Research Article

Impact of Time Interval between PGF2α Administration and Semen or Blood Plasma Collection on Spermatozoa Quality, Testosterone Level, cAMP, and Plasma ATP Level in Rats

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Abstract | The effect of prostaglandin F2α (PGF2α) on spermatozoa quality improvement still shows inconsistent results. In addition, the mechanism leading to increased spermatozoa concentration after administration of PGF2 has not been clearly explained. Therefore, this research aimed to assess the impact of the time interval between PGF2α administration and semen collection on the enhancement of spermatozoa quality, testosterone, cyclic adenosine monophosphate (cAMP), and adenosine triphosphate (ATP) levels. A total of 15 rats (*Rattus norvegicus*) categorized into five treatment groups (n=3) were used in this study. In the control group (P0), the semen and blood samples were collected 30 minutes after a 0.5 ml NaCL injection. In groups P1, P2, P3, and P4, semen and blood samples were collected 30, 60, 90, and 120 minutes, respectively, after intraperitoneal injection of 2.5 mg PGF2α/kg BW. Upon treatment completion, all the rats were euthanized with Zoletil at 40 mg/kg BW. Microscopic examination of spermatozoa quality, including motility, concentration, viability (survival), and abnormalities, was conducted using spermatozoa from cauda epididymis. Simultaneously, testosterone, cAMP, and ATP were also assessed using blood plasma from blood samples. Administration of PGF2α significantly affected spermatozoa concentration ($p<0.05$) at the 90th minute (P3), increased motility ($P<0.05$) overall treatment times, enhanced viability ($P<0.05$) at the 30th and 60th minutes (P1 and P2), and reduced abnormalities ($P<0.05$) in all treatment groups, but it did not affect testosterone levels ($P>0.05$). The peak of cAMP concentration occurred at P1, while that of ATP occurred at P4 ($P<0.05$). The time interval between PGF2α administration and sample collection affected spermatozoa concentration, viability, abnormalities, ATP, and cAMP, whereas spermatozoa motility and testosterone concentration remained unaffected.

Keywords | cAMP/ATP; PGF2α; Spermatozoa; Testosterone; Wistar rat

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INTRODUCTION

The success of artificial insemination programs significantly depends on the quality of the produced frozen semen (Isnaini et al. 2019a; Isnaini et al., 2019b). To optimize frozen semen quality, it is necessary to obtain high-quality spermatozoa from animals. Recent research indicates that the addition of prostaglandin F2α (PGF2α) holds the potential for enhancing such semen quality attribute both *in vivo* (Armansyah et al., 2018; Husnurrizal

et al., 2021; Sari et al., 2021) and *in vitro* (Aswadi et al., 2021; Prestiya et al., 2020). Prostaglandin F2 alpha can act directly or indirectly to enhance spermatozoa quality (Şen and Akcay, 2015; Husnurrizal et al., 2024). According to Hafizuddin et al. (2023) and Panjaitan et al. (2024), this improvement is due to enhanced testosterone release. Testosterone, a crucial hormone in the spermatogenesis process, is influenced by PGF2α, which contributes to luteinizing hormone (LH) secretion (Haynes et al., 1978). Prostaglandin F2 alpha stimulates the hypothalamus to produce gonadotropin-releasing hormone (GnRH), subsequently prompting the pituitary gland to produce interstitial cell-stimulating hormone (ICSH) or LH (Luteinizing hormone) that stimulates Leydig cells, leading to increased testosterone production (Rahmawati et al., 2015).

The relationship between the enhancement of spermatozoa quality and testosterone production after PGF2α administration remains inconclusive. In Kacang goats, administering PGF2α *in vivo,* two days before semen collection, increased testosterone concentration but did not significantly enhance spermatozoa quality (Armansyah et al., 2018). A similar pattern was observed in Bali cattle (Sari et al., 2019), while in Aceh bulls it enhanced spermatozoa quality without a simultaneous rise in testosterone levels (Sari et al., 2021). These inconsistent results might be attributed to variations in the examination time of spermatozoa quality after PGF2α administration.

