Effect of platelet rich plasma lysate and fibroblast growth factor 2 on stallion sperm motility

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Abstract

Growth factors (GFs) are known to modulate cell function and their presence in semen could be advantageous to sperm. In humans and mice, fibroblast growth factor 2 (FGF2) improved semen motility. Platelet rich plasma (PRP), rich in GFs including FGF2, reduced post mating inflammatory response within the uterus; however, its effects on sperm are not known. PRP lysate (PRPL) is much purer than PRP and contains higher concentrations of GFs. Hence, the objective of the present study was to evaluate the effect of supplementing fresh and cool-stored stallion sperm with either PRPL at 1, 2.5, 5, and 10% (also containing 1 IU/ml of heparin), or FGF2 at 0.1, 1, 10, and 100 ng/ml. Motility parameters were evaluated using computer assisted semen analysis at 0, 0.5, 1, 1.5, 6, and 24 hours after treatment. For both PRPL and FGF2 treatments, there were no differences in total and progressive motility among groups. Concentrations of PRPL > 5% induced sperm agglutination via head-to-head attachment, starting at hour 1 and was more pronounced for 10% PRPL than 5% PRPL, suggesting a dose-dependent characteristic. Direct addition of PRPL to semen extender at doses < 5% might not substantially affect sperm motility, whereas doses > 5% might affect sperm motility due to head-to-head attachments. Addition of FGF2 at the concentrations studied may not affect sperm characteristics.

Keywords: Stallion semen, platelet-rich plasma lysate, fibroblast growth factor 2, sperm motility

Introduction

Assisted reproductive techniques aim to disseminate the genetics of an animal by maximizing production of live offspring compared to natural breeding. For that to occur, sperm survival, fertilization, implantation of the embryo, and delivery of a live offspring should be controlled to prevent losses. Artificial insemination (AI) allows the ejaculate to be evaluated, to use a minimum number of progressive motile sperm, and to breed at an optimal time frame to maximize fertilization. Additionally, AI allows the use of several breeding doses from an ejaculate, maximizing stallion use. Appropriate nutrients and/or stimulatory proteins in association with antibiotics are used to preserve sperm during shipment and/or storage. Breeding success is influenced by semen quality and the mare's immunologic response to semen. Therefore, compounds that improve semen quality and reduce post mating endometritis, are of great value.

Sperm motility and viability can be improved by semen extenders. Uterine inflammatory response can be reduced by decreasing contamination and supporting uterine clearance by using uterine lavage, ecbolic agents, and antiinflammatory compounds. New techniques to improve semen quality in conjunction with the control of post breeding uterine inflammation would maximize sperm survival in the female reproductive tract and provide an optimal uterine environment for the embryo to establish pregnancy. Recently, platelet rich plasma (PRP) was used to reduce inflammation in mares susceptible to persistent mating-induced endometritis (PMIE)[1] and in mares with chronic degenerative endometritis (CDE).[2] Mares are defined as susceptible to PMIE when they are unable to overcome the inflammatory process within 12 - 24 hours after breeding[3] and CDE is a degenerative process in the endometrium characterized by fluid retention and a reduced ability to clear inflammation. In PMIE susceptible mares, uterine infusion of PRP 24 hours prior to AI substantially reduced intrauterine inflammatory cells and when infused either 24 hours prior to or 4 hours after AI, lowered COX-2 labeling.[1] Additionally, PRP treatments increased pregnancy rates in PMIE susceptible mares (30% without PRP infusion versus 60% with PRP infusion). In CDE mares, PRP treatment (4 hours after AI) decreased intrauterine fluid accumulation and decreased neutrophil numbers (24 hours after AI) in the uterine lumen; however, it did not decrease nitric oxide release.[2] PRP treatment before AI had a more pronounced reduction in inflammatory cell influx within the uterus,[1] suggesting better efficacy in controlling inflammation since it acts prior to the inflammatory insult. However, direct effects of PRP on sperm characteristics remain unknown.

