# Effect of Platelet Rich Plasma Lysate and Fibroblast Growth Factor 2 on Sperm Motility in Stallions

by

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## Abstract

Semen extenders are continually tested to improve sperm motility, longevity, and, consequently, improve fertility. Growth factors (GFs) modulate cell function, which could be advantageous to sperm by improving motility. In humans and mice, fibroblast growth factor 2 (FGF2) improved sperm motility. Platelet rich plasma (PRP), which is rich in growth factors (GFs) including FGF2, reduced post-mating inflammatory response within the uterus when infused 24 hours before or after artificial insemination. Effect of PRP on sperm has not been determined. In this study, PRP lysate was used instead of PRP since it is more purified and highly concentrated in GFs. The objective was to evaluate the effect of adding either pooled PRP lysate (PRPL) or recombinant equine FGF2 (reFGF2) at varying concentrations to sperm extended in commercial semen extender (INRA 96) in the stallion. Eight aliquots were tested using concentrations of 1, 2.5, 5, and 10% of PRPL containing 1 IU/ml of heparin, and 0.1, 1, 10, and 100 ng/ml of reFGF2 and compared to control groups with and without 1 IU/ml of heparin. Heparin use was based on a pilot study to prevent gel formation from PRPL reaction with semen extender, precluding motility analysis. Motility parameters were evaluated with samples standardized to 50 million sperm/ml using computer assisted semen analysis (CASA) at hours 0, 0.5, 1, 1.5, 6, and 24 after treatment. For both PRPL and reFGF2 treatments, there were no differences ($p = 0.99$) in motility among groups at any time point, with ranges from 60 to 66% at hour 0 and 46 to 49% at hour 24. Interestingly, results revealed that concentrations of PRPL > 5% induced sperm agglutination via head-to-head attachment (HHA), starting at hour 1, whereas PRPL at concentrations below 2.5% did not induce HHA nor affect sperm motility. In addition, HHA was objectively detected by decreases in total number of cells counted per field (total cells) and estimated concentration measured by CASA, assuming that only free sperm were counted, whereas sperm entrapped by HHA were not counted

by CASA. Decreases in total sperm number and concentration were different ($p < 0.0001$) for 5 and 10% PRP groups, being more pronounced for 10% PRPL group, suggesting a dose-dependent characteristic of HHA induced by PRPL. One important finding was that PRPL induced HHA but did not kill sperm. Although no motility improvement was observed, results suggests that direct addition of PRPL in semen extender at doses below 5% could be used without substantially affecting sperm motility. However, further research on uterine inflammatory response is needed to test whether this would have a beneficial effect in the endometrium.

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| | |
|---|---|
| PRP | Platelet rich plasma |
| PRPL | Platelet rich plasma lysate |
| FGF2 | Fibroblast growth factor 2 |
| GF | Growth factor |
| HHA | Head-to-head attachment |
| TOTCELLS | Total number of cells counted |
| CONC | Concentration |
| TMOT | Total motility |
| PMOT | Progressive motility |
| DCL | Distance curved line |
| DAP | Distance average path |
| VCL | Velocity curved line |
| VAP | Average path |
| ALH | Amplitude of lateral head displacement |
| AOC | Average orientation change |
| DSL | Distance straight line |
| VSL | Velocity straight line |
| BCF | Beat cross frequency |
| STR | Straightness |
| WOB | Wobble |
| LIN | Linearity |
| SLOWMOT | Slow motility |

## 1. Introduction

Assisted reproduction techniques in horses aim to disseminate an animal's genetics by improving methods of breeding, sperm survival, fertilization of the oocyte, and implantation of the embryo. An example of a commonly used assisted reproduction technique is artificial insemination (AI), which allows allocation of sperm into several breeding doses and provision of appropriate nutrients and/or stimulatory proteins that support sperm cells outside the uterus during shipment and/or storage. Breeding success is influenced by many factors, including semen quality, which can be influenced by extender composition, and the mare's response to the semen post-breeding. Therefore, new clinically useful approaches to improving semen quality and reducing post-mating endometritis of fresh or cooled transported semen are needed.

Sperm motility and viability can be improved by the composition of semen extender. Uterine inflammatory response can be reduced by decreasing contamination and supporting uterine clearance, achieved through intra-uterine antibiotic infusion, uterine lavage, ecbolic agents, and anti-inflammatory compounds. Therefore, new techniques to improve semen quality in adjunction to the control of post-breeding uterine inflammation would have a good value for conception rates, especially in mares susceptible to endometritis.

Recently, a blood-derived biologic compound named platelet rich plasma (PRP), proved useful in reducing post-breeding endometritis (1, 2). Uterine infusion of PRP post-breeding significantly reduced the uterine inflammatory response as measured by a reduction of inflammatory cells in the uterus of mares that previously experienced prolonged post-breeding endometritis. Consequently, a significant increase in confirmed pregnancies (30% without PRP infusion vs. 60% with PRP infusion) was observed. However, there were no studies evaluating the effects of PRP supplementation on sperm characteristics.

Platelet rich plasma is a portion of plasma that contains a greater number of platelets compared to whole blood and platelets are a natural reservoir of multiple proteins, cytokines, growth factors, adhesive proteins, clotting factors, fibrinolytic factors, proteases and antiproteases, and membrane glycoproteins (GFs) (3, 4). GFs contained within α granules of platelets aid in the regulation of the inflammatory niche of a wound through chemo attraction, cell proliferation and maturation, matrix synthesis, and angiogenesis (5).

Platelet lysate (PL) is an acellular serum substitute obtained after lysis of platelet concentrates and is composed of growth and chemotactic factors that promote cell proliferation and recruitment (6). Previous report of platelet pheresis in horses showed a 1 to 2.3 higher concentrations of platelets in PRP lysate than whole blood platelet count (7). In addition, platelet lysates can be stored at -20$^{o}$C for at least five months, while maintaining stability in growth factor content (8).

Fibroblast growth factor (FGF2), one of the key growth factors in PRP (9), has been shown to improve the characteristics of human and murine sperm (10-12). Sperm cells incubated with recombinant human FGF2 (rhFGF2) showed an increase in the percentage of motile cells and enhanced sperm kinematics using human (11) and murine (12) models. These previous experiments tested rFGF2 concentrations (0.1 to 1 ng/mL) within the range reported to be present in human PRP (0.15 to 0.30 ng/mL) (9). In addition, FGF2 concentration in the ejaculate was positively correlated with total sperm number, percentage of motile cells, and total motile spermatozoa, which was supported by the incubation of the ejaculate with rFGF2 that led to an increase in both total number and motile sperm recovered by the swim up technique (10).

Therefore, the aims of this project were to determine if PRPL or FGF2 treatments enhance sperm motility and affect sperm survival. The following hypotheses were tested: (1)

Platelet rich plasma lysate and FGF2 added to semen extender will enhance sperm motility and (2) sperm characteristics will be maintained when PRPL or FGF2 are added to semen extender. Positive results will allow further research involving the effect of PRPL and FGF2 on sperm capacitation, the effect of PRPL added to semen extender on the uterine inflammatory response post-breeding, and possible fertility trials for clinical application.

## 2. Literature review

### 2.1.Cool shipped semen

Artificial insemination (AI) in horses is a widely used tool in horse reproduction (13). Assisted reproduction techniques, such as AI and intracytoplasmic sperm injection (ICSI), require the storage of sperm for its application either by chilling or cryopreserving the sperm (14). During storage, it is inevitable to have cell death which can be delayed or even prevented through temperature-induced metabolic restriction by chilling or cryopreserving the sperm cell. The use of chilled or cryopreserved semen depends on the length of time desired before insemination.

Sperm chilling is the most widely used technique for the transport and storage of stallion spermatozoa. Commercially available passive coolers are used to achieve a controlled chill rate to slowly cool extended semen to a temperature of between 4 and 10$^{o}$C and reduce sperm metabolism sufficiently to maintain sperm functionality for up to 72 hours (15). Milk-based extenders are routinely incorporated into media for chilled semen due to their membrane-stabilizing effects (16). Cold shock can occur when the sperm cell is not able to survive the cooling process and is usually due to an improper cooling rate or lack of cell protection. In stallions with a low tolerance for cooling, cholesterol-loaded cyclodextrins (CLCs) have been shown to improve membrane fluidity and ameliorate cold shock (16-18), although with an effect on delaying sperm capacitation (17). Other compounds used for cooling semen are coenzyme Q10 (a-tocopherol) (19) and superoxide dismutase (20). Antioxidant treatments have been attempted, but disappointing results were obtained (21) and it is hypothesized that antioxidants have an interaction or interference with milk constituents in the extenders (22).

The restriction of metabolic rate by cooling can also reduce the production of toxic metabolic by-products, such as hydrogen peroxide, lipid aldehydes, and carbon dioxide (23, 24). Decreased temperature slows the depletion of factors associated with the maintenance of homeostasis, such as adenosine triphosphate (ATP) (23, 24). The temperature-induced metabolic restriction can also reduce the production of reactive oxygen species (ROS) and slow-down the acidification of the storage medium through the accumulation of lactic acid and $CO_2$ from glycolysis and oxidative phosphorylation.

As one of the constituents of the ejaculate, seminal plasma has been shown to be beneficial to the viability and acrosome integrity of spermatozoa from boars, bulls, and rams (25). However, studies have also shown that high concentrations of seminal plasma can be detrimental to the motility and membrane integrity of stallion spermatozoa over time (26-28). In stallions, a small amount of seminal plasma (0.6%–20%) is beneficial to both sperm parameters and fertility (29, 30). Seminal plasma has an inhibitory effect on the binding of polymorphonuclear neutrophils, reducing sperm phagocytosis within the female reproductive tract, and enhancing fertility when there is underlying uterine inflammation (31).

Poor quality semen, which contains increased amount of abnormal and/or non-viable sperm, is believed to have higher amounts of ROS produced by non-viable cells (32). These non-viable cells will accelerate the demise of the remaining sperm population during storage. In addition, enzymes that generate ROS can also affect the amino acid substrates found in animal protein-based semen extenders, such as milk based and egg yolk–based semen extenders (33). One factor shown to be released primarily by dead spermatozoa is L-amino acid oxidase and, when left in the insemination dose during semen processing and storage, a quick decline in motility and viability will occur (34). Therefore, the reduction of ROS in stored sperm is desired,

as low levels of oxidative stress results in decreased oxidative damage of DNA and maintain the sperm cell's ability to participate in fertilization (35).

Sperm motility can be evaluated subjectively by a visual estimation of the proportion of motile and non-motile sperm or objectively using computer assisted semen analysis (CASA), which is able to trach the sperm movement and calculate the proportion of motile sperm and also changes on movement and velocity. Subjective evaluation is expected to have 30-60% variation among observers or laboratories (Coetzee 1999, Davis 1992). The CASA evaluation prevents this variation of subjective analysis caused by skill of the observer, the method of specimen preparation, and number of cells evaluated. Motility parameters calculated by the CASA include total (MOT, %) and progressive (PROG, %) 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, %). In addition, other parameters can be includes as the total number of cell counted (TOTCELL, cell number), distance curved line (DCL, μm), distance average path (DAP, μm), distance straight line (DSL, μm), wobbling (WOB, VAP/VCL), slow motility (SLOWMOT, %), non-progressive motility (NPMOT, %), average orientation change (AOC, degree), and concentration (CONC, cells/mL).

Proportion of moving sperm versus non-moving sperm is calculated by TMOT. Variations on sperm motion are detected by PMOT, PMOT, ALH, BCF, STR, LIN, WOB, and NPMOT. Variations on sperm path can be detected by DCL, DAP, DSL, and AOC, and changes on sperm velocity can be detected by VAP, VSL, VCL, and SLOWMOT. In addition, concentration can be converted using TOTCELL divided by the volume of the microscopic fields

counted, represented by CONC. A representation of the measurements is shown in Following figure by (36).

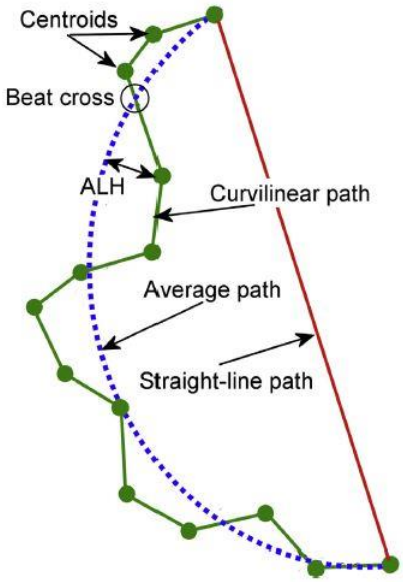



Figure from (36). Illustration showing CASA terminology. Initial image processing provides a centroid for each spermatozoon in the first frame of a scene, and for each cell location of the most probable centroid in successive frames is deduced. Connecting the centroids for a spermatozoon provides its actual trajectory, termed curvilinear path. The time-averaged velocity along this trajectory is termed curvilinear velocity (VCL; mm/s). The average path is computed, and time-averaged velocity along this trajectory is termed average path velocity (VAP; mm/s). A straight-line path from the first to last position of a sperm head is plotted, and velocity along this trajectory is termed straight line velocity (VSL; mm/s). For each centroid location there is a deviation from the average path, and this is termed the amplitude of lateral head displacement (ALH; mm). Similarly, there are points where the curvilinear path intersects the average path, and the number of such intersections is termed beat-cross frequency (BCF; number per second). CASA, computer-assisted sperm analysis.

In the present study, motility analysis will be performed using CASA to evaluate changes on sperm motility during storage after *in vitro* treatments of semen samples. Improved sperm motility is the first step for detection of a potential treatment for sperm activity and survival for storage. If positive effects are observed, molecular analysis for specific events for sperm fertilization as cell membrane stability and capacitation are the next steps.

### 2.2.Platelet rich plasma and platelet lysate

Platelets are cellular fragments of megakaryocytes in peripheral blood that are known primarily for their role in hemostasis. The basic cytokines identified in platelets include transforming growth factor–β (TGFβ), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-I, IGF-II), fibroblast growth factor (FGF), epidermal growth factor, vascular endothelial growth factor (VEGF), endothelial cell growth factor, zinc (Zn), and superoxide dismutase (SOD) that are stored in a dense membranous system, the alpha granules (3, 37-40). Other molecules inside platelet α-granules include adhesive proteins, clotting factors, fibrinolytic factors, proteases and anti-proteases, membrane glycoproteins serotonin, histamine, dopamine, calcium, and adenosine (4). The processing of whole blood to concentrate platelets should be done under anti-coagulation factors because in coagulated blood, platelets become part of the clot, are lysed, and lose their content.

One of the benefits of PRP therapy includes the minimally invasive harvest from the patient, rapid and relatively easy preparation, and low relative cost. Production of PRP can be performed by concentration of platelets using either centrifugation (41) or filtration (42). Centrifugation is the most common processing method and the product can be obtained manually or using commercially available kits. Several commercial systems are available to extract PRP from whole blood, but there is great variability in the final platelet, leukocyte, and red blood cell concentration. One of the filtration methods is plateletpheresis which has the benefit of collection of large volumes of platelet concentrate from standing and awake horses and can then be used to produce PRP (7). Another benefit of plateletpheresis is that the process generally involves fewer steps than a protocol from whole blood and may decrease the likelihood of contamination (7).

Prior to use of PRP, platelets need to release GFs from alpha granules by the process of activation (41) which allows a greater concentration of growth factors to be available when compared to non-activated PRP (43). However, this process still leaves intact platelets, white blood cells, fibrinogen, and fibrin which can induce immunogenic activity after application (44). Natural activation of platelets results from exposure to collagen, but physical or chemical activation may be performed in vitro. The most common activation methods include mechanical disruption of the platelet through freezing and thawing, or chemical disruption through exposure to bovine thrombin or calcium chloride ($CaCl_2$) (43, 45). The most feasible activation method is the freeze and thaw involving a snap freeze and storage in a -20°C freezer. The aim of the freeze/thaw activation is to physically injure the platelets to initiate degranulation, but there is no consensus regarding the number of freeze/thaw cycles necessary for complete degranulation. Many protocols recommend four freeze/thaw cycles for *in vitro* studies. As one example of freeze/thaw activation for experimental use, PRP is frozen, then thawed in a 37°C water bath and microcentrifuged at 21,000 x g for 10 minutes. The supernatant, which contains the factors of lysed platelets, is collected and considered activated PRP. (43).