Guan et al. (2018) reported that administration of PGF2α directly affects testosterone synthesis in Leydig cells by inducing the production of cyclic adenosine monophosphate (cAMP), a common intracellular signaling molecule (second messenger) in eukaryotic cells (Campbell and Reece, 2015). This messenger triggers the synthesis of protein kinase A that is required for the transport of cholesterol from the cytoplasm to mitochondria (Hardie, 2022). During the process, a steroidogenic acute regulatory protein (StAR) and peripheral benzodiazepine receptor (PBR) facilitate cholesterol transport from the outer membrane of mitochondrial to the inner membrane (Tugaeva and Sluchanko, 2019). The initiated cholesterol transport, guided by StAR and PBR, passes through the membrane gate. Furthermore, the P450scc (side-chain cleavage) enzyme situated in the inner mitochondrial membrane, converts cholesterol into pregnenolone. Pregnenolone is then transported to the smooth endoplasmic reticulum (SER) where testosterone is synthesized through enzymatic steroidogenic steps (Papadopoulos and Miller, 2012; Zirkin and Papadopoulos, 2018). Moreover, the increase in cAMP concentration is expected to enhance testosterone production (Ho et al., 2016; Kaspul, 2011).

Furthermore, PGF2α induces the formation of cAMP, a cyclic ring-shaped molecule derived from adenosine triphosphate (ATP), serving as a ubiquitous intracellular signaling molecule (second messenger) in eukaryotic cells. Consequently, the presumed association between the increase in testosterone and cAMP, following PGF2α administration, is linked to heightened ATP production. Adenylate cyclase stimulation for cAMP production is influenced by the intensity of Ca^{2+} entering the spermatozoa membrane (Sun et al., 2017), and the energy required for spermatozoa motility is sourced from intracellular stores of ATP produced by the spermatozoa tail fibers (Sengupta et al., 2020; Susilawati, 2011). In instances where ATP and ADP stores are depleted, spermatozoa fibril contractions will cease. To sustain before ongoing spermatozoa motility, the regeneration of ADP and ATP must occur (Susilawati, 2011). This research aimed to assess the impact of the time interval between PGF2α administration and semen or blood plasma collection on the enhancement of spermatozoa quality, testosterone, cAMP, and ATP levels in rat.

MATERIAL AND METHODS

Animals and Ethics Approval

This study was conducted with approval from the Ethics Commission responsible for use of Experimental Animals, Faculty of Veterinary Medicine, Universitas Syiah Kuala with certificate number: 169/KEPH/IX/2022. This study used 15 male rats (*Rattus norvegicus*), aged 12 weeks, with weight of 200-250 g. All the rats were subjected to a 2-week adaptation period and were fed on Hi-Gro 555SP complete feed (PT. Charoen Pokphand Indonesia Tbk.) at 10% of their body weight.

All the rats were divided randomly into five groups, namely P0, P1, P2, P3, and P4 (each n=3). The control group (P0) involved semen and blood collection 30 minutes after a 0.5 ml NaCl 0.9% injection. The semen and blood samples in groups P1, P2, P3 and P4 were collected at 30, 60, 90, and 120 minutes, respectively, after intraperitoneal injection of 2.5 mg PGF2α/ kg BW (Lutalyse, Zoetis). The doses used were based on those of Suripto et al. (2000), including the dose (2.5 mg) of PGF2α. However, we changed the PGF2α dose to 2.5mg PGF2α/kg BW by single injection. At the end of experiment, all the rats were euthanized using zoletil 40 mg/kg BW (Ferrari et al., 2005 which has been modified)

Testis and Cauda Epididymis Collection

Immediately after termination, rats were placed in the dorsal recumbency and then an incision was made on the surface of the scrotal skin to remove the testicles from the scrotum. The testicles were separated from the cauda epididymis, rinsed with physiological sodium chloride (NaCl) and soaked in a petri dish.

Semen Collection and Spermatozoa Quality Examination

A sample was collected from the cauda epididymis and immediately examined to obtain the data on spermatozoa quality. Sample examination was carried out using a light microscope (Olympus, Tokyo, Japan) to determine motility, concentration, viability (survival), and abnormalities of spermatozoa. The procedure for spermatozoa quality evaluation followed those of Nora et al. (2024).

Blood Sample Collection and Preparation

Blood samples from each group were collected from the heart, immediately after a rat was terminated, using a disposable syringe (3 cc, 22 gauge). The samples obtained was dispensed into vacutainer tubes containing ethylene diamine tetra acetic acid (EDTA) and homogenized. The homogenized samples were placed into a cool box and transported to the laboratory. To obtain the plasma sample, 3 mL blood sample was centrifuged at 3000 rpm for 15 minutes, and the plasma was transferred to a microtube and stored in the freezer at -20 °C before testosterone, cAMP, and ATP measurements.