Platelet rich plasma contains more platelets compared to whole blood.[4,5] Platelets are a natural reservoir of multiple proteins, cytokines, growth factors, adhesive proteins, clotting factors, fibrinolytic factors, proteases and antiproteases, and

membrane glycoproteins.[6,7] Platelet lysate (PL) is an acellular serum substitute obtained after lysis of platelet concentrates and is comprised of growth and chemotactic factors that promote cell proliferation and recruitment.[8] The higher purity of PL and acellularity imply higher GF concentrations compared to PRP.[9] The acellularity characteristic of PL is suggested to have a low potential for immune reaction when used autologously; however, immune reactions caused by heterologous versus autologous PL have not yet been characterized. Additionally, studies comparing PL with PRP regarding their growth factor contents and their stimulatory potential on cell function are lacking. Platelet pheresis in horses[10] resulted in 1-2.3 fold (1.8-fold average) higher concentration of platelets in PRP lysate than whole-blood platelet count. Furthermore, platelet lysates can be stored at -20°C up to 5 months and still maintain growth factor content stability.[11]

Fibroblast growth factor (FGF2), one of the key growth factors in PRP,[12] improved characteristics of human and mouse sperm[13-15] and increased percent motile sperm with higher kinematics after incubation with recombinant human FGF2.[14,15] The rhFGF2 concentration (0.1-1 ng/ml) used was similar to reported doses used with human PRP (0.15 - 0.30 ng/ml).[12] The concentration of FGF2 in human ejaculates was positively correlated with total sperm number and motility. Ejaculates exposed to exogenous FGF2 prior to sperm recovery (swim up technique) increased recovery of motile sperm.[13]

Objectives of this study were to determine if equine PRPL or FGF2 treatments would enhance sperm motility (sperm motion characteristics) and improve sperm survival in uterus by reducing semen-induced uterine inflammation.

Material and methods

Animals

Number of animals used was based on a power analysis of 80% and by applying preliminary data from humans,[13,14] wherein sperm motility increased from 42 ± 4 to 55 ± 4% after treatment with 100 ng/ml of rcFGF2 in 5 subjects. Selection of mature stallions was based on breeding soundness examination, presence of a normal external and internal reproductive tracts via visual examination and transrectal palpation, and fertility based on previous breeding season performance when a minimum of 30% total motility and > 1 x 109 progressively motile sperm were used. All procedures were performed in accordance with the institutional animal care and use committee at Auburn University.

Semen collection

Two separate ejaculates were collected from each stallion (n = 7). Collection was performed using a Missouri style artificial vagina with stallion exposed to an estrus or ovariectomized mare and mounting a breeding phantom. Semen analysis was performed by the same person within 30 minutes after collection. After first analysis and immediate dilution with semen extender, semen samples were maintained at 37°C until assigned to a treatment group (< 45 minutes from collection)

Platelet rich plasma lysate.

The PRPL was prepared as described.[10] Briefly, platelet concentrates (from 5 donor horses) were obtained following plateletpheresis (COBE Spectra Dual-Needle). Platelets were fractured using 2 freeze-thaw cycles followed by 3 centrifugation cycles. The PRPL was then filtered through a 40-µm cellulose acetate membrane (EMD Millipore, Billerica, MA) to remove cellular debris. An equal portion of lysate from each horse was combined to obtain a pooled product. Samples of PRPL (aliquots of 1 ml) were stored in a freezer at -80°C for further use.

Recombinant fibroblast growth factor 2

Equine rcFGF2 was produced by bacterial expression (R&D Systems, Minneapolis, MN). As recommended by the manufacturer, FGF2 was reconstituted to a maximum concentration of 250 µg/ml in water containing at least 0.1% bovine serum albumin. After reconstitution, diluted FGF2 was added to the ejaculate to achieve the desired concentrations of 0, 0.1, 1, 10, and 100 ng/ml. Treatment doses were formulated based on recombinant FGF2 treatments of human and mouse semen.[14,15] Reconstituted FGF2 was stored under sterile conditions between -20 to -70°C and used within 3 months (expiration date).

Semen sample preparation

Sperm concentration was measured using a NucleoCounter® SP-100™ (Chemometec, Allerod, Denmark). A volume containing 600 x 106 sperm was placed in 50 ml conical tubes, diluted 1 part semen to 4 parts of standard native phosphocaseinate-based extender (INRA 96®) and centrifuged at 900 x g for 10 minutes. The supernatant was removed and the sperm pellet re-suspended to a final concentration of 50 x 10^6 sperm per ml (confirmed via NucleoCounter).