Although currently not easily available for equine practitioners, platelet rich plasma lysate (PRPL) is an acellular substitute for PRP obtained after the lysis of platelet concentrates by plateletpheresis. It is like PRP, but with a higher concentration of growth and chemotactic factors that have been shown to promote cell proliferation and recruitment (6). Most studies using both PRP and PRPL involve the use as therapeutics for healing wounds, soft tissues injuries, and a variety of musculoskeletal injuries in both human and veterinary patients (46, 47). Since it is acellular, PRPL has been used as an alternative to bovine fetal serum for the expansion of stem cells in a variety of experimental and clinical cell transplantation studies (6, 48, 49). In

human medicine, PRPL presence in culture media induced mesenchymal cell expansion and maintained their differentiation potential and immunophenotypical profile (50, 51). Another benefit of PRPL is its use in a allogenic manner (pooled), due to its lower immunogenicity while still preserving a higher concentration of growth factors and cytokines compared with PRP (7, 48, 52-66). In addition to the ability to pool PRPL, it can be stored for at least 1 year (67), which allow the possibilities of standardizing PRPL for use in regenerative medicine and other applications.

The mechanism of action for both PRP and PRPL is the release of GFs from α granules and effect on the regulation of inflammation. (5). For that reason, most of the applications of PRP in equine practice include the inflammatory control of joints, bursa, and soft tissue injuries (e.g., tendonitis, tenosynovitis, and skin wounds) (68-71). In equine reproduction, the first mention of blood derivatives on post-mating endometritis was using autologous plasma in association with antibiotic therapy to successfully improve pregnancy rates in lactating and barren mares (72). The mechanism that blood derivatives and plasma or PRP act in the endometrium and control inflammation is not yet well elucidated. Studies using PRP have shown that the anti-inflammatory action of PRP is due to its ability to suppress the expression of COX-2, metalloproteinase-3 (MMP-3), TNFα, IL1, and vascular adhesion molecules (73-75). In mares suffering from chronic endometritis, treatments of PRP at the time of breeding were able to reduce uterine inflammation and increase pregnancy rates (2). As previously shown in humans and horses, platelet granules containing antimicrobial peptides (RANTES, platelet factor 4, and thymosin beta-4), may contribute to PRP's known bactericidal activity against Staphylococcus aureus, Escherichia coli, and Klebsiella pneumoniae (76-83), which are bacteria known to cause endometritis in mares (84). In horses, PRP has been shown to display antibiofilm properties and

restore antimicrobial activity against synovial fluid biofilms and has synergism with amikacin in reducing bacterial load in synovial fluid (85).

The only study using PRP supplementation with sperm was performed recently in humans (86). Supplementation of semen with PRP showed an improvement on sperm motility and protection against oxidation as shown by a decrease in ROS levels, increase of sperm viability, reduction in sperm head vacuolization and decrease in DNA fragmentation (86). Interestingly, doses of 2% and 5% PRP, but not 10% PRP, were able to improve sperm motility and protection against oxidative stress when sperm were challenged with hydroxide peroxide. On the other hand, 10% PRP was deleterious for both sperm motility and viability. Moreover, better outcomes were obtained with 2% PRP compared with 5%PRP.

In horses, no studies have been performed using PRP or PRPL supplementation in semen. The high amount of GF present in PRP and the positive results in other species with the use of both PRP and GFs on the improvement of sperm motility and/or oxidative stress protection raises promising outcomes on its use. In addition, the synergic action of PRP on the modulation uterine inflammation would be an additional benefit on its clinical application.

### 2.3.Uterine inflammatory response modulation post-mating

As the major cause of subfertility, endometritis is considered the third most common disease affecting mares (87, 88). A transient uterine inflammatory response occurs in all mares approximately 30 minutes after natural or artificial breeding. The goal of the transient uterine inflammatory response is to eliminate seminal plasma, microorganisms, debris, and excess sperm, preparing the uterine lumen for the arrival of an embryo (89). Mares are classified as susceptible or resistant to persistent breeding-induced endometritis (PBIE) based on their ability

to clear this inflammation by 48 hours post-breeding (90, 91). In PBIE mares, the inflammation can persist for more than 96 hours impairing embryonic survival and establishment of pregnancy (92-94). The main cause of PBIE is poor uterine clearance often due to poor vulvar conformation and a pendulous uterus (95) found most in older multiparous mares.

The diagnosis of endometritis involves multiple approaches combined with thorough history evaluation. Endometrial culture, cytology, and biopsy are the most common tools employed to diagnose endometritis in mares (96). Mares susceptible to PBIE may have a history of accumulating intrauterine fluid before and after breeding, recurrent embryonic loss, early return to estrus, failure to become pregnant despite good breeding management, and vulvar discharge. Currently, the lack of response to traditional therapy and the increasing prevalence of antimicrobial-resistant pathogens has led to the development of alternative therapies to treat mares suffering from chronic endometritis (97).

Therapies to control uterine inflammation include a combination of uterine lavage, ecbolic agents, anti-inflammatories, and antibiotics, which can fail in some mares (84, 97). Mares with defective reproductive anatomy (e.g., poor vulvar conformation, torn vestibulovaginal sphincter, pendulous uterus, impaired uterine contractility, incompetent cervix, and atrophied endometrium folds) are more prone to aspirate air or accumulate fluid or urine in the vaginal vault and uterus, which makes the mares more prone to endometritis (84, 98). Mares with a competent immune response and functional reproductive anatomy are able to clear infections spontaneously (i.e., mares are resistant to endometritis), whereas mares with a deficient immune response may be unable to combat the development of an infection or may have persistent inflammation (99-102). The most recent post-mating endometritis treatment

protocols include ecbolics, antibiotics, uterine lavage, biofilm treatment, immunomodulatory agents, lactoferrin, platelet-rich plasma, and stem cells (103).

As described in the previous section of this literature review, PRP showed promising improvement of PBIE, but the mechanism of action on the inflammatory response within the uterus is not yet understood. For the present study, PRPL could also act as a modulator of uterine inflammatory response if directly diluted in the semen dose. Additionally, if PRPL acts negatively on the sperm, then it should not be used in the uterus close to insemination.

### 2.4.Heparin effect on semen

Heparin-binding proteins have been isolated from the seminal plasma of bulls (104), boars (105-107), stallions (108) and dogs (109). Heparin is a glycosaminoglycan and has been reported to capacitate sperm in rabbit (110) and bull (111). Glycosaminoglycans are present in uterine and oviductal secretions, and follicular fluid and have been shown to stimulate capacitation, acrosome reaction and fertilization in bull spermatozoa (111-115). Heparin can also be used *in vitro* to enhance capacitation and induce acrosome reactions in bovine (115).

Stallion and boar spermatozoa respond to heparin by increasing intracellular calcium concentration, suggesting that heparin may play a role in the fertilizing ability of the spermatozoa through inducing capacitation (109, 116). Although heparin is a component of bovine oviductal fluid (117), the ability of oviductal cell monolayers to decrease calcium uptake in ejaculated equine spermatozoa have not been associated with heparin, as shown in heparin supplemented media, which did not increase intracellular calcium concentrations (118). Glycolytic substrates, such as glucose, have been reported to block heparin-induced capacitation in bull spermatozoa (119). In stallion semen, virtually no fructose is present and spermatozoa

rely only on glucose as an energy source (120). Compared to bulls, stallion spermatozoa may be more tolerant to the presence of glucose in the environment. A possible explanation for the block of heparin-induced capacitation by glucose is that glycolysis or other similar substrates leads to an acidification of bovine sperm that blocks heparin-induced capacitation (121).

In the stallion, heparin has been used to facilitate capacitation and acrosome reaction (122-124). A statistically significant increase in the percentage of acrosome reacted spermatozoa occurred at a dose of 100 µg/ml of heparin (equivalent to 50 IU/mL), when incubated for 4 h, without a decrease on sperm viability (122). Other report using a lower dose of 10 µg/ml (equivalent to 5 IU/ml) of heparin also induced a significant increase in the percentage of acrosome reacted sperm 4h after incubation (124). Yet another study concluded that only doses higher than 50 µg/mL (equivalent to 25 IU/ml) would induce acrosome reaction (123), showing inconsistency on heparin induced sperm capacitation in stallions among laboratories. In contrast, other reports showed no effect or detrimental effect of heparin on *in vitro* fertilization of equine spermatozoa (125, 126). The apparent insensitivity of stallion spermatozoa to lower concentrations of heparin and inability of low concentrations of heparin to support *in vitro* fertilization (125, 126) may reflect differences in heparin binding sites or binding affinity for heparin to stallion spermatozoa in contrast with the bull, in which 10 µg/ml of heparin can induce capacitation and acrosome reaction (111, 113, 119, 127). However, bull spermatozoa capacitated with heparin demonstrates intense head-to-head attachment (HHA) (111, 128) which interfere with motility analysis resulting in lower estimations of motility when, in fact, motility may have been similar between heparin and non-heparin-treated spermatozoa (123). In stallion, HHA was minimal after heparin treatment of sperm and motility evaluation was not affected (123).

For the present experiment, heparin treatment for either PRP and PRPL was necessary to prevent a gel reaction of milk-based extender and likely fibrinogen present in the PRPL. The possibility of sperm capacitation induced by heparin is important to consider, even with a low dose. To check the occurrence of capacitation, a comparison of hyperactivated sperm motility between treated and non-treated groups would be an easy and feasible measurement, that can be confirmed by a molecular analysis using pisum sativum Agglutinin labeled with fluorescein isothiocyanate and Hoechst dye Nu33258 (double fluorescence) (FITC-PSA) (Risopatron et al. 2001; de Andrade et al. 2018) to measure the proportion of capacitated sperm.

### 2.5.Fibroblast growth factor 2 effect on semen

Fibroblast growth factor (FGF2) is one of the growth factors present in human PRP (9) and has been shown to enhance certain characteristics of sperm in humans and mice. Ejaculates incubated with recombinant FGF2 had an increase in the percentage of motile cells and enhanced sperm kinematics using human (11) and murine (12) models. In humans, samples incubated with 100 ng/mL of FGF2 had significantly higher percentages of total and progressive motility, as well an increased linearity (LIN), amplitude of lateral head displacement (ALH), beat/cross frequency (BCF), and velocity parameters, which represents advanced sperm functionality. This effect of FGF2 was confirmed by preincubation with BGJ398 (FGF2 receptor inhibitor) which inhibited the motility stimulation response (11).

In mice, sperm motility increased 30 min after treatment with 0.1 and 1 ng/mL of rFGF2 followed by a decrease after 60 and 90 min (12). Intracellular $Ca^{2+}$ levels and acrosomal exocytosis, known to occur during sperm capacitation, increased after FGF2 treatment (12). Important consideration is that the experiment in mice was performed using sperm harvested

from the epididymis, while in humans, it was performed using natural ejaculates which contains seminal fluid. Therefore, the absence or presence of seminal plasma should be considered as a confounding effect for the sperm response to FGF2.

Concentrations of FGF2 in human ejaculate are positively correlated with total sperm number, percentage of motile cells, and total motile spermatozoa (10, 129, 130). Human spermatozoa contain receptors for fibroblast growth factor (FGF) 1, 2, 3, and 4 (131), which are present at the flagellum (middle and principal pieces) (11) and acrosomal region (10). The exposure of sperm to FGF2 led to an increase in the phosphorylation of flagellar FGF receptors and to the activation of extracellular-signal-regulated kinase (ERK) and protein kinase B signaling pathways (11). Furthermore, FGF2 has also been measured in the bovine oviduct, oocyte, and cumulus cells (132), suggesting that FGF2 may also play a role in sperm transport in the female reproductive tract. These findings support a possible physiological effect of FGF2 on stimulation of sperm cell motility in the female reproductive tract (129, 130, 133, 134). Therefore, FGF2 shows promising characteristics that would qualify it as a potential supplement to ejaculates for sperm selection techniques (10).

In addition to the observed increase in motility, the incubation of human ejaculates with 10 ng/mL of FGF2 led to an increase in both total number and motile sperm recovered by the swim up technique, in comparison with 0 and 100 ng/mL (10). The apparent loss of effect at 100 ng/mL was suggested to be due to a saturation of FGF receptors and inhibition of complex receptor/ligand formation, preventing rFGF2 stimulation of sperm motility. This phenomenon is defined as a biphasic-dose response, previously observed in cellular response to FGF (135). Sperm agglutination was observed when semen was diluted in an ascending gradient solution of FGF2, but not when diluted in a solution with a homogeneous concentration (12). This

phenomenon supports the chemokinetic action of FGF2, resulting in the increment of sperm velocity and cell to cell migration (12).

Other GFs were also able to improve semen motility in other species than horses. In rams, EGF was able to increase sperm motility and progressive movement at a dose-dependent manner, which started 30 minutes after treatment and was maintained for a longer period (48 h) after cooling storage (136, 137). In human, VEGF has been suggested to have a positive effect on sperm motility parameters, but not survival, in a concentration-dependent manner in vitro (138). One suggested hypothesis is that VEGF may act in on the stabilization of mitochondrial membrane potential and prevention of oxidative damage via activation of the nuclear factor erythroid 2 – related factor 2 (NrF2) pathway (139). In bovine, IGF I and II increased sperm motility parameters and indicated an involvement in the initiation and maintenance of bovine sperm motility (140), suggested to be related to the stimulation of adenylate cyclase and cAMP production.

More specific with factors present on PRP, sperm motility improvement was also observed on *in vitro* treatment containing TGF-ß (141), FGF (12), VEGF (138), ZN (142), and SOD (143). However, other studies reported no effect on sperm motility by IGF-I (144) and PDGF (145). In addition to motility improvement, TGF- ß had also anti-inflammatory properties with a potential role on the interaction between female reproductive tract and sperm after mating (141). The antioxidant Zn/Cu SOD enzyme had also a protective role on sperm motility besides improvement, which was mainly related with the maintenance of membrane integrity, reduction of DNA fragmentation, and inhibition of lipid peroxidation (LPO) of sperm (142-144).

Following the rationality of previous studies, the first idea for the present experiment was to test if sperm would survive after supplementation with PRP and used directly in the semen to

treat post-mating endometritis. Since GFs have been shown to improve sperm motility in other species, the focus of the study was adjusted to examine the direct effect of PRP on semen motility in the stallion. For that, a more purified and active form of PRP, the PRPL, was selected and an additional experiment was planned with one GF alone, the FGF2. Therefore, the present study would be able to test the effect of PRPL factors on sperm motility and an additional test would be performed to test the effect of FGF2 alone. Moreover, we could define a maximal dose of PRPL to be added in the semen for a potential treatment for post-mating endometritis.

## 3. Objectives

The objectives for the have study are:

1- Determine if PRPL or FGF2 treatments enhance sperm motility.

2- Evaluate if PRPL or FGF2 treatments affect sperm survival over 24 hours and during the cooling process.

## 4. Hypothesis

The following hypotheses were tested in the have study:

1- Treatment of semen with PRPL or FGF2 enhances sperm motility.

2- Sperm motility enhancement is more pronounced after treatment with FGF2 when compared with PRPL.

3- Sperm survival will be maintained when PRPL or FGF2 are added to semen extender.

## 5. Design, Methods, and Analysis

### 5.1.Animals

Seven stallions deemed healthy by physical examination were used for semen collection. Experimental number was based on a power analysis of 80% using preliminary data from humans (10, 11), which showed an increase from 42 ± 4% to 55 ± 4% in sperm cell motility after treatment with 100 ng/mL of recombinant human FGF2 in 5 subjects. Selection of stallions was based on a breeding soundness examination including, normal reproductive tract by visual and palpation examination and presence of at least 30% progressively motile sperm in the ejaculate and more than 50% morphologically normal sperm. All procedures were performed in accordance with the Institutional Animal Care and Use Committee (IACUC), protocol 2018-3226.