Testosterone, cAMP and ATP Level Assays

The examination of testosterone, cAMP and ATP concentration used the enzyme-linked immunosorbent assay (ELISA) method. The measurement of testosterone concentration adhered to the ELISA kit protocol (DRG Testosterone ELISA EIA-1559, DRG Instruments GmbH, Marburg, Germany). The measurement of cAMP and ATP levels followed the Bioenzy ELISA kit protocols for cAMP (ELISA Kit 96 wells BZ-08188920-EB) and ATP (ELISA Kit 96 wells BZ-08190290-EB).

Data Analysis

Data on spermatozoa quality, cAMP, ATP, and testosterone concentration in rats after *in vivo* PGF2α administration were analyzed using a one-way analysis of variance and were subsequently subjected to Duncan's test (SPSS 24 IBM™).

RESULTS AND DISCUSSION

The administration of PGF2α had a significant effect on spermatozoa concentration ($P < 0.05$) at the 90th minute interval between PGF2α administration and sample collection (P3). Increased spermatozoa motility was found in all treatment groups (P1, P2, P3, P4) compared to the control group (P0) ($P<0.05$), but there were no significant differences between groups ($P>0.05$). Furthermore, PGF2α administration also had an effect on increasing spermatozoa viability ($P<0.05$) in the 30 and 60 minutes interval between administration and semen collection (P1 and P2), and reducing spermatozoa abnormalities ($P<0.05$) in all treatment interval groups (P1, P2, P3, and P4) as presented in Table 1.

PGF2α administration for 90 minutes resulted in a higher spermatozoa concentration compared to the control (P0) and the 30, 60, and 120 minute groups. The concentration in the control group was lower over other groups treated with PGF2α for 30, 60, 90, and 120 minutes. Essentially, PGF2α administration for 90 minutes before collection affected spermatozoa concentration ($P<0.05$), while the treatment for 30, 60, and 120 minutes did not show a significant effect ($P>0.05$). This result indicated that the timing of PGF2α administration affected spermatozoa concentration. The optimal time interval between PGF2α administration and spermatozoa concentration measurement in rats may be up to 90 minutes.

The current results were in line with the differences observed in previous reviews (Armansyah et al., 2018; Sari et al., 2019). Administering PGF2α injection two days before semen collection in Kacang goats numerically increased testosterone levels, but did not result in a significant improvement in spermatozoa concentration and motility (Armansyah et al., 2018). Similar results were reported by Sari et al. (2019) in Bali cattle. Different results were reported in Aceh cattle that the administration of PGF2α at 30 minute before semen collection shows the improved quality of spermatozoa but at the same time there was no increase in testosterone concentration (Sari et al., 2021).

The administration of PGF2α had no significant effect ($P>0.05$) on spermatozoa motility in all groups (0, 30, 60, 90, and 120 minutes). The results were supported by those previous studies suggesting that the improved spermatozoa motility after the treatment was more likely associated with *in vitro* rather than *in vivo* application. The enhancement in spermatozoa quality resulting from the addition of PGF2α *in vitro*, in a diluent medium, was reported in Nubian goats (Prestiya et al., 2020) and Waringin sheep (Husnurrizal et al., 2021). The improvement mechanism resulting from *in vitro* PGF2α administration consisted of its impact on the contractile elements of spermatozoa, leading to increased motility (Şen and Akcay, 2015). According to Kowalczyk et al. (2021), prostaglandin E2 present in ejaculated semen acted through prostaglandin E2 EP1 (PTGER1) and prostaglandin E2 EP3 (PTGER3) receptors. This modulation influenced spermatozoa motility, capacitation, acrosome reaction, and enhanced fertilization capacity by mediating increased intracellular calcium concentration. Another report indicated that *in vivo* PGF2α administration in cattle did not affect frozen semen motility (Masoumi et al., 2011).