The PRPL treatment groups consisted of the following: extended semen (CTRL), extended semen with 1 IU/ml of heparin (CTRL Hep), extended semen with 1 IU/ml of heparin and 1% PRPL (1% PRPL, extended semen with 1 IU/ml of heparin and 2.5% PRPL (2.5% PRPL), extended semen with 1 IU/ml of heparin and 5% PRPL (5% PRPL), and extended semen with 1 IU/ml of heparin and 10% PRPL (10% PRPL). The FGF2 groups consisted of the following: extended semen (CTRL), extended semen with 0.1 ng/ml of FGF2 (FGF2-0.1), extended semen with 1 ng/ml of FGF2 (FGF2-1), extended semen with 10 ng/ml of FGF2 (FGF2-10), and extended semen with 100 ng/ml of FGF2 (FGF2-100).

Semen analysis

Semen analysis was performed at 0, 0.5, 1, 1.5, 6, and 24 hours. Samples were cooled to 5 °C and stored in a standard semen shipping container after hour 1 analysis. Motion characteristics of sperm were assessed objectively after warming using a computer assisted sperm analysis (Sperm Vision® SAR, CASA software with PC monitor, Minitube, Verona, WI) with light threshold between 180 - 255, field-of-view depth of 20 µm, pixel ratio of 16 - 100 µm,2 and assessment requirements of 5000 sperm or 7 fields. Warmed aliquots of each treatment were placed in a 20 µm standard count analysis chamber (SC20.01. FA; Leja®, Nieuw-Vennep, The Netherlands). Parameters selected for analysis[14] were total (TMOT %) and progressive (PMOT %) motility, average path (VAP µm/s), straight-line (VSL µm/s) and curvilinear (VCL µm/s) velocities, amplitude of lateral head displacement (ALH µm); beat/cross frequency (BCF Hz); straightness (STR %), and linearity (LIN %). Additional measurements were performed including the total number of cells counted (TOTCELL number), distance curved line (DCL µm), distance average path (DAP µm), distance straight line (DSL µm), wobbling (WOB, VAP/VCL), slow motility (SLOWMOT %), nonprogressive motility (NPMOT %), average orientation change (AOC degree), and concentration (CONC cells/ml). Sperm morphology was subjectively assessed in the initial ejaculate by evaluating 100 sperm stained with eosin/nigrosin stain. The following abnormalities were assessed: abnormal head shape, abnormal midpiece, detached head, proximal droplet, distal droplet, bent tail, coiled tail, and premature germ cells.[16]

Data analyses

Data were analyzed separately for each experiment (PRPL and FGF2). Differences among groups, time, and interaction between groups and time were tested. Data were examined for normality using the Shapiro-Wilk test and if data were not normally distributed, they were transformed into natural logarithm or rank prior to each analysis. The SAS MIXED procedure (Version 9.4; SAS Institute, Cary, NC) was used with a repeated statement to account for correlation between sequential measurements and differences were detected by Tukey's test. A probability of $p \leq 0.05$ indicated that the difference was significant.

Results

Heparin effect on semen

To monitor effects of heparin on PRPL-treated samples, one heparin-only treated group was compared to a nontreated control. There was no effect of group, time, or interaction between group and time, and between heparin treated and control groups for all motility variables.

Platelet rich plasma lysate

For TOTCELLS and CONC, there were differences among groups, time, and interaction between group and time. Both variables had a decrease starting at hour 1 in groups 5 and 10% PRPL. Values for groups 5 and 10% PRPL remained lower than all other groups starting at hour 1.5, and values for group 10% PRPL were lower than group 5% PRPL starting at hour 1.5. In both groups, head-to-head attachment (HHA) was observed on the screen; however, it was not possible to objectively quantify.