### 5.2.Semen Collection

Two separate ejaculates were collected from each of the 7 stallions. Collection was performed using a Missouri style artificial vagina (AV) with stallion exposed to an estrus or ovariectomized mare and mounting a breeding phantom. Semen analysis was performed within 30 min of collection by the same unblinded investigator. After first analysis and immediate dilution with semen extender, semen samples were maintained at 37 $^{o}$C until allocation into the appropriate treatment groups and start of motility analysis.

### 5.3.Trials with platelet rich plasma and heparin

The first trial was performed using platelet rich plasma (PRP) at concentrations of 0, 5, 10, 25, and 50% added to semen diluted in three different extenders: Dulbecco′s modified eagle′s

medium (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany); E-Z Mixin® - "BF" Semen Extender Standard Formula (ARS, Ontario, CA, USA); and INRA 96 (IMV technologies, L'Aigle, France). In all semen extenders used, a dose dependent gel formation was observed. Therefore, a test was performed to detect if the PRP was reacting with the unextended semen or pure extender. For that, pure INRA 96 was diluted with 50% PRP as well as unextended semen at 50% PRP and gel formation was monitored visually. No gel formation was visualized when semen alone was diluted with PRP, which confirmed that this reaction was between the semen extender and PRP, and independent of the presence of semen. This gel formation was like the one reported with use of PRP *in vitro* for cell culture and could be prevented by supplementation with heparin (146). Of extenders tested, INRA96 showed better preservation of sperm motility characteristics and is the extender most used in our lab. Therefore, INRA96 was used for further testing of gel formation. Based on the previous literature, heparin at doses of 0, 0.5, 0.7, and 0.9 IU/mL were added to extender containing 50% of PRP (without semen). The concentration of 0.7 IU/mL of heparin prevented gel formation during hours 0, 1, 2, 3, and 20 after treatment. At doses 0 and 0.5 IU/mL, gel was visualized at hours 1 and 20, respectively. Concentrations of heparin greater than 0.7 IU/mL were able to prevent gel formation at all time points and could be used for further sperm motility analysis in the experiment. Therefore, 1 IU/mL was selected to be used for being above 0.7 IU/mL and 5 times below the expected concentration to cause sperm capacitation (5 IU/mL, (124)) which is an unwanted confounding effect for the experiment.

Further trials were performed using extended semen with 1 IU/mL of heparin and 0, 5, 10, 25, and 50% of PRP. For this trial, frozen semen was thawed and resuspended in 1 part of semen to 4 parts of extender (INRA 96), centrifuged and diluted to a standard concentration between 30 to 50 million/mL for computer assessed sperm analysis (CASA). No gel formation

was noted, but agglutination via head to head attachment (HHA) occurred and resulted in a decrease of free-swimming sperm starting at hours 1 for 10% and 25% PRP, and hours 3 for 5% PRP (Table 1). For 50% PRP, most of sperm cells lost motility in less than one hour and HHA was not visualized.

Based on these findings, another trial was performed using fresh semen and lower percentages of PRP (0, 1, 2.5, 5, and 10%) with more frequent analyses (hours 0, 1, 6, 12, and 24). Additionally, samples were aliquoted, cooled, and maintained in an Equitainer at 5$^{o}$C after the first hour for better preservation of sperm characteristics and more closely follow the equine industry process. For each successive analysis, 25µL of each sample was warmed to 37 $^{o}$C prior to each analysis. Results showed that HHA occurred at 6 hours at 10% PRP and at 12 hours at 5% PRP. Due to an increase in HHA in groups 5% and 10% PRP, motility percentage analyzed by the computer was falsely increased and the data was deemed unreliable. Below 10% PRP, no effect on motility was apparent when compared with the control throughout the 24-hour period analyzed. For the main experiment PRP was switched to PRP lysate (PRPL) for being an acellular substitute for PRP obtained after the lysis of platelet concentrates by plateletpheresis, able to be pooled with different donors for standardization of treatments, and present less contamination.

### 5.4.Preparation of PRPL

Platelet rich plasma lysate samples were provided by the laboratory of Dr. John F. Peroni from University of Georgia. The preparation of PRPL was performed as previously described (7). Briefly, platelet concentrates were obtained following plateletpheresis (COBE Spectra Dual-

Needle) in five-mixed breed horses belonging to the University of Georgia equine blood donor herd. The platelets were fractured using two freeze-thaw cycles followed by three centrifugation cycles. The PRPL was then filtered through a 40-μm cellulose acetate membrane (EMD Millipore, Billerica, Massachusetts) to remove cellular debris. An equal portion of lysate from each horse was combined to obtain a pooled product. Samples of PRPL in aliquots of 1 mL were stored in a freezer at – 80$^{o}$C for further use.

### 5.5.Recombinant FGF2

Recombinant equine FGF basic protein (FGF2) was produced by bacterial expression system and commercially obtained (R&D Systems, Minneapolis, MN). As recommended for the manufacturer, FGF2 was reconstituted to a maximum concentration of 250 μg/mL in water containing at least 0.1% of bovine serum albumin (BSA). After reconstitution (250 μg/mL), different amounts of diluted FGF2 were added to the ejaculate to achieve the desired concentrations of 0, 0.1, 1, 10, and 100 ng/mL of FGF2. Treatment doses were formulated based on previous studies using recombinant FGF2 in human and mice semen (11, 12). After reconstitution under sterile conditions, FGF2 was stored between -20 to -70$^{o}$C and is stable for at least 3 months.

### 5.6.Sample Preparation

The ejaculate obtained was analyzed for sperm concentration using the Sperm Cell Counter (NucleoCounter® SP-100™, Chemometec) and a volume containing 600 million total sperm was placed in 50 mL conical tubes, diluted 1 part of semen to 4 parts of standard milk

based extender (INRA 96®) and centrifuged at 900xG for 10 minutes. The supernatant was withdrawn to a volume of 3ml and the pellet obtained was re-suspended. The concentration was then recounted with the NucleoCounter to confirm concentrations between 100 to 200 million sperm/mL, as illustrated in figure 1.

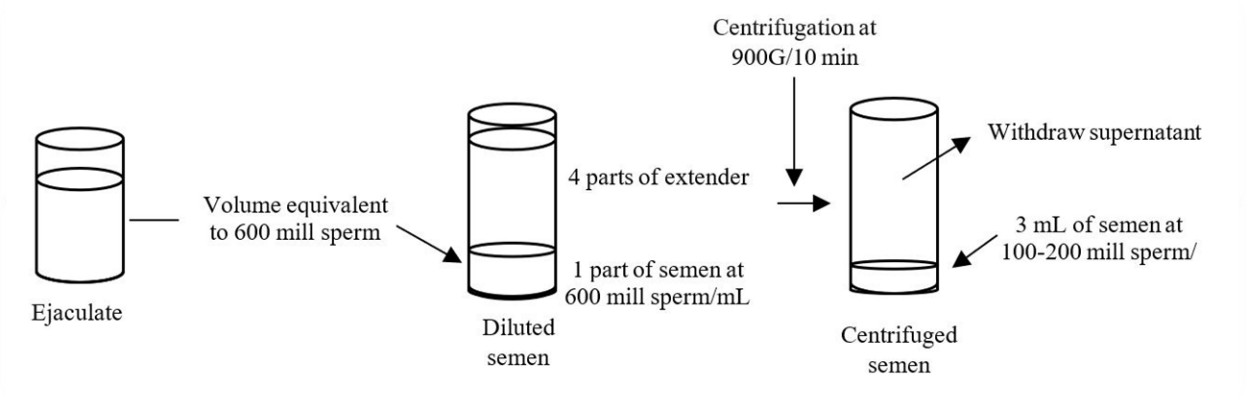



**Figure 1.** Illustration of preparation of the ejaculate prior to treatments.

Prior to the addition of treatments, the volume of the centrifuged ejaculate was adjusted to a final concentration of 50 million/mL in all samples. For that, PRPL treatment groups had the following components with 50 million sperm per ml in all treatment groups.

1- Extended semen only (CTRL; control without heparin)

2- Extended semen with 1 IU/mL of heparin (CTRL Hep; control with heparin)

3- Extended semen with 1 IU/mL of heparin and 1% PRPL (1% PRPL)

4- Extended semen with 1 IU/mL of heparin and 2.5% PRPL (2.5% PRPL)

5- Extended semen with 1 IU/mL of heparin and 5% PRPL (5% PRPL)

6- Extended semen with 1 IU/mL of heparin and 10% PRPL (10% PRPL)

For FGF2 treatments groups contained the following components with 50 million sperm per ml in all groups:

1- Extended semen only (CTRL)

2- Extended semen with 0.1 ng/mL of FGF2 (FGF2-0.1)

3- Extended semen with 1 ng/mL of FGF2 (FGF2-1)

4- Extended semen with 10 ng/mL of FGF2 (FGF2-10).

5- Extended semen with 100 ng/mL of FGF2 (FGF2-100).

Experimental design is represented in Figure 1:

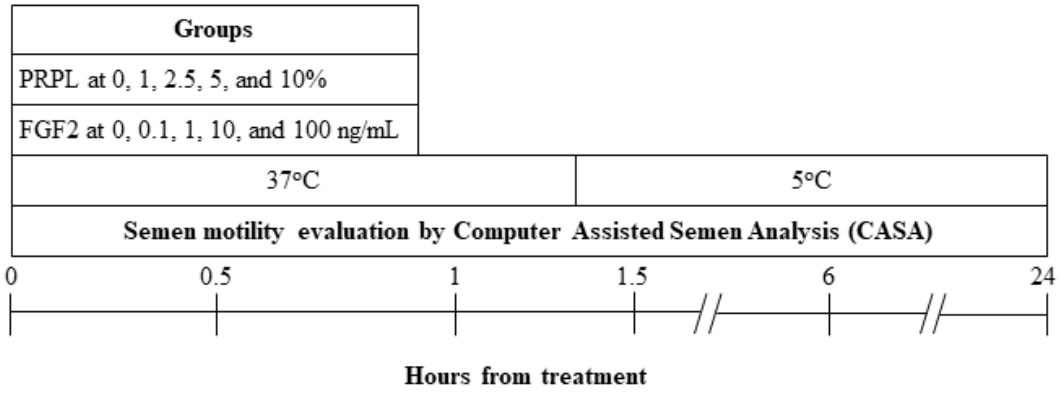



**Figure 2.** Experimental design.

The rationale of the doses of PRPL were established during the initial trials leading up to the present study and heparin was added to prevent gel formation. The rationale of FGF2 concentrations used was based on previous studies in human and mice using the same doses (11, 12). In all treated samples the final concentration of sperm was standardized to 50 million sperm per mL in all groups to allow motility evaluation by the computer assisted sperm analysis (CASA, Minitube SAS).

### 5.7.Semen Analysis

Motion characteristics of the sperm were assessed objectively after warming using a computer assisted sperm analysis (CASA, Minitube SAS). Aliquots of 5µL of each treatment were placed in a 20µm standard count analysis chamber (SC20.01. FA; Leja®, Nieuw-Vennep, The Netherlands). Seven fields were selected for analysis of total (MOT, %) and progressive (PROG, %) 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, %), as previously described (147). Additional measurements were performed including total number of cell counted (TOTCELL, cell number), distance curved line (DCL, µm), distance average path (DAP, µm), distance straight line (DSL, µm), wobbling (WOB, VAP/VCL), slow motility (SLOWMOT, %), non-progressive motility (NPMOT, %), average orientation change (AOC, degree), and concentration (CONC, cells/mL). Sperm morphology was assessed on the initial ejaculate by evaluating 100 sperm cells stained with eosin nigrosine 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. Aliquots of extended semen under the outlined experimental conditions were cooled to 5°C and stored in a standard semen shipping container after motility analysis after hour one. For each analysis time point (0, 0.5, 1, 1.5, 6, and 24 hours), 25 µL of the samples were warmed to 37ºC prior to semen motility evaluation. Experiment design is represented in Figure 1.

### 5.8.Data Analysis

Data was analyzed separate for each experiment, PRPL and FGF2. Differences among groups, time, and interaction between group and time were tested. Data was examined for

normality using a Shapiro-Wilk and if the data was not normally distributed it was transformed into natural logarithm or rank. The SAS MIXED procedure (Version 9.4; SAS Institute) was used with a repeated statement to account for correlation between sequential measurements. A probability of $P \leq 0.05$ indicated that a difference was significant.

## 6. Results

### 6.1. Heparin effect on semen

To monitor the effect of heparin on the PRPL treated samples, one heparin treated group was compared with a non-treated control. No effect ($P < 0.05$) of group, time, or interaction between group and time was found between heparin treated and control groups for all motility variables obtained by the CASA.

### 6.2. Platelet rich plasma lysate (PRPL)

For TOTCELLS and CONC, differences among groups, time, and interaction between group and time were noted ($P < 0.05$) (Fig. 1). Both variables showed a decrease ($P < 0.05$) starting at hour 1 in groups 5% and 10% PRPL. Values for groups 5% PRPL and 10% PRPL remained lower ($P < 0.05$) than all other groups starting at hour 1.5, and values for group 10% PRPL were lower ($P < 0.05$) than group 5% PRPL starting at hour 1.5. In both groups, head-to-head attachment was visually observed on the screen, but not able to be objectively evaluated.

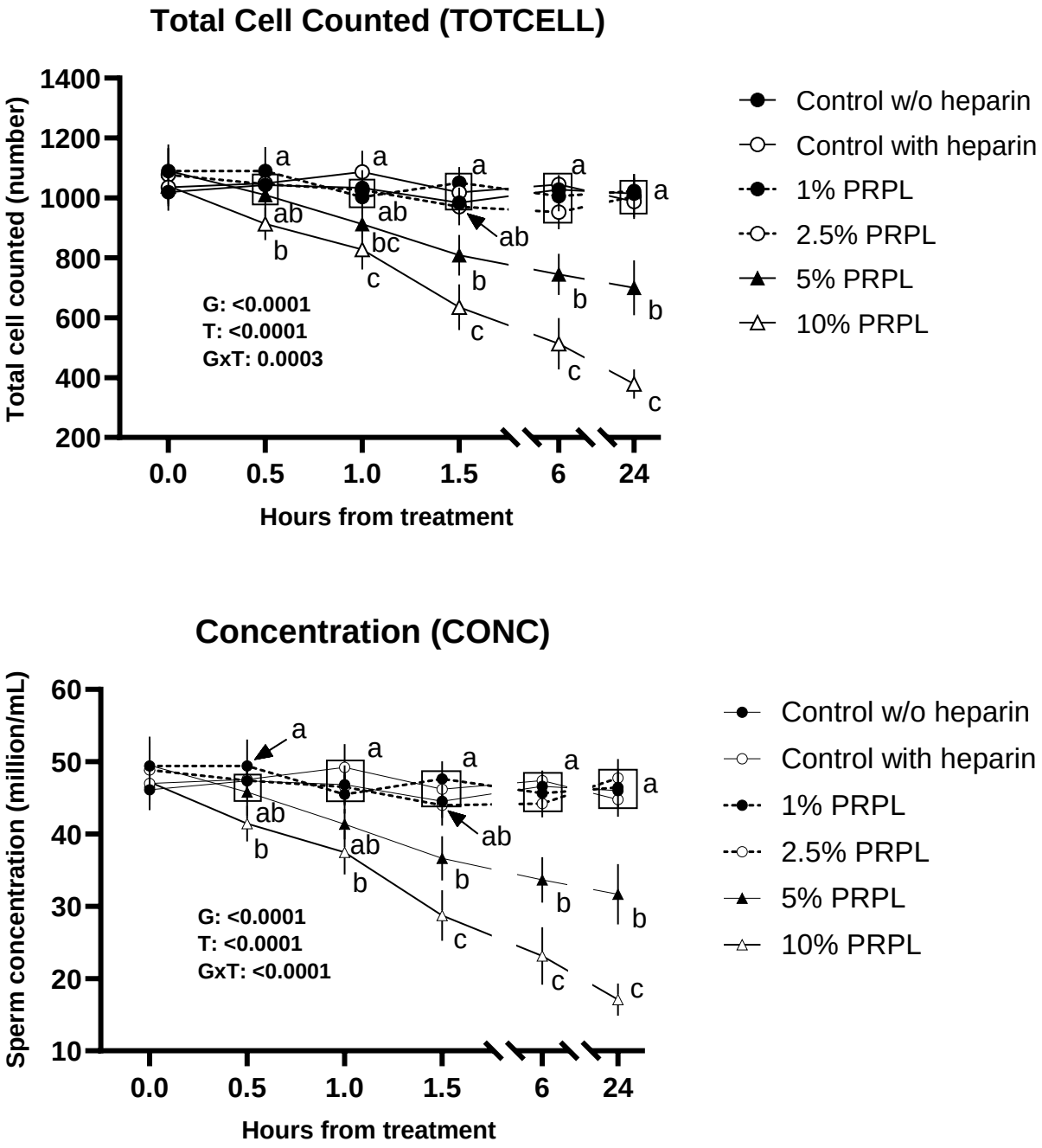



**Figure 3.** TOTCELLS and CONC obtained by CASA during hours 0, 0.5, 1, 1.5, 6, and 24 in a control group and with 0 ,1, 2.5, 5, and 10% of PRPL in 1 IU/mL of heparin. Each group contained 7 stallions with 2 ejaculates each. Significant differences ($P < 0.05$) were noted among groups, time, and interaction between groups and time. Groups 5% and 10% PRPL had a gradual decrease from hour 0 to 24. 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).