The viability of spermatozoa in the group administered with PGF2α showed a significant increase after 30 (P1)

Table 1: Spermatozoa quality of rats after PGF2α injection at different time intervals

Variable	P0 (n=3)	P1 (n=3)	P2 (n=3)	P3 (n=3)	P4 (n=3)
Concentration (x 106 cells/mL)	42.33±16.01[a]	64.67±5.86[a,b]	64.67±2.52[a,b]	82±18.25[b]	79±18.36[a,b]
Motility (%)	48.94±16.03[b]	71.08±4.89[a]	74.14±2.51[a]	77.92±21.48[a]	64.01±17.99[a]
Viability (%)	38.97±14.67[a]	74.14±2.51[b]	77.16±4.14[b]	66.15±8.2[a,b]	59.74±28.51[a,b]
Abnormalities (%)	22.24±24.38[b]	3.18±1.73[a]	6.09±5.35[a]	5.38±4.46[a]	15.38±15.56[a]

[a,b] Different superscripts in the same row indicate significant differences (p<0.05).
P0 (Control / 0.9% NaCl injection group); P1 (PGF2α injection group, semen collected after 30 minutes); P2 (PGF2α injection group, semen collected after 60 minutes); P3 (PGF2α injection group, semen collected after 90 minutes); P4 (PGF2α injection group, semen collected after 120 minutes).

Table 2: The concentration of cAMP, ATP, and calcium in rats after injection with PGF2α

Variable	P0 (n=3)	P1 (n=3)	P2 (n=3)	P3 (n=3)	P4 (n=3)
Testosterone (ng/mL)	1.17±0.56[a]	1.05±0.5[a]	1.73±1.09[a]	1.24±0.34[a]	0.78±0.05[a]
cAMP (ng/mL)	26.42±3.32[c,d]	28.95±1.56[d]	23.00±1.47[b,c]	19.73±0.35[a,b]	18.12±2.88[a]
ATP (ng/mL)	130.87±25.12[a]	142.94±5.64[a,b]	124.38±20.11[a,b]	156.1±27.73[a,b]	172.99±27.95[b]

[a,b,c,d] Different superscripts in the same row indicate significant differences (p<0.05).
P0 (Control/0.9% NaCl injection group); P1 (PGF2α injection group, examined after 30 minutes); P2 (PGF2α injection group, examined after 60 minutes); P3 (PGF2α injection group, examined after 90 minutes); P4 (PGF2α injection group, examined after 120 minutes).

and 60 minutes (P2) compared to the control group. This result contrasted with those of Sari et al. (2019), who observed no significant difference in spermatozoa viability in Aceh cattle with or without PGF2α. The variations in results might have been attributed to the timing of semen examination. In this research, spermatozoa viability did not significantly increase at 90 and 120 minutes after PGF2α administration. It was suspected that in Aceh cattle, the examination interval of 30 minutes after PGF2α administration, as reported by Sari et al. (2019), might not have been sufficient to enhance spermatozoa viability.

PGF2α administration at the intervals of 30, 60, 90, and 120 minutes before semen collection did not affect spermatozoa abnormalities (P>0.05). Numerically, the treatment for 30 minutes showed a decrease in spermatozoa abnormalities compared to the control (P0) and the 60, 90, and 120-minute time groups. The spermatozoa abnormalities in the control was higher than those in group injected with PGF2α.

The variation in spermatozoa quality after PGF2α administration was associated with an increase in testosterone. The mechanism of testosterone elevated through cAMP should have preceded the rise in spermatozoa, often resulting in asynchrony between testosterone concentration and spermatozoa quality when examined simultaneously. Statistically, PGF2α administration did not lead to a significant increase in testosterone concentration (P>0.05), as outlined in Table 2. Despite the lack of statistical significance, PGF2α administration had a rise in testosterone at 60 minutes, preceding the increase in spermatozoa concentration at the 90 minutes. The results supported the assertion that the enhancement of spermatozoa quality was linked to an increase in testosterone. This research was in line with the observation of Armansyah et al. (2018) that PGF2α administration in Kacang goats increased testosterone concentration to 18.51±19.46 ng/mL compared to the control group given physiological NaCl (10.27±5.42 ng/mL). Saifudin (2004) documented an increase in testosterone levels in local sheep after PGF2α administration a week before sample collection. According to Kiser et al. (1978), PGF2α administration for 90 minutes before sample collection increased testosterone levels in cattle. On the other hand, Siregar et al. (2014) reported different results that the treatment did not increase testosterone levels in white rats. The inconsistent results might be attributed to differences in the animal species used and the collection interval with the treatment. In general, this study found that an increase of spermatozoa quality occurred at a 90 minutes administration interval, which was preceded by an increase in testosterone at a 60 minutes administration interval. The results of this study are relatively difficult to compare with other studies because reports of PGF2α administration at different time intervals have never been reported in other species.