Significant differences over time were noted for TMOT, PMOT, DCL, DAP, VCL, VAP, ALH, AOC, DSL, VSL, BCF, STR, WOB, LIN, and SLOWMOT; however, there were no differences among groups nor interaction between groups and time. Therefore, groups were combined for each variable to evaluate changes over time. TMOT and PMOT gradually decreased from 0 - 1.5 hours, followed by a plateau in TMOT and PMOT after hour 1.5 (Figure 1). Distance straight line (DSL) and VSL had a gradual decrease from hours 0 - 1, a plateau from hours 1 - 1.5, and a gradual decrease until hour 24. BCF, STR, WOB, LIN, and

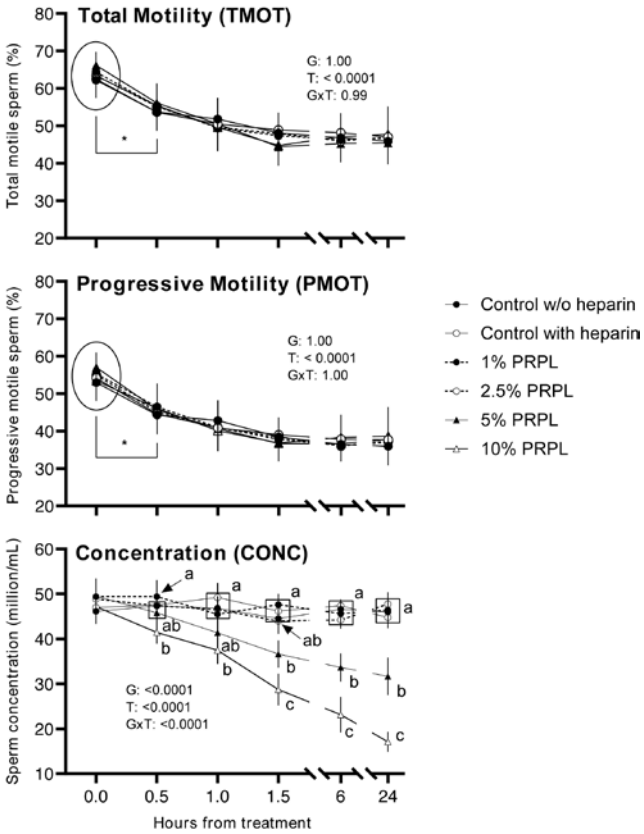

Figure 1. Total motility (TMOT), progressive motility (PMOT), and concentration (CONC) at 0, 0.5, 1, 1.5, 6, and 24 hours in the control group and with 0, 1, 2.5, 5, and 10% of PRPL in 1 IU/ml of heparin. Significant time changes in direction (increase or decrease) for groups combined are represented by a circle and the first significant change on direction for time in the groups combined is indicated by an asterisk (*). Group differences within a time point are represented by lowercase letters (a, b, and c). Groups with statistically similar values at a specific time point are grouped by a square and represented by a lowercase letter (a, b, and c).

SLOWMOT had an increase from hour 0 - 0.5, a decrease from hours 0.5 and 1, a plateau from hours 1 - 1.5, and a gradual decrease until hour 24.

Fibroblast growth factor 2

There were no differences between PRPL and FGF2, and no effect of group or interaction between groups and time for all FGF2 treated groups. However, a significant effect of time was noticed for all variables, except for TOTCELLS and CONC that had constant values throughout the experiment. Therefore, all groups were combined and changes among times had a similar pattern to PRPL experiment. The variables TMOT and PMOT had a gradual decrease from hours 0 - 6 followed by motility maintenance until hour 24 (Figure 2).

The variables DCL, DAP, VCL, VAP, and ALH decreased from hours 0 - 1, and AOC decreased from hours 0 - 6. There was an increase was on DCL, VCL, and ALH starting at hour 0.5, and with DAP, VAP, and AOC starting at hour 6. The variables DSL and VSL had a gradual decrease starting at hour 0 until hour 24. A decrease was present with BCF, STR, and LIN, starting at hour 0.5 and on WOB and SLOWMOT starting at hours 1 and 1.5, respectively. There were no significant effects of group, time or interaction between group and time between PRPL or FGF2 treatments on any sperm motility variables.