Significant differences ($P < 0.05$) over time were noted for TMOT, PMOT, DCL, DAP, VCL, VAP, ALH, AOC, DSL, VSL, BCF, STR, WOB, LIN, and SLOWMOT, but no difference ($P > 0.05$) among group or interaction between groups and time were noted. Therefore, groups were combined for each variable to evaluate changes over time.

Total motility (TMOT) and PMOT gradually decreased ($P < 0.05$) from 0 to 1.5 hours followed by a plateau in TMOT and PMOT after hour 1.5 (Fig. 2).

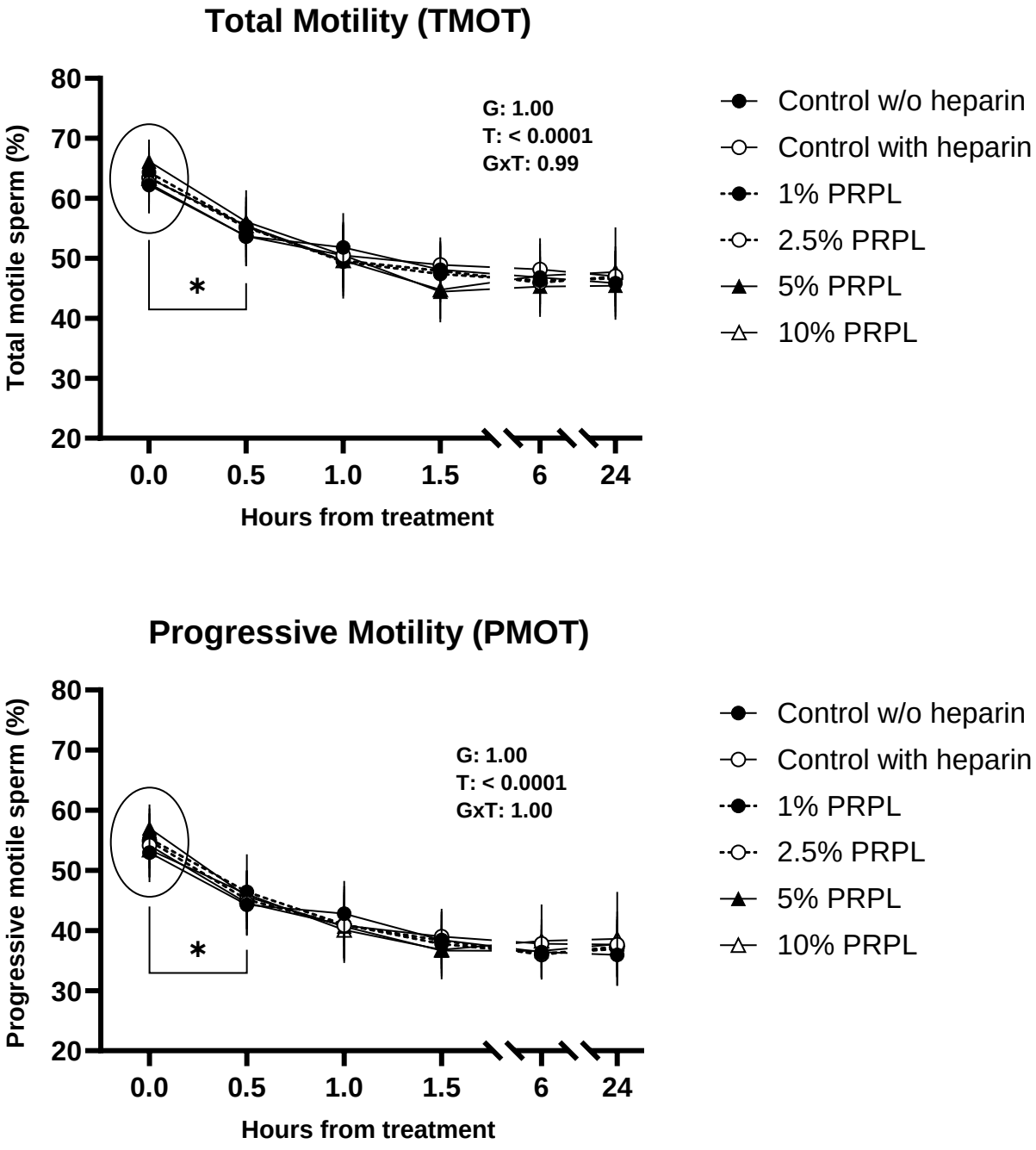
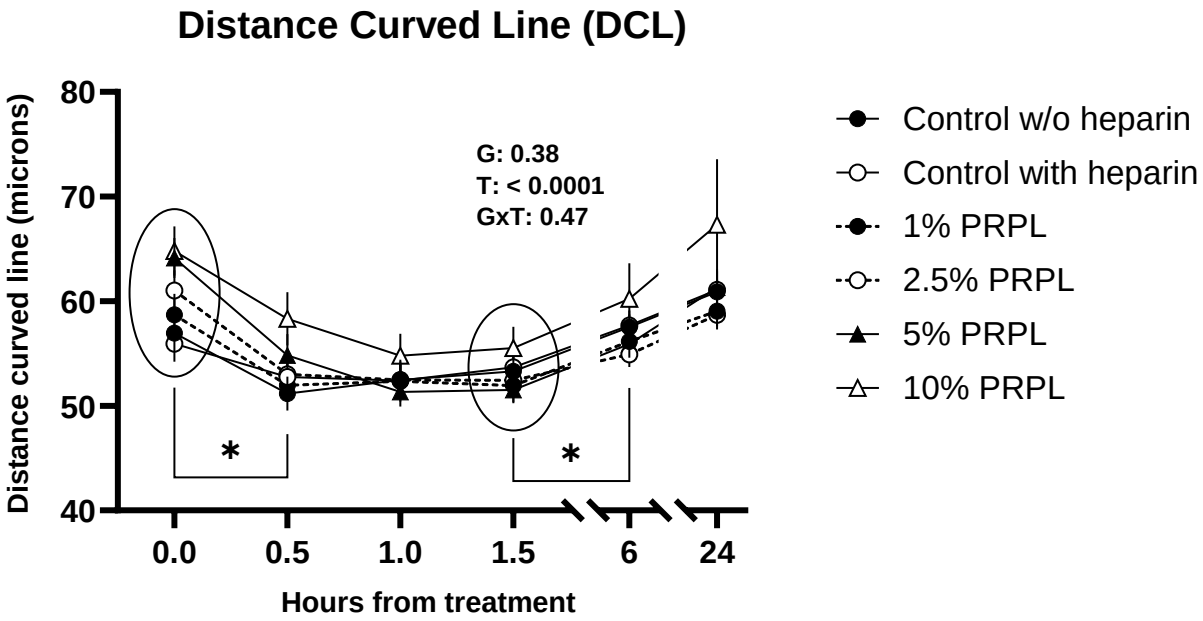
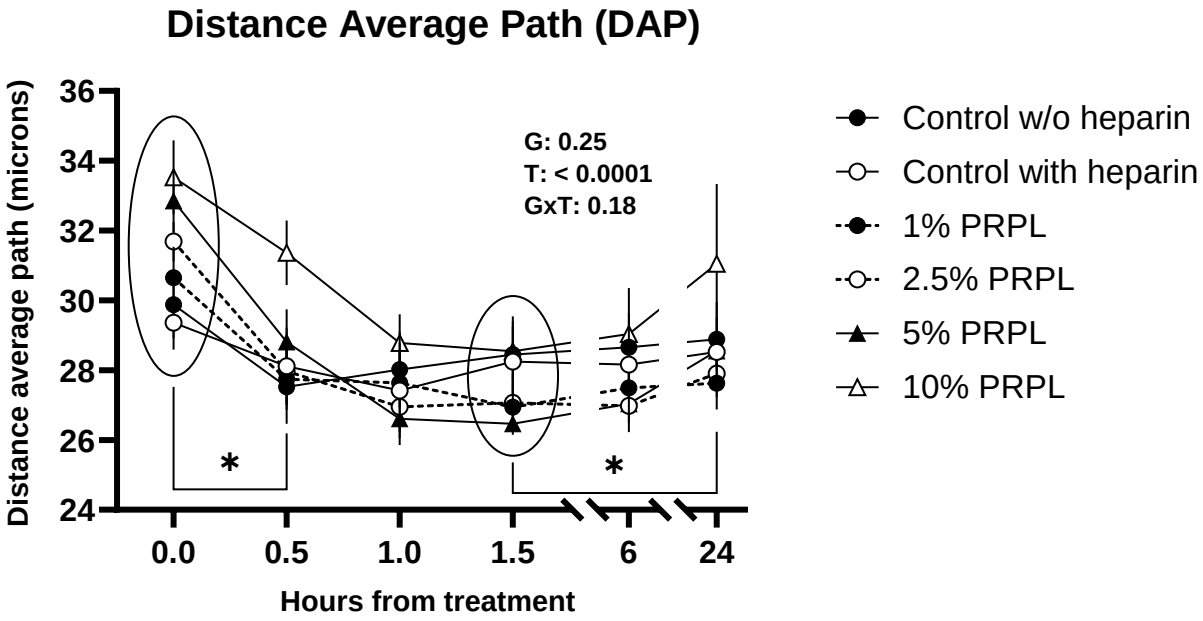
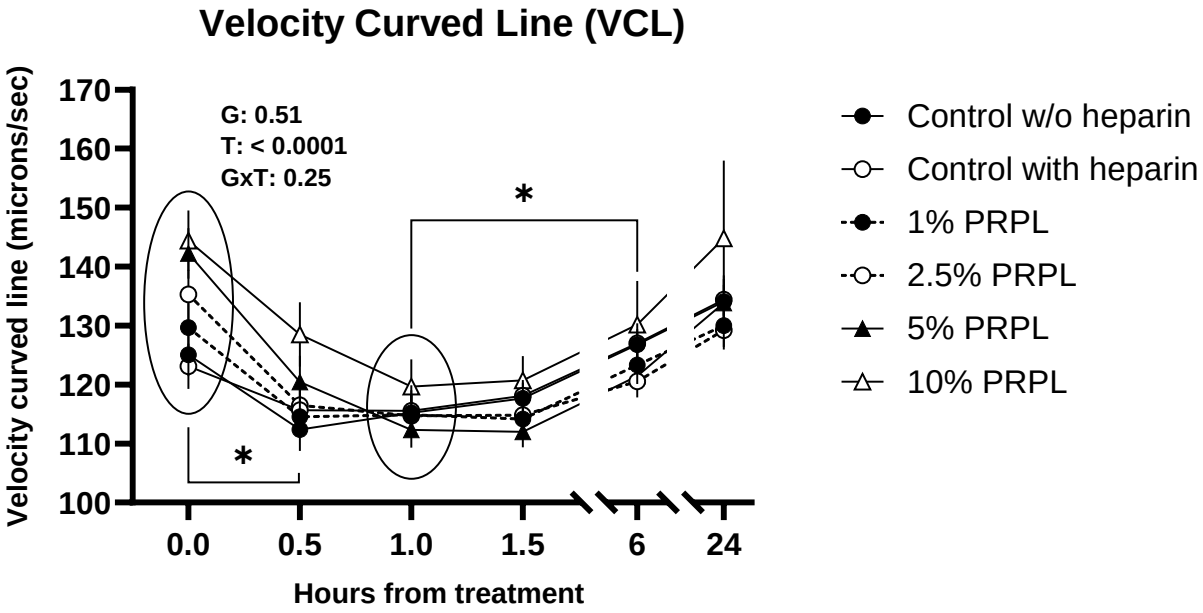


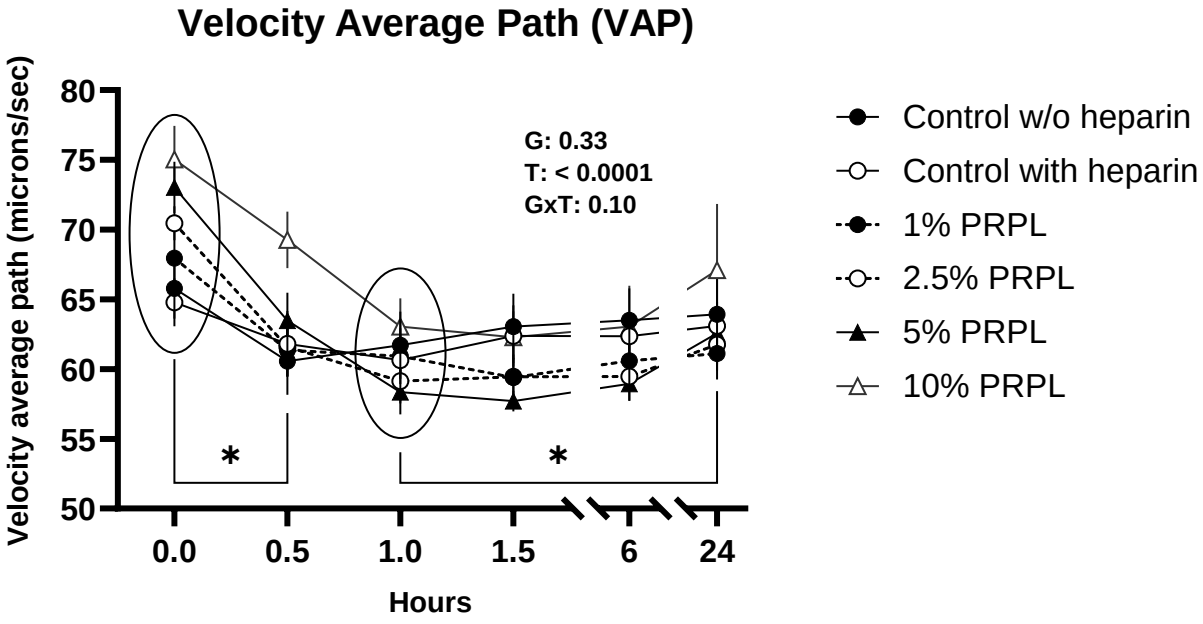

**Figure 4.** TMOT and PMOT obtained by CASA during hours 0, 0.5, 1, 1.5, 6, and 24 in the control group and with 0, 1, 2.5, 5, and 10% of PRPL in 1 IU/mL of heparin. Each group contained 7 stallions with 2 ejaculates each. Significant effect of time ($P < 0.05$) was noted, represented by a gradual decrease in all groups from hours 0 to 1.5 when groups were combined. Significant ($P < 0.05$) time changes in direction (increase or decrease) for groups combined are represented by a circle. The first significant ($P < 0.05$) change on direction for time in the groups combined are indicated by an asterisk (*).

Distance curved line (DCL), DAP, VCL, VAP, ALH, and AOC showed significant decreases ($P < 0.05$) from hours 0 to 1 followed by a gradual increase ($P < 0.05$) up to 24 hours (Fig. 3).