PGF2α is believed to boost testosterone levels through two pathways: direct and indirect. In direct action, the treatment had effects similar to steroids and induced local contraction in the lumen muscles of the male reproductive system (Capitan et al., 1990). Indirectly, PGF2α contrib-

uted to luteinizing hormone (LH) secretion (Haynes et al., 1978). The treatment stimulated the hypothalamus to generate GnRH, which further prompted the pituitary to release ICSH or LH. LH subsequently stimulated Leydig cells, leading to increased testosterone production (Rachmawati et al., 2014). In this study, the increase in spermatozoa quality is thought to occur through the pathway of increasing testosterone as previously described, although LH concentration was not measured.

PGF2α directly participated in the testosterone formation process within Leydig cells by stimulating the formation of cAMP, a ring-shaped molecule derived from ATP. cAMP functioned to be a common intracellular signaling molecule (second messenger) in eukaryotic cells, including vertebrate endocrine cells. Furthermore, it facilitated the synthesis of protein kinase A, a crucial element for transporting cholesterol from the cytoplasm to the mitochondria (Sharma et al., 2023). StAR and PBR facilitated the transfer of cholesterol from the outer membrane to its inner part (Bogan et al., 2007; Haider, 2007). Although the increase in cAMP concentration was not statistically significant (P>0.05), it has been documented to precede the rise in testosterone concentration, consistent with the mechanism described by Haider (2007). The elevation in cAMP was believed to boost testosterone production (Kaspul, 2011). The non-significant increase (P>0.05) after PGF2α administration between the control and P1 groups was in line with the observation of Li et al. (2021), who reported that PGE2 was more effective in increasing cAMP concentration. Specifically, PGE2 could elevate cAMP by 80 times, while PGF2α achieved a fivefold increase in the UMR-106 osteosarcoma cell line. The distinct receptors in tissues for PGE2 and PGF2α were suspected to contribute to these observed differences.

The time of ATP increase after PGF2α administration in this research in accordance with the cAMP increase at the 30 minute interval. In contrast to cAMP, which experienced a relative decrease after the 30th minute, ATP concentration showed fluctuations. The fluctuations suggested that PGF2α administration might not have directly impacted ATP concentration. However, the available ATP influenced cAMP production, testosterone, and spermatozoa quality. According to Lestari and Ismudiono (2014), mitochondria served as the site for ATP energy synthesis used for spermatozoa movement, converting chemical energy into kinetic energy.

Disruption in ATP production resulted in low spermatozoa motility, given their susceptibility to reactive oxygen species (ROS) due to membrane composition rich in polyunsaturated fatty acids (PUFAs) and single electrons. ROS readily bound to the membrane, initiating an extensive lipid peroxidation chain reaction, damage cell biochemistry, and causing structural harm to cell and mitochondrial membranes. Mitochondrial damage disrupted ATP production, thereby contributing to decreased spermatozoa motility (Bansal et al., 2011; Gharagozloo et al., 2011). The results in this present study are in accordance with the aforementioned statement, indicating insignificant differences in ATP and spermatozoa motility. The increased spermatozoa motility observed in Nubian goats after PGF2α administration, as documented by Prestiya et al. (2020), was likely unrelated to ATP increase. However, it was related to a direct action of PGF2α on spermatozoa because the administration was performed *in vitro*.

CONCLUSION

In conclusion, the interval time between PGF2α administration and semen or blood analysis impacted spermatozoa concentration, viability, abnormalities, ATP, cAMP, and testosterone. In general, an increase spermatozoa quality (at 90 minutes) is preceded by increase in cAMP (at 30 minutes), and followed by an increase in testosterone (at 60 minutes). The study was limited by the small sample size, which may affect the level of validity of the data obtained. The results of this study are expected to be used as a reference for determining the best time for the effect of PGF2a on spermatozoa quality which does not always coincide with the increase in testosterone.

CONFLICT OF INTEREST

The authors have no conflicts of interest regarding this investigation.

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NOVELTY STATEMENT

Determination of the best time interval for PGF2α administration to improve sperm quality, testosterone, cAMP, and ATP.

AUTHORS CONTRIBUTION

HR, SW and CNT participated in performing, selecting samples, sample collection, performed practical experiments, wrote the initial manuscript, performed manuscript revision, and data analysis. TNS and HZ developed the

original idea and protocol, conducted the research and revised the final manuscript.

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