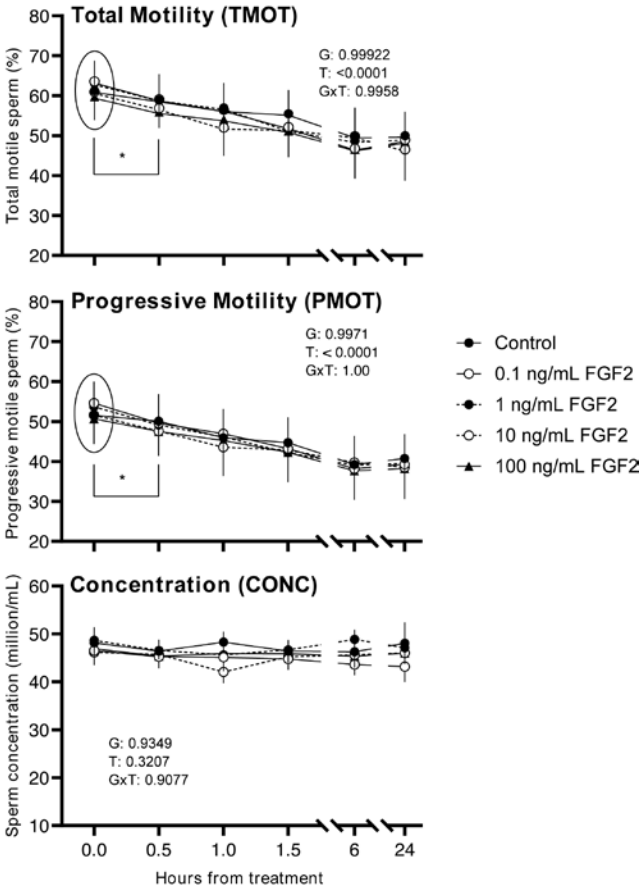

Figure 2. Total motility (TMOT), progressive motility (PMOT), and concentration (CONC) during 0, 0.5, 1, 1.5, 6, and 24 hours in the control group and after treatments with recombinant equine FGF2 at concentrations 0.1, 1, 10, and 100 ng/ml. Significant time changes in direction (increase or decrease) for groups combined are represented by a circle. The first significant change on direction for time in the groups combined are indicated by an asterisk (*)

Discussion

Treatment with PRPL at concentrations > 5% resulted in sperm HHA, identified on gross microscopic examination by decreased TOTCELLS and CONC values (note: CASA does not identify agglutinated cells as individual motile sperm). Head to head agglutination could be considered an artifactual reaction to components of PRPL (i.e. fibrin) rather than an important physiological event. It is possible that PRPL contributed to slight amounts of gel formation entrapping sperm and resulting in HHA. Regardless of the doses of PRPL, HHA was observed. Since the heparin dose remained constant in this study, further work may evaluate increasing amounts of heparin and observe the incidence of HHA.

Use of serum in bull sperm resulted in HHA in immotile sperm.[17] Several compounds (heparin, serum or follicular fluid in monkeys,[18] bulls,[19,20] and pigs[21]) induced HHA. In contrast, HHA was not due to heparin addition in our study. In rabbits, HHA was detected (in utero) when motile sperm came in contact with uterine luminal secretions.[22] Furthermore, HHA was induced by dibutyril-cAMP and due to dilution, washing, cold shock, extreme pH, or certain osmotic conditions.[23,24] However, these conditions were controlled across groups in the current study.

Besides the HHA after PRPL treatment, and in contrast with reports in human and mouse,[13,14,25] there was no effect on motility by either PRPL or FGF2 treatments. However, these studies used various media and commercially available semen extenders (with no mention of components for patent purposes). It is reasonable to consider that factors present in semen extenders could potentially interfere or even mask effects of additional PRPL and FGF2 on sperm motility improvement. Future studies may determine the effect of a nonnative phosphocaseinate-based extender (could be beneficial for stallions that do not have an adequate sperm preservation with native phosphocaseinate-based extenders). However, the complete absence of extender would compromise the lifespan and viability of sperm during storage due to lack of energy source, buffer, and antibiotics.