Velocity Curved Line (VCL)
Velocity curved line (microns/sec)
170
160
150
140
130
120
110
100
G: 0.51
T: < 0.0001
GxT: 0.25
*
*
0.0
0.5
1.0
1.5
6
24
Hours from treatment
Control w/o heparin
Control with heparin
1% PRPL
2.5% PRPL
5% PRPL
10% PRPL


Velocity Average Path (VAP)
Velocity average path (microns/sec)
80
75
70
65
60
55
50
G: 0.33
T: < 0.0001
GxT: 0.10
*
*
0.0
0.5
1.0
1.5
6
24
Hours
Control w/o heparin
Control with heparin
1% PRPL
2.5% PRPL
5% PRPL
10% PRPL

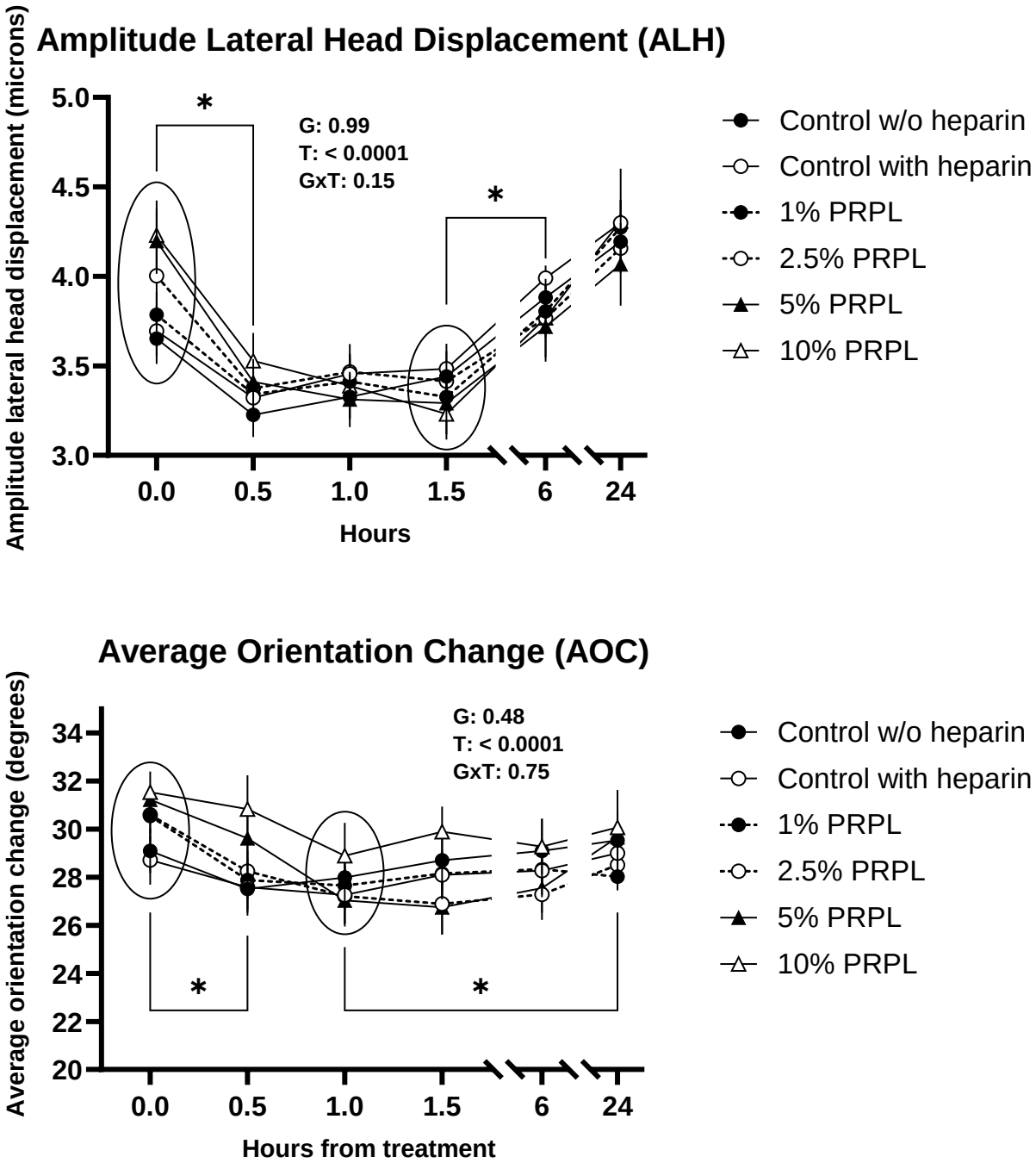



**Figure 5.** DCL, DAP, VCL, VAP, ALH, and AOC obtained by CASA during hours 0, 0.5, 1, 1.5, 6, and 24 in the control group and with 0, 1, 2.5, 5, and 10% of PRPL in 1 IU/mL of heparin. Each group contained 7 stallions with 2 ejaculates each. Significant effect of time ($P < 0.05$) was noted, represented by a gradual decrease from hours 0 to 1 followed by a gradual increase until hour 24 when groups were combined. Significant ($P < 0.05$) time changes in direction (increase or decrease) for groups combined are represented by a circle. The first significant ($P < 0.05$) change on direction for time in the groups combined are indicated by an asterisk (*)

Distance straight line (DSL) and VSL showed a gradual decrease ($P < 0.05$) from hours 0 to 1, a plateau from hours 1 to 1.5, and a gradual decrease ($P < 0.05$) until hour 24 (Fig. 4).

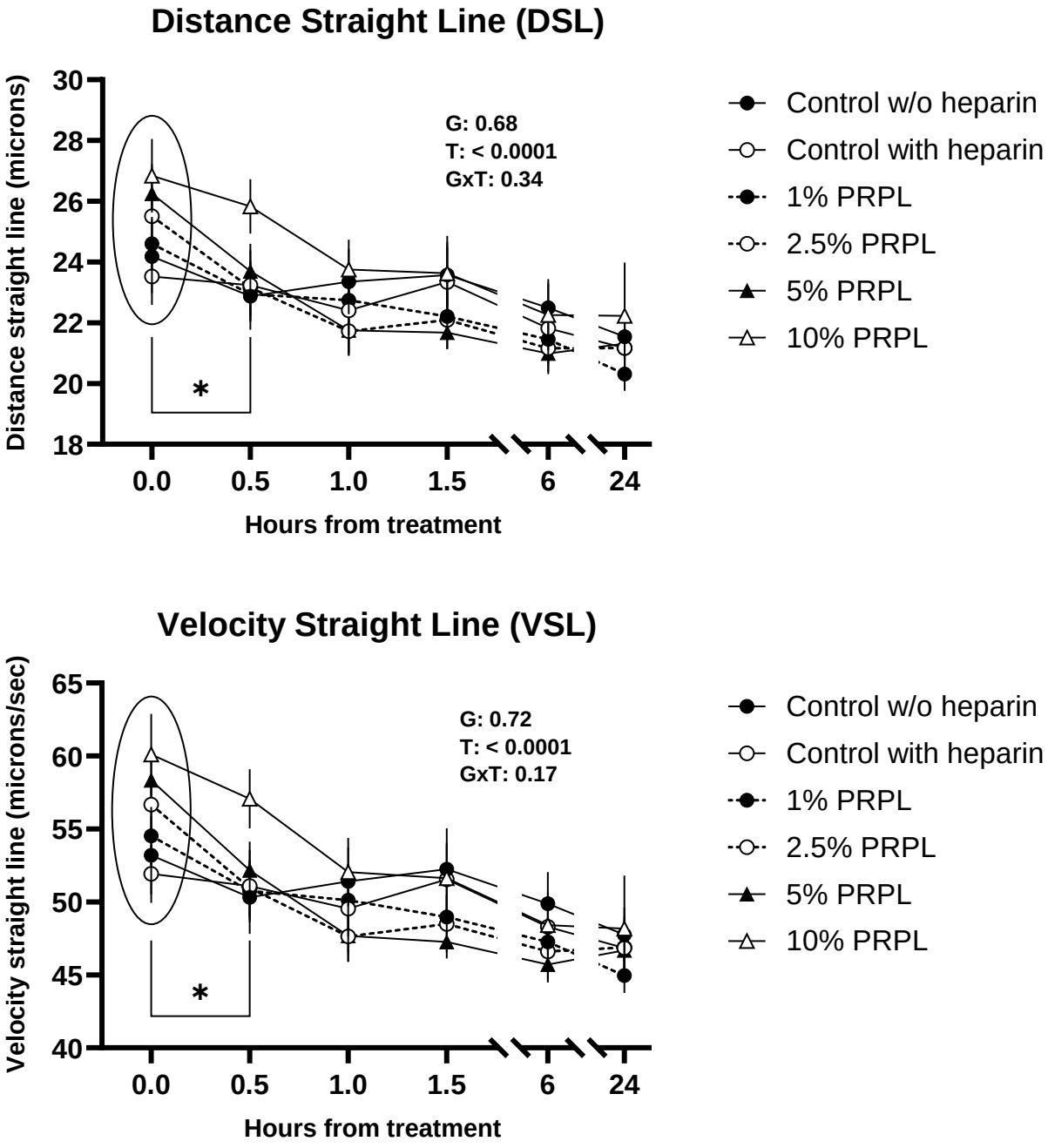
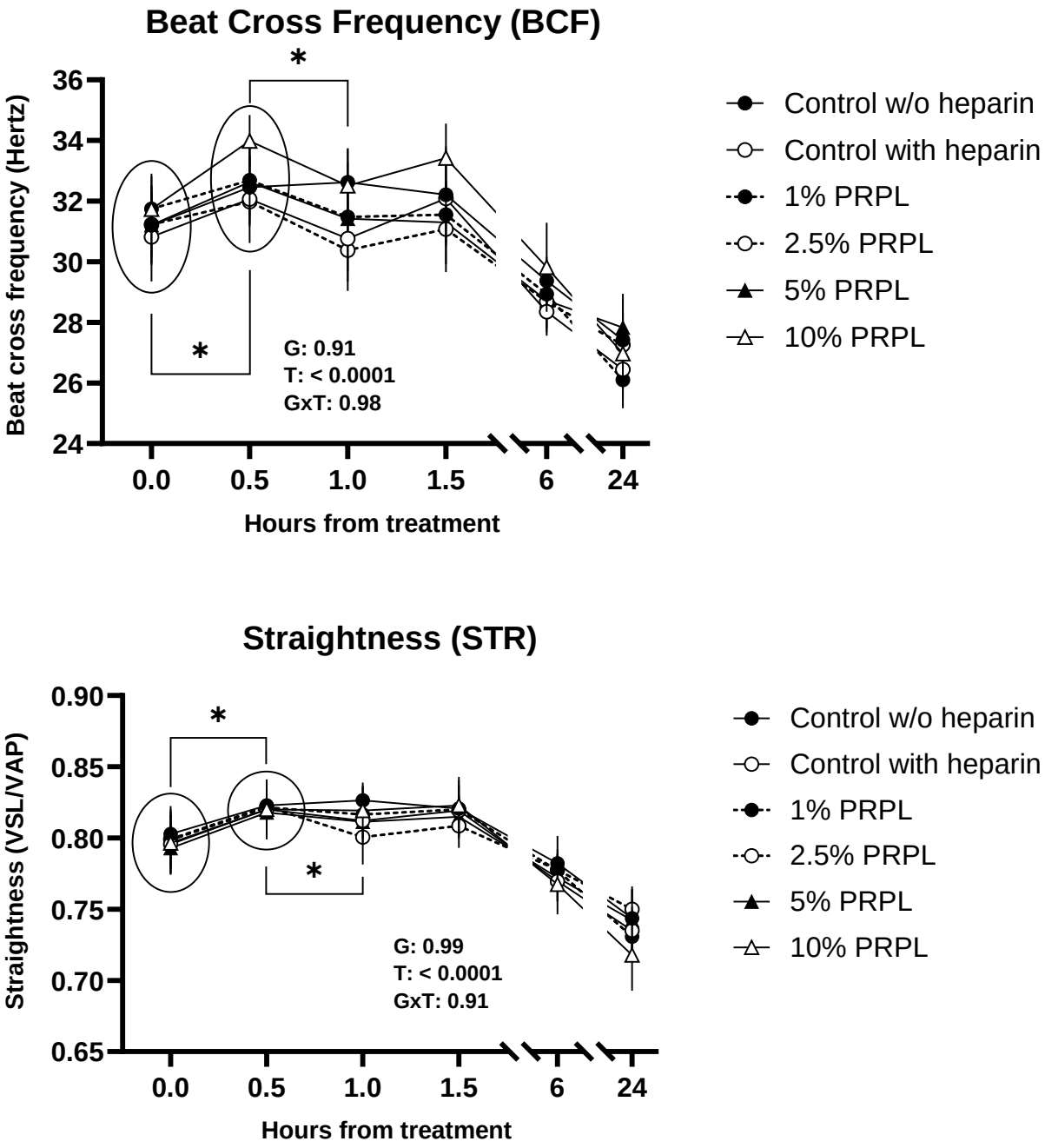
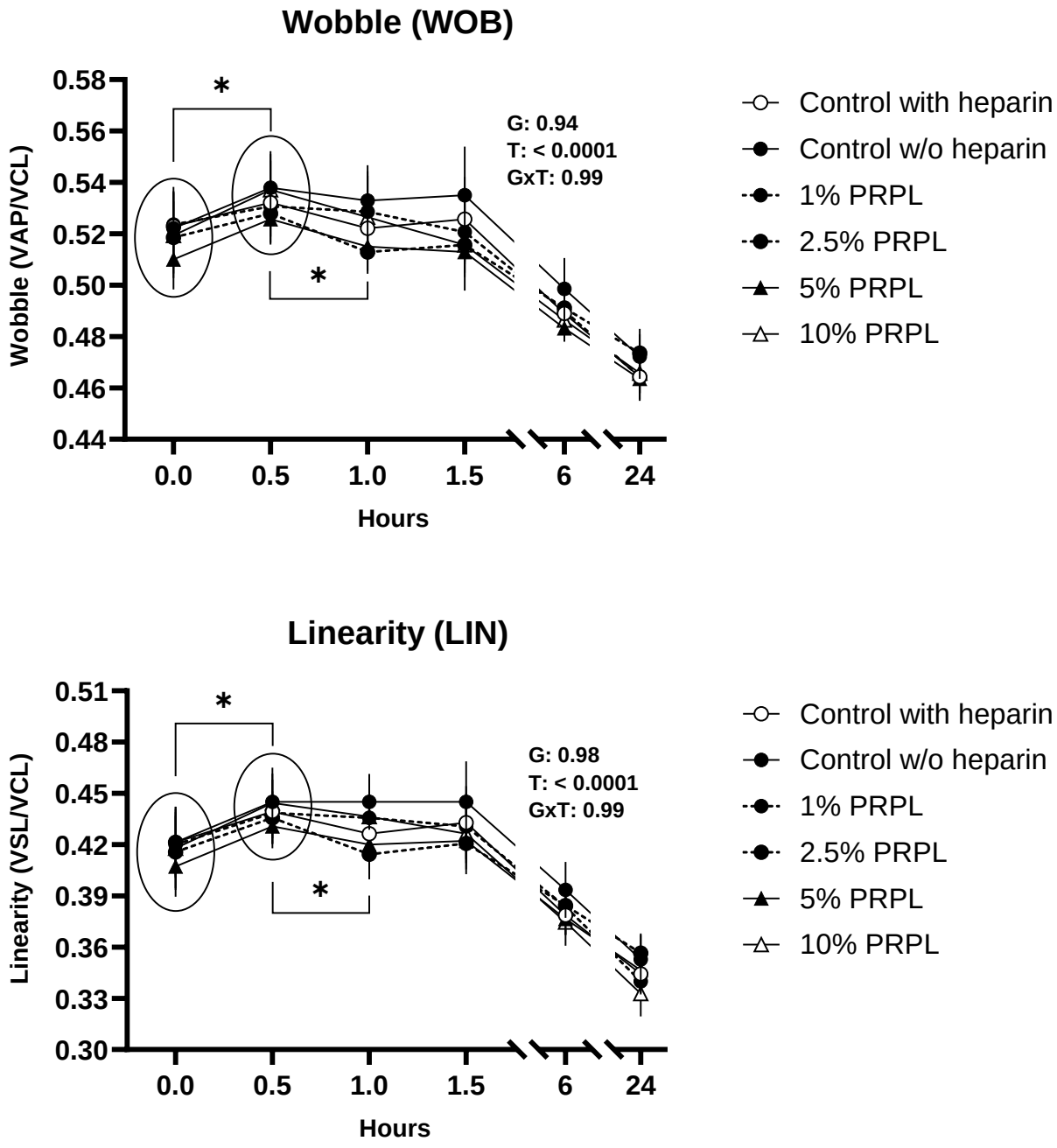



**Figure 6**. DSL and VSL obtained by CASA during hours 0, 0.5, 1, 1.5, 6, and 24 in the control group and with 0, 1, 2.5, 5, and 10% of PRPL in 1 IU/mL of heparin. Each group contained 7 stallions with 2 ejaculates each. Significant effect of time ($P < 0.05$) was noted, represented by a gradual decrease from hours 0 to 1, a plateau from hours 1 to 1.5, and a gradual decrease until hour 24. Significant ($P < 0.05$) time changes in direction (increase or decrease) for groups combined are represented by a circle. The first significant ($P < 0.05$) change on direction for time in the groups combined are indicated by an asterisk (*).

