of PGF$_{2\alpha}$ destroyed sperm membrane integrity which resulted in a large leakage of aminotransferase. They discovered that there was almost no aminotransferase leakage when PGF$_{2\alpha}$ concentration was at 300 ng·mL^{-1} or lower. Furthermore, low concentrations of PGF$_{2\alpha}$ improved sperm fertilization.

In our study Giant Panda post-thawed semen was treated with PGF$_{2\alpha}$ at 50, 100 and 200 ng·mL^{-1}. The results suggested that sperm motility in the groups with higher concentrations of PGF$_{2\alpha}$ (100 and 200 ng·mL^{-1}) was lower but the difference wasn't significant. Sperm survival time in the groups with higher concentrations of PGF$_{2\alpha}$ (100 and 200 ng·mL^{-1}) was significantly lower than that in the control group ($P < 0.05$). At the same time, the groups with higher concentrations of PGF$_{2\alpha}$ resulted in a lower membrane integrity rate. All of these indicated that PGF$_{2\alpha}$ would damage sperm membrane. After sperm were treated for 1 hour with 200 ng·mL^{-1} PGF$_{2\alpha}$, their penetrated-egg capabilities were almost completely lost, possibly due to a very low acrosomal reaction rate. Sperm motility and penetrated-egg capability hardly changed in post-thawed sperm treated with 50 ng·mL^{-1} PGF$_{2\alpha}$. After post-thawed sperm were treated with 50 ng·mL^{-1} PGF$_{2\alpha}$ for 4 hours, sperm penetrated-egg rate still reached 40.66%, which was significantly higher than that in the control group ($P < 0.01$), but the rate of sperm membrane integrity in the 50 ng·mL^{-1} PGF$_{2\alpha}$ treated group wasn't significantly different with that of the control group. The results suggested that PGF$_{2\alpha}$ can improve post-thawed fertilizing capability *in vitro* but high concentration of PGF$_{2\alpha}$ may cause damage to the sperm. This conclusion is in accordance with that of Fayed (1996).

The semen used for artificial insemination is usually directly diluted or removed from seminal plasma by centrifugation. Dilution and seminal plasma removal may decrease the concentration of PGF$_{2\alpha}$ in semen, and thus weaken the stimulus to the female genital tract. This is why PGF$_{2\alpha}$ is usually added into the diluted semen to increase fecundation rate for artificial insemination (Maes et al., 2003; Kos and Bilkei, 2004). In our study, PGF$_{2\alpha}$ helped improve Giant Panda's post-thawed sperm motility, but there was no significant influence on the fertility of Giant Panda sperm. In addition, further studies are necessary to evaluate the roles of PGF$_{2\alpha}$ and its optimal dosage if applied to Giant Panda fertilization.

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