The hypothesis that PRPL treatment is not deleterious for sperm survival was supported at concentrations < 10%. However, HHA occurred in concentrations > 5%. Sperm survival can be measured by the maintenance of sperm motility over a period that decreased at a similar rate in all groups. Since there was no difference in sperm motility between treated versus control groups, it could be suggested that direct PRPL addition to the

ejaculate does not negatively affect sperm motility parameters. Addition of heparin to sperm has been shown to induce sperm capacitation at higher doses than was used in this study.[26] Evaluation of sperm kinetic analysis showed no differences between groups in lateral/side to side head movement and tail beat frequency (indicating hyperactivity). Hyperactivation can be an indicator of sperm capacitation.[27] As in bulls,[8] both hyperactivation and acrosome reaction during capacitation are processes that can be independently stimulated.

Although addition of PRPL had no apparent benefit to sperm, it may be beneficial in modulating post-mating endometritis, since the dense alpha granules of platelets secrete a multitude of chemokines, cytokines, and bioactive molecules with regenerative capacity, rather than just growth factors.[29] In PMIE mares, there was a down-regulation of mRNA expression in endometrial proinflammatory cytokines (IL-1b, IL-6, IL-8, and iNOS)[30] when autologous PRP was infused 24 - 36 hours before AI. Furthermore, fluid retention was reduced and pregnancy rates improved.[31] However, there was no study that used PRPL instead of PRP to evaluate the overall volume and concentration of PRPL necessary to provide uterine immunomodulation after breeding in mares. Infusion of 20 ml of PRP either 24 hours before or 4 hours after AI in PMIE mares was successful in controlling the inflammatory response and increased pregnancy rates.[2] However, use of PRPL has to be tested in the uterus for its immunomodulatory effect and immune responses after autologous versus heterologous origin.

The hypotheses that FGF2 added to semen extender enhances sperm motility was not supported, but the hypothesis that sperm motility would be maintained was supported. There were no significant changes in sperm motility after FGF2 treatment. Sperm motility was maintained throughout the experiment and the decrease of motility over 24 hours was similar in all FGF2-treated groups. Therefore, there was no beneficial or detrimental effect of FGF2 on sperm motility. This finding was contrary to human studies where recombinant human FGF2 treatment (incubation with 10 ng/ml of FGF2) increased both total number and motile sperm recovery and improved sperm VCL, VSL, and LIN values.[13] This suggests that the FGF2/FGFRs system is involved in motility regulation similar to results observed in mice.[14,32] In the present experiment, there was no significant effect on sperm characteristics. There was also no effect of FGF2 at the concentrations tested that would encourage the clinical use of FGF2 for improvement of stallion sperm motility. One important difference from the present experiment and others performed in humans and mice is the media used for the analysis.[13,14,32] In the present experiment, the extender used has a broad variety of factors that are beneficial to sperm. Therefore, as suggested in the PRPL experiment, the presence of the extender could interfere with FGF2 actions or even mask positive effects on sperm motility. Additionally, sperm capacitation effect of FGF2 should be considered, as in the murine model (FGF2 treatment increased sperm motility, velocity, enhanced intracellular Ca2+ concentrations, and acrosomal exocytosis).[32]

In conclusion, use of PRPL or FGF2, at the concentrations studied, did not have apparent detrimental effects on sperm over 24 hours. Doses of PRPL < 10% did not interfere with sperm survival and PRPL doses > 5% induced HHA. The physiological relevance HHA observed after treatment with PRPL is uncertain and is more suggestive of an artifactual reaction between sperm and components of PRPL and the semen extender. For further investigation, the use of raw semen would be preferred to test the effect of both compounds without extender interference. For sperm capacitation, the addition of PRPL and FGF2 without the extender component should be tested with the molecular evaluation of either acrosomal exocytosis, Ca2+ intracellular influx, and sperm hyperactivation. Clinically, PRPL could be added to extended semen prior to breeding, focusing on uterine inflammatory control. However, studies are needed to control HHA and to characterize the immunomodulatory effect of PRPL within the uterus.

Acknowledgements

Authors thank Dr. John F. Peroni (University of Georgia) for PRPL samples and Marti McCoy for assistance in stallion management and handling. Research supported by the Theriogenology Foundation and the Department of Clinical Sciences (Auburn University).

Conflict of interest

No conflict of interest to declare.

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