Beat cross frequency (BCF), STR, WOB, LIN, and SLOWMOT showed an increase ($P < 0.05$) from hour 0 to 0.5, a decrease ($P < 0.05$) from hours 0.5 and 1, a plateau from hours 1 to 1.5, and a gradual decrease ($P < 0.05$) until hour 24 (Fig. 5).




Wobble (WOB)
Wobble (VAP/VCL)
0.58
0.56
0.54
0.52
0.50
0.48
0.46
0.44
*
*
G: 0.94
T: < 0.0001
GxT: 0.99
0.0
0.5
1.0
1.5
6
24
Hours
Control with heparin
Control w/o heparin
1% PRPL
2.5% PRPL
5% PRPL
10% PRPL
Linearity (LIN)
Linearity (VSL/VCL)
0.51
0.48
0.45
0.42
0.39
0.36
0.33
0.30
*
*
G: 0.98
T: < 0.0001
GxT: 0.99
0.0
0.5
1.0
1.5
6
24
Hours
Control with heparin
Control w/o heparin
1% PRPL
2.5% PRPL
5% PRPL
10% PRPL

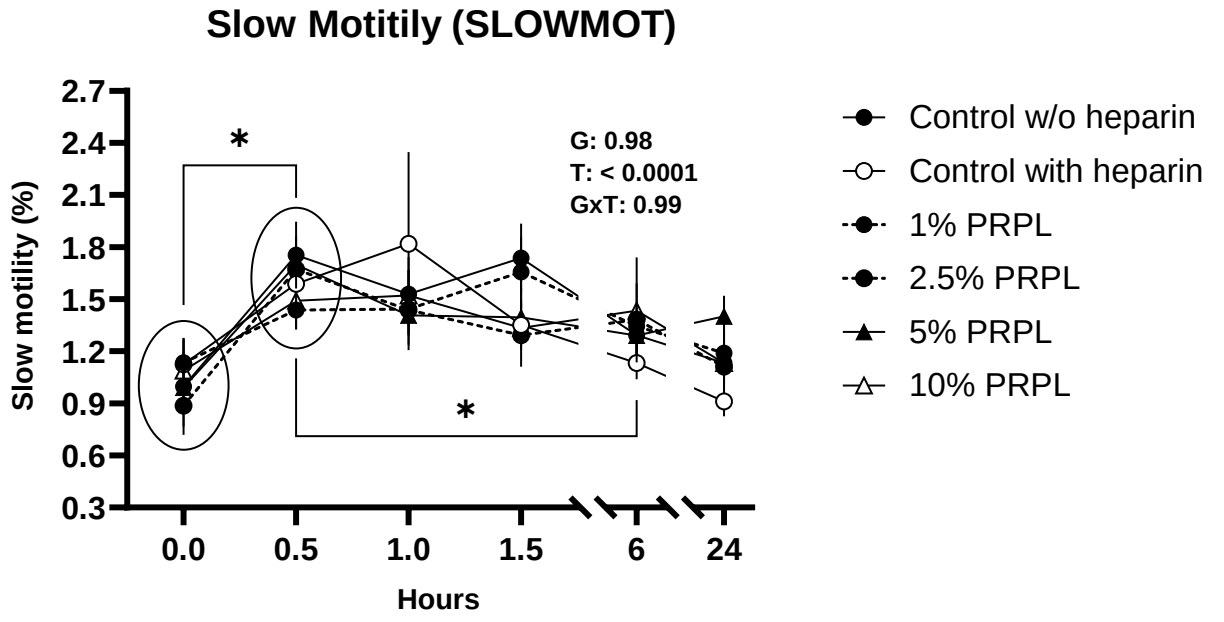



**Figure 7.** BCF, STR, WOB, LIN, and SLOWMOT obtained by CASA during hours 0, 0.5, 1, 1.5, 6, and 24 in the control group and with 0, 1, 2.5, 5, and 10% of PRPL in 1 IU/mL of heparin. Each group contained 7 stallions with 2 ejaculates each. Significant effect of time ($P < 0.05$) was noted, represented by an increase from hour 0 to 0.5, a decrease from hours 0.5 and 1, a plateau from hours 1 to 1.5, and a gradual decrease until hour 24 when groups were combined. Significant ($P < 0.05$) time changes in direction (increase or decrease) for groups combined are represented by a circle. The first significant ($P < 0.05$) change on direction for time in the groups combined are indicated by an asterisk (*).

### 6.3.Fibroblast growth factor 2 (FGF2)

No differences ($P < 0.05$) were noted between PRPL and FGF2 variables. No effect ($P < 0.05$) of group or interaction between group and time was present for all FGF2 treated groups. However, significant effect of time ($P < 0.05$) was noticed for all variables, except for TOTCELLS and CONC which showed constant values throughout the experiment (Fig. 6).

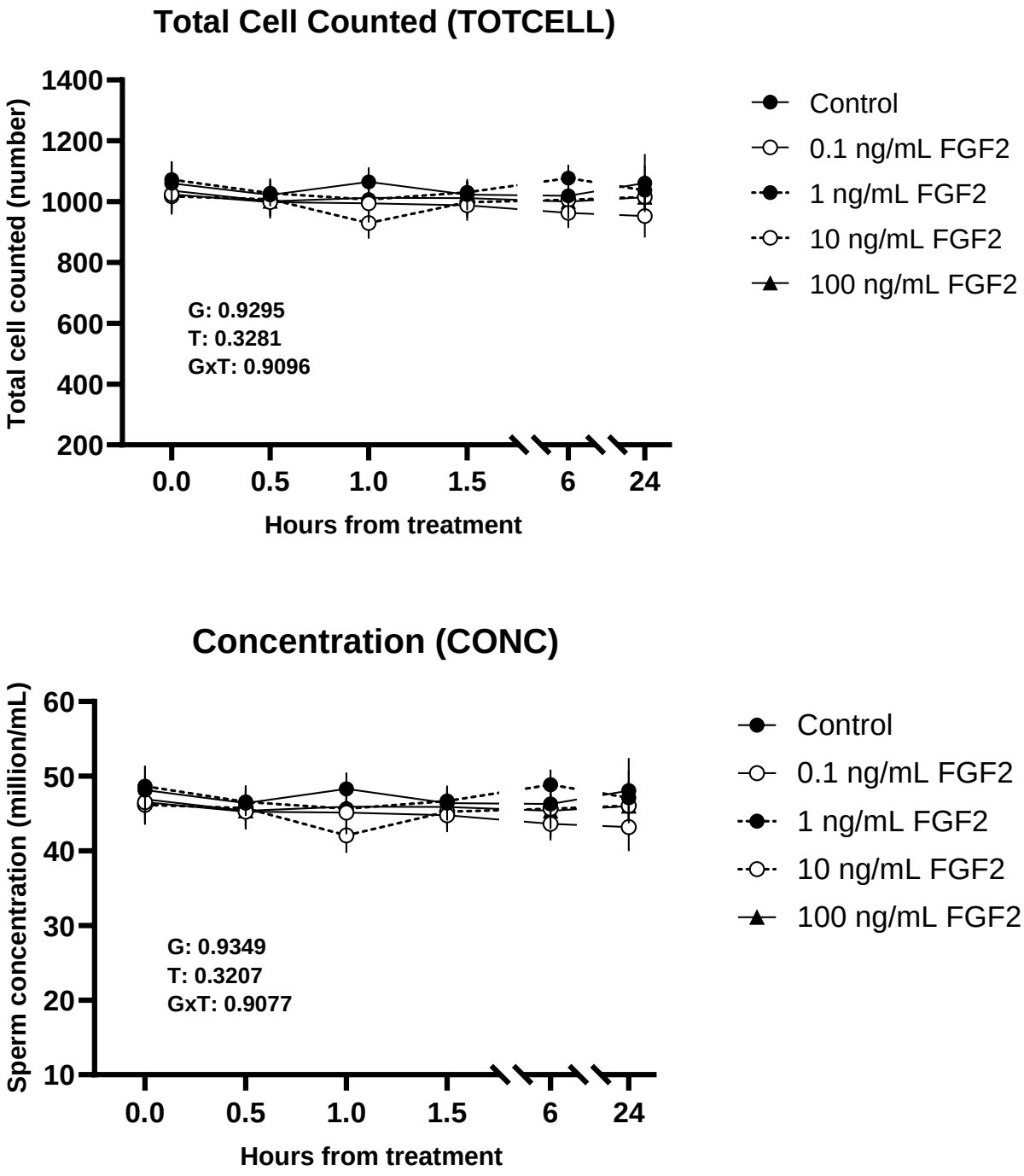



**Figure 8.** TOTCELL and CONC obtained by CASA during hours 0, 0.5, 1, 1.5, 6, and 24 in the control group and after treatments with recombinant equine FGF2 at concentrations 0.1, 1, 10, and 100 ng/mL. Each group contained 7 stallions with 2 ejaculates each. No significant difference ($P < 0.05$) was noted among groups, time, or interaction between groups and time.

Therefore, all groups were combined and changes among times showed a similar pattern to PRPL experiment. The variables TMOT and PMOT showed a gradual decrease ($P < 0.05$) from hours 0 to 6 followed by motility maintenance until hour 24 (Fig. 7).

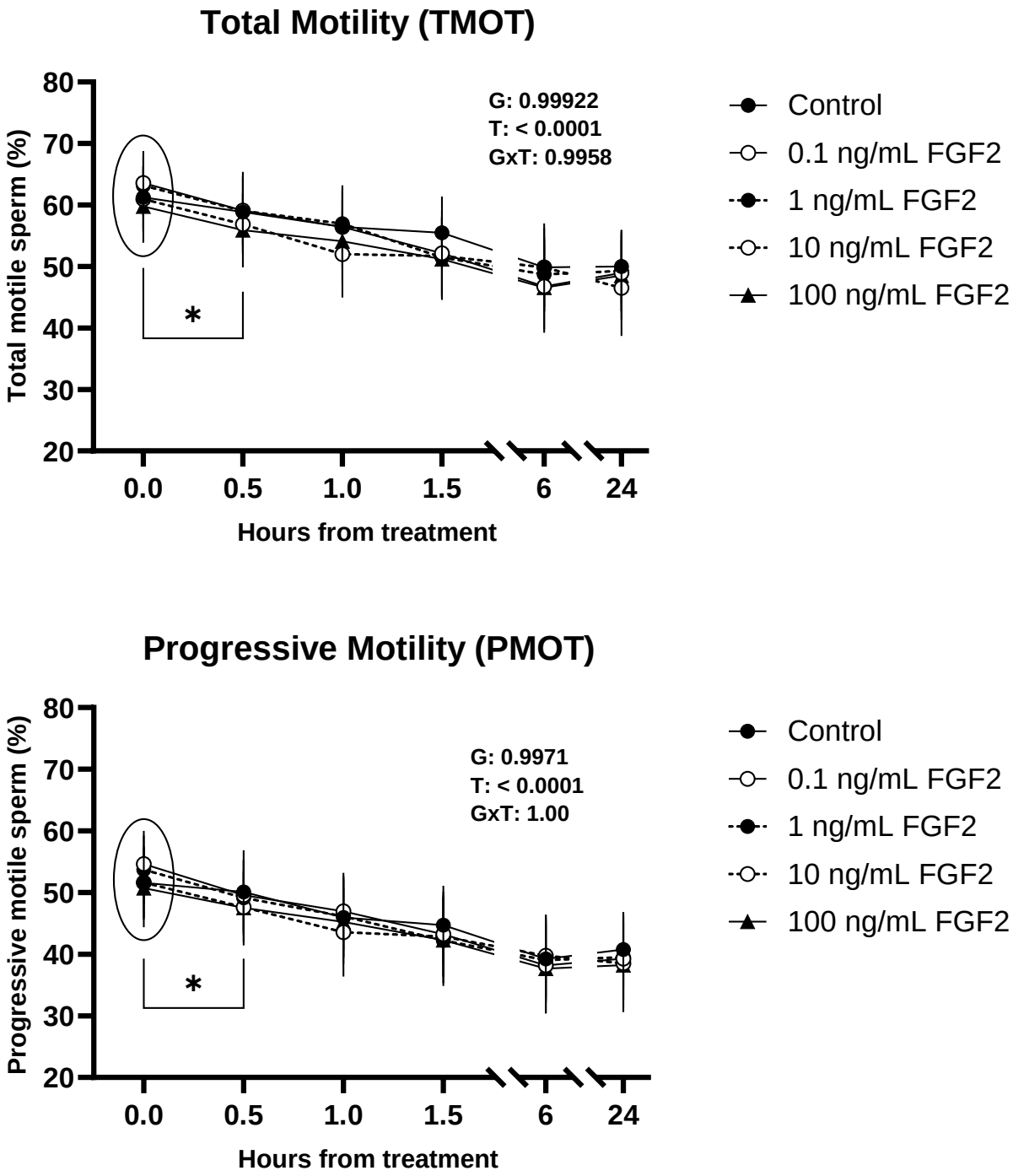
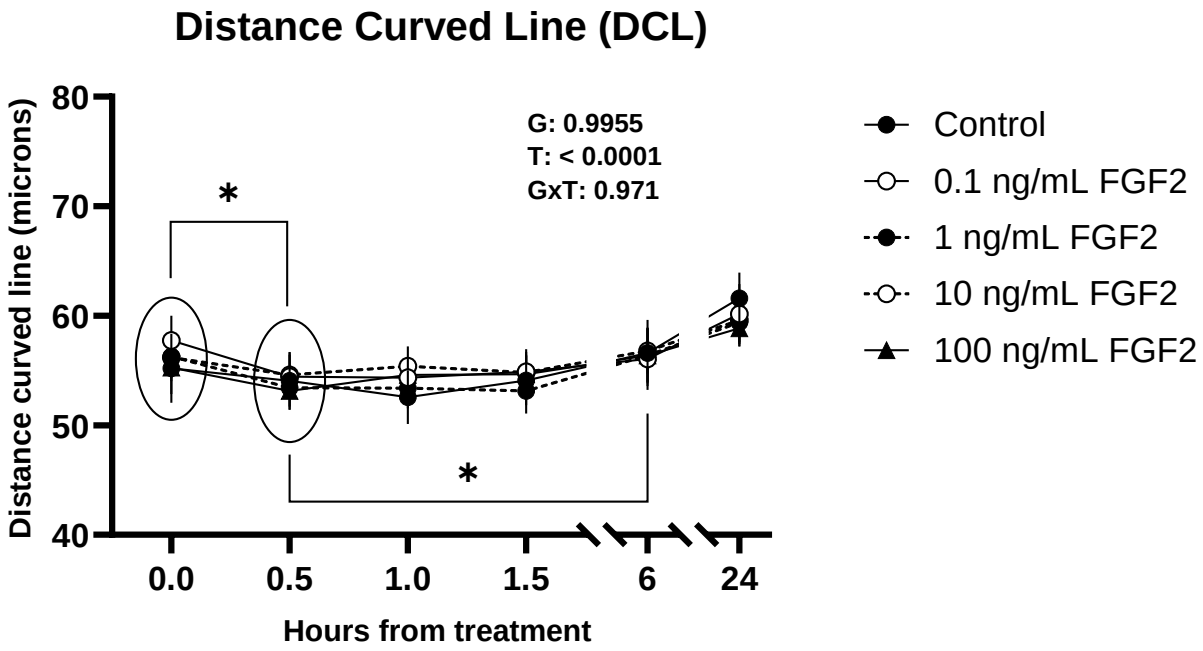
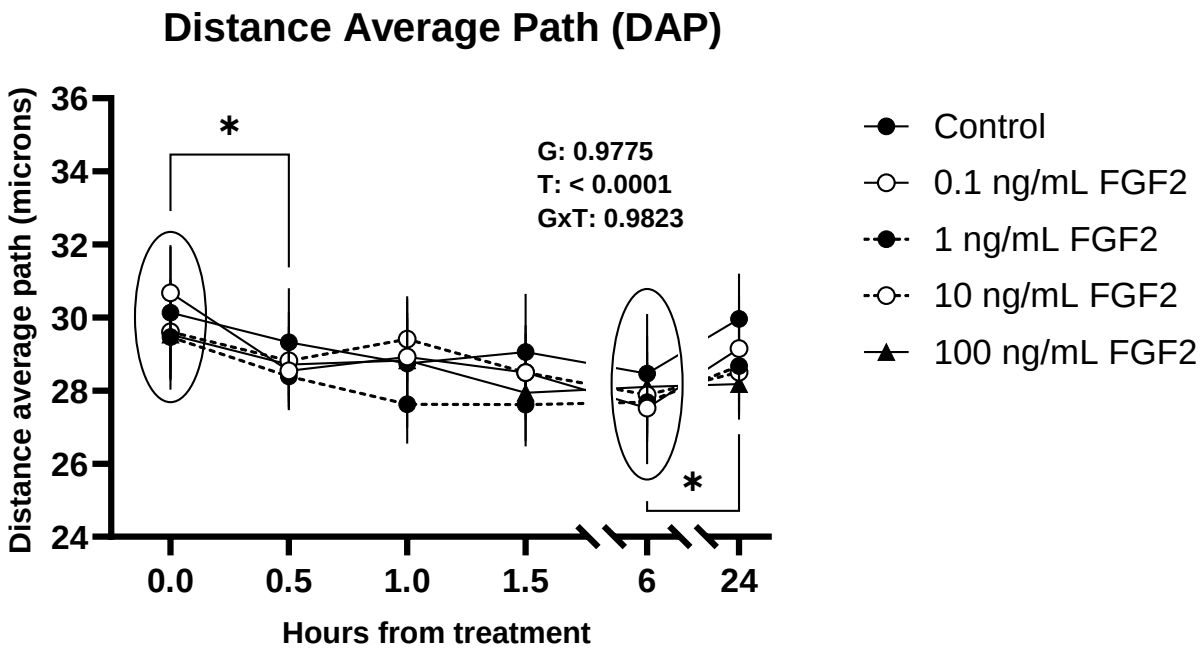
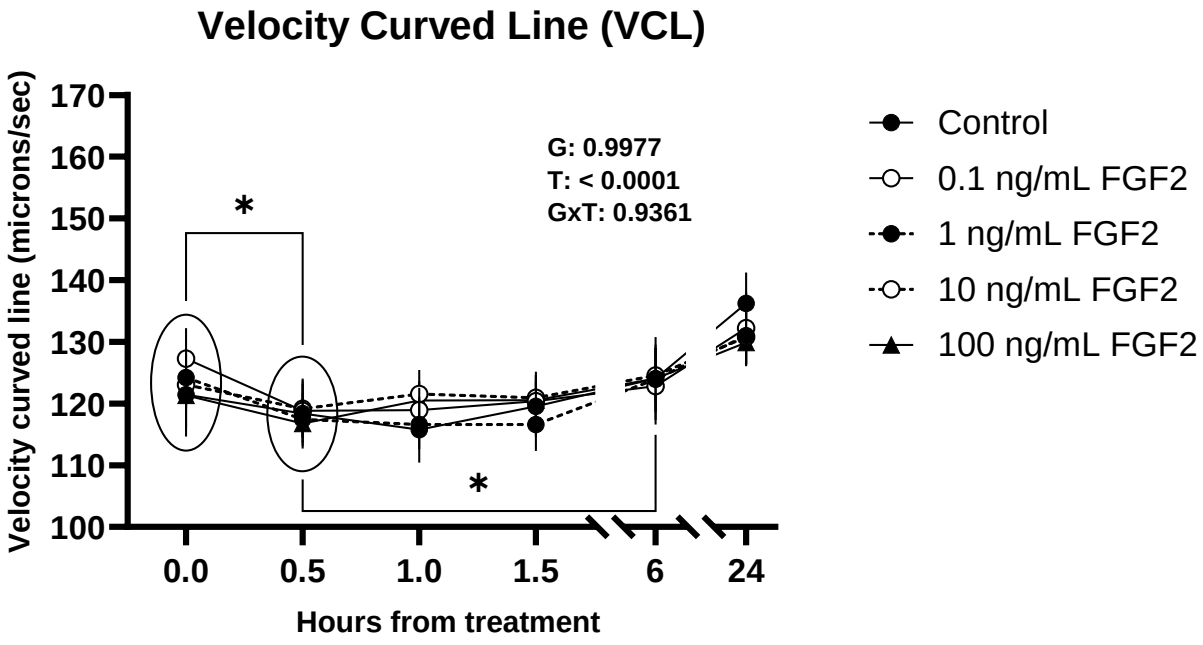


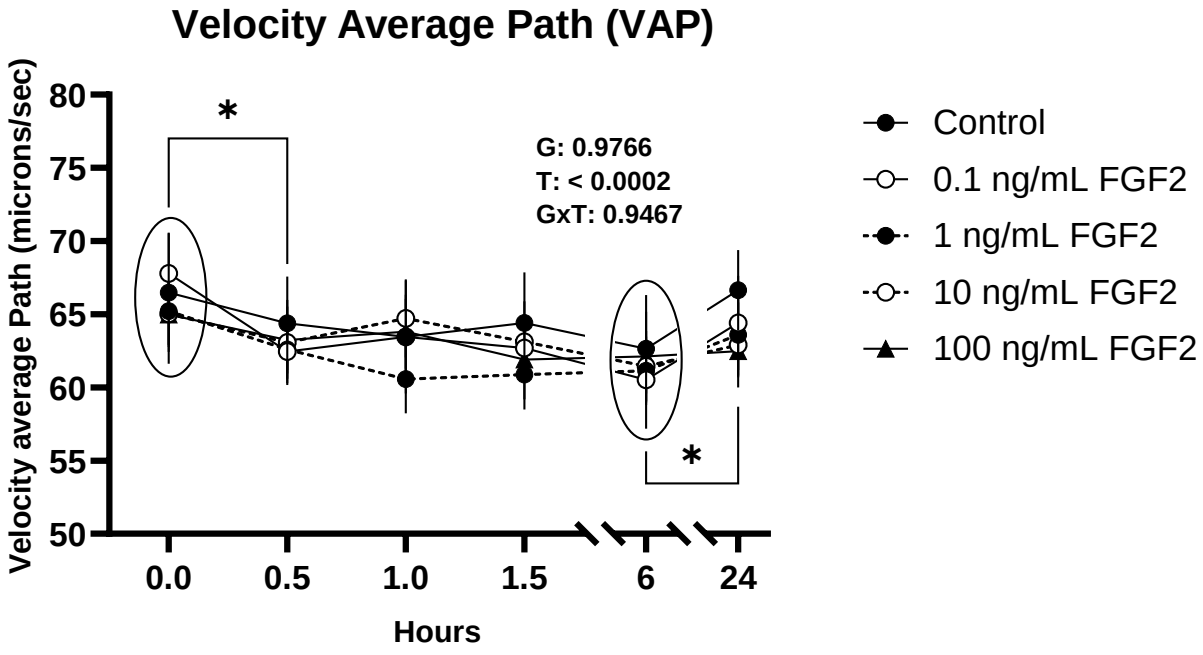
**Figure 9.** TMOT and PMOT obtained by CASA during hours 0, 0.5, 1, 1.5, 6, and 24 in the control group and after treatments with recombinant equine FGF2 at concentrations 0.1, 1, 10, and 100 ng/mL. Each group contained 7 stallions with 2 ejaculates each. Significant effect of time ($P < 0.05$) was noted, represented by a gradual decrease in all groups. Significant ($P < 0.05$) time changes in direction (increase or decrease) for groups combined are represented by a circle. The first significant ($P < 0.05$) change on direction for time in the groups combined are indicated by an asterisk (*).

The variables DCL, DAP, VCL, VAP, and ALH, decreased ($P < 0.05$) from hours 0 to 1, and AOC decreased ($p < 0.05$) from hours 0 to 6. An increase ($P < 0.05$) was noted on DCL, VCL, and ALH starting at hour 0.5, and with DAP, VAP, and AOC starting at hour 6 (Fig. 8).


Distance Curved Line (DCL)
Distance curved line (microns)
80
70
60
50
40
G: 0.9955
T: < 0.0001
GxT: 0.971
*
*
0.0
0.5
1.0
1.5
6
24
Hours from treatment
Control
0.1 ng/mL FGF2
1 ng/mL FGF2
10 ng/mL FGF2
100 ng/mL FGF2


Distance Average Path (DAP)
Distance average path (microns)
36
34
32
30
28
26
24
G: 0.9775
T: < 0.0001
GxT: 0.9823
*
*
0.0
0.5
1.0
1.5
6
24
Hours from treatment
Control
0.1 ng/mL FGF2
1 ng/mL FGF2
10 ng/mL FGF2
100 ng/mL FGF2


Velocity Curved Line (VCL)
Velocity curved line (microns/sec)
170
160
150
140
130
120
110
100
G: 0.9977
T: < 0.0001
GxT: 0.9361
*
*
0.0
0.5
1.0
1.5
6
24
Hours from treatment
Control
0.1 ng/mL FGF2
1 ng/mL FGF2
10 ng/mL FGF2
100 ng/mL FGF2


Velocity Average Path (VAP)
Velocity average Path (microns/sec)
80
75
70
65
60
55
50
G: 0.9766
T: < 0.0002
GxT: 0.9467
*
*
0.0
0.5
1.0
1.5
6
24
Hours
Control
0.1 ng/mL FGF2
1 ng/mL FGF2
10 ng/mL FGF2
100 ng/mL FGF2

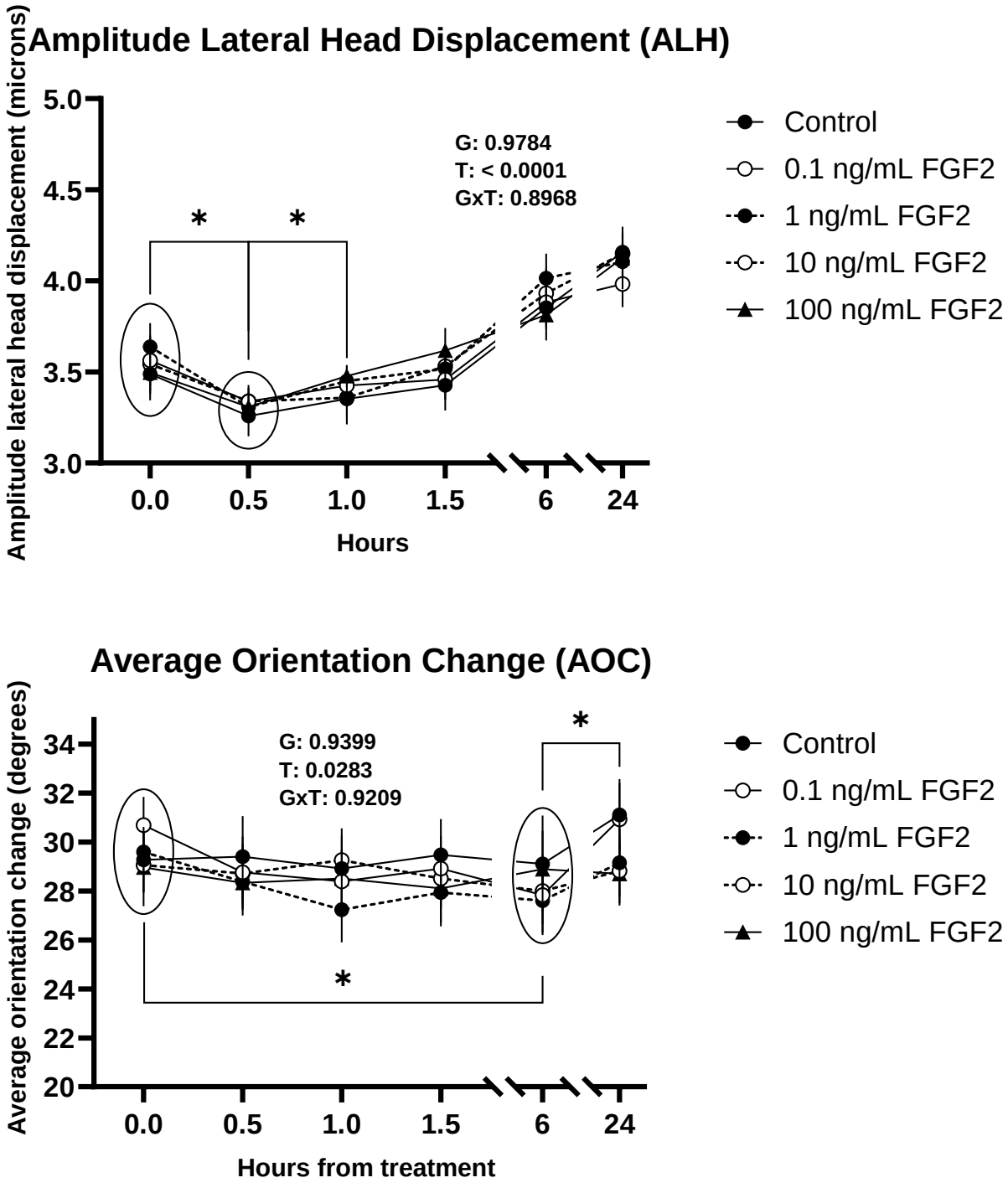



**Figure 10.** DCL, DAP, VCL, VAP, ALH, and AOC obtained by CASA during hours 0, 0.5, 1, 1.5, 6, and 24 in the control group and after treatments with recombinant equine FGF2 at concentrations 0.1, 1, 10, and 100 ng/mL. Each group contained 7 stallions with 2 ejaculates each. Significant effect of time ($P < 0.05$) was noted in all variables represented by decrease followed by an increase at different hour 0.5 for the variables DCL, VCL, and ALH, and at hour 6 for the variables DAP, VAP, and AOC. Significant ($P < 0.05$)

time changes in direction (increase or decrease) for groups combined are represented by a circle. The first significant ($P < 0.05$) change on direction for time in the groups combined are indicated by an asterisk (*).

The variables DSL and VSL showed a gradual decrease ($P < 0.05$) starting at hour 0 until hour 24 (Fig. 9).

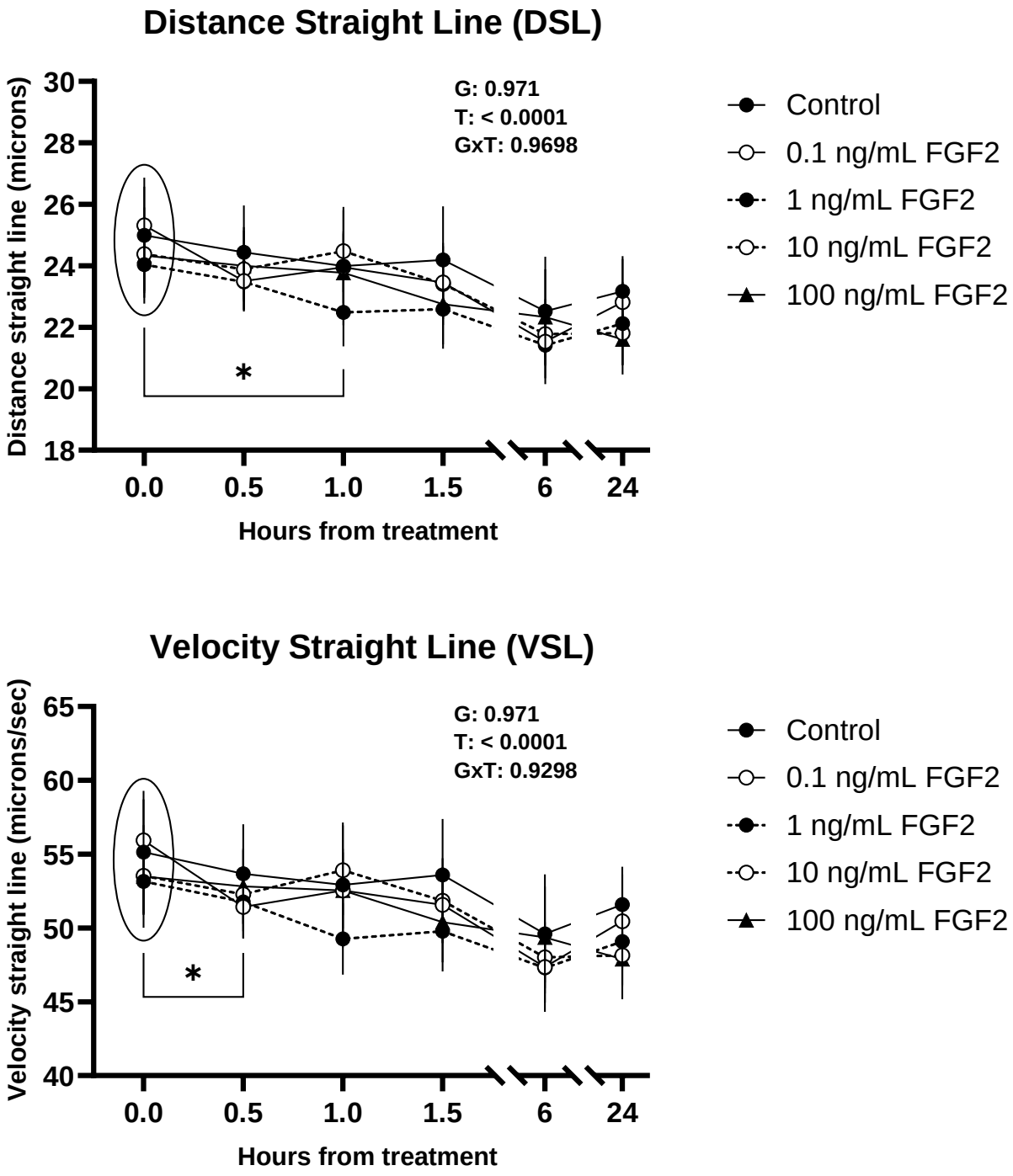
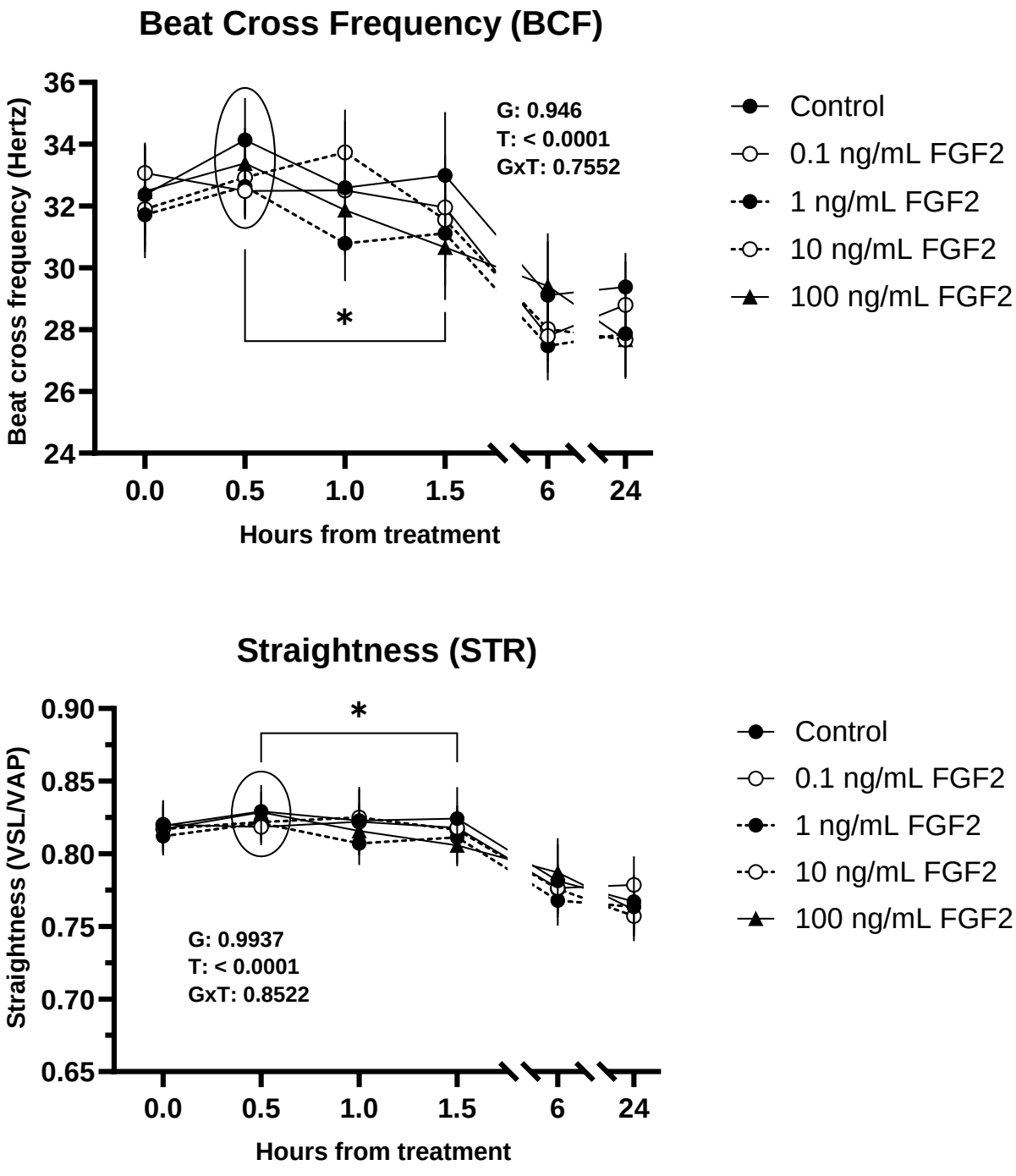
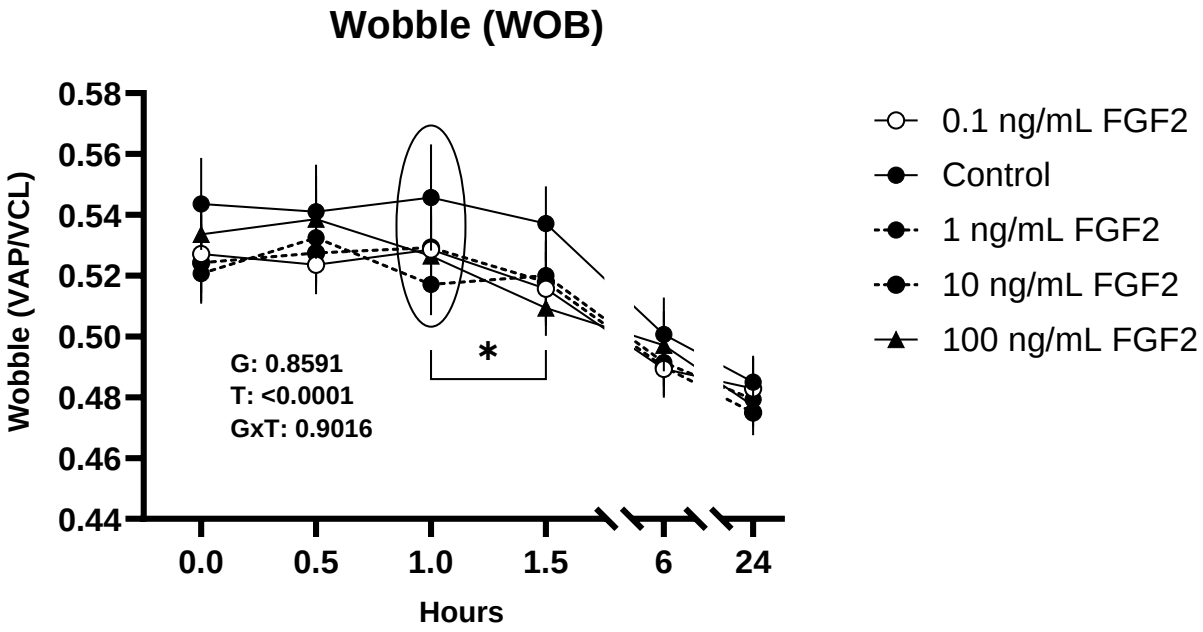
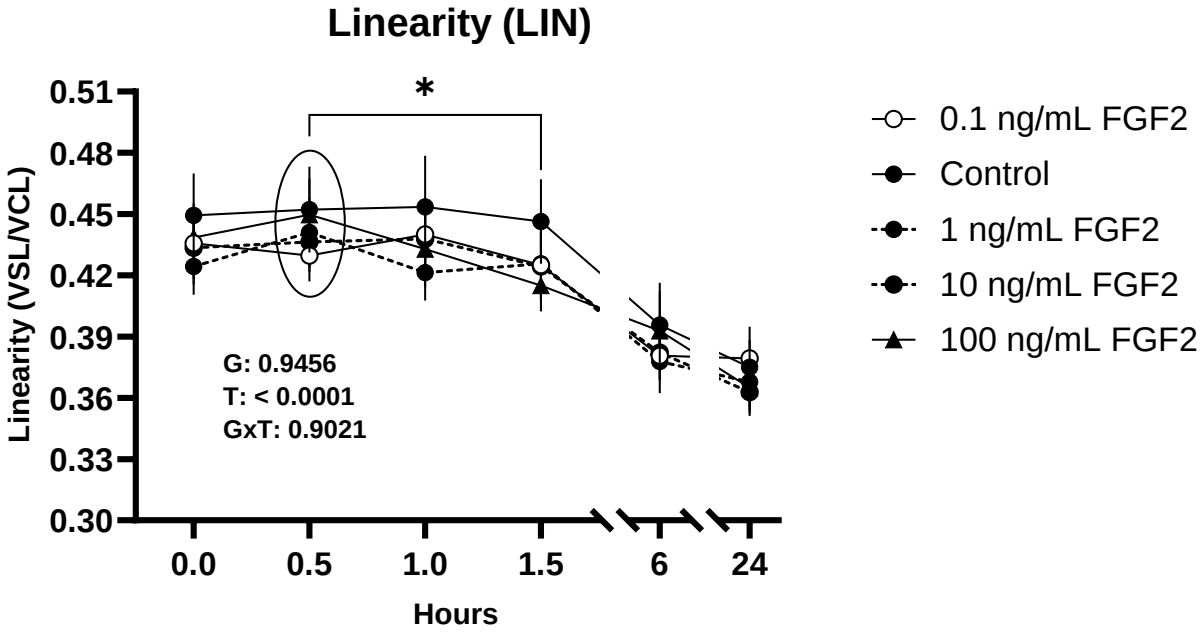



**Figure 11.** DSL and VSL obtained by CASA during hours 0, 0.5, 1, 1.5, 6, and 24 in the control group and after treatments with recombinant equine FGF2 at concentrations 0.1, 1, 10, and 100 ng/mL. Each group contained 7 stallions with 2 ejaculates each. Significant effect of time ($P < 0.05$) was noted, represented by a gradual decrease from hours 0 to 1, a plateau from hours 1 to 1.5, and a gradual decrease until hour 24. Significant ($P < 0.05$) time changes in direction (increase or decrease) for groups combined are represented by a circle. The first significant ($P < 0.05$) change on direction for time in the groups combined are indicated by an asterisk (*).

A decrease ($P < 0.05$) 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 (Fig. 10).




Wobble (WOB)
Wobble (VAP/VCL)
0.58
0.56
0.54
0.52
0.50
0.48
0.46
0.44
G: 0.8591
T: <0.0001
GxT: 0.9016
*
0.0
0.5
1.0
1.5
6
24
Hours
0.1 ng/mL FGF2
Control
1 ng/mL FGF2
10 ng/mL FGF2
100 ng/mL FGF2


Linearity (LIN)
Linearity (VSL/VCL)
0.51
0.48
0.45
0.42
0.39
0.36
0.33
0.30
*
G: 0.9456
T: < 0.0001
GxT: 0.9021
0.0
0.5
1.0
1.5
6
24
Hours
0.1 ng/mL FGF2
Control
1 ng/mL FGF2
10 ng/mL FGF2
100 ng/mL FGF2

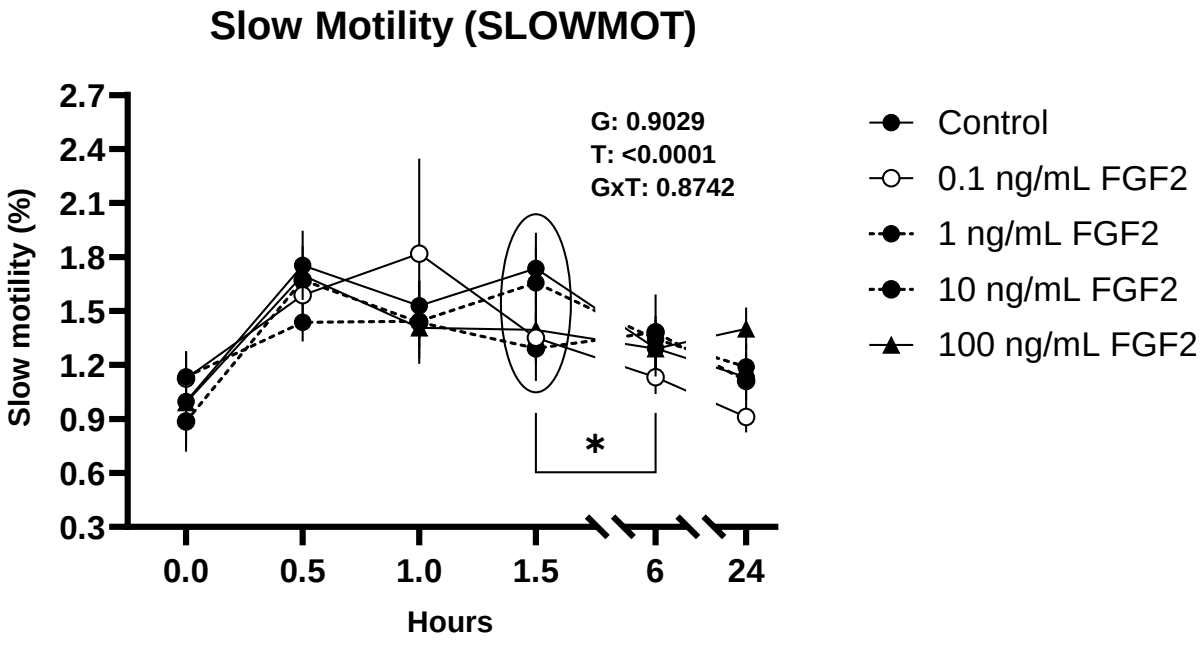



**Figure 12.** BCF, STR, WOB, LIN, and SLOWMOT obtained by CASA during hours 0, 0.5, 1, 1.5, 6, and 24 in the control group and after treatments with recombinant equine FGF2 at concentrations 0.1, 1, 10, and 100 ng/mL. Each group contained 7 stallions with 2 ejaculates each. Significant effect of time ($P < 0.05$) was noted, observed by a decrease starting at hour 0.5 for the variables, BCF, STR, and LIN, at hour 1 for the variable WOB, and at hour 1.5 for the variable SLOWMOT. Significant ($P < 0.05$) time changes in direction (increase or decrease) for groups combined are represented by a circle. The first significant ($P < 0.05$) change on direction for time in the groups combined are indicated by an asterisk (*).

No significant ($P < 0.05$) effects of group, time, or interaction between group and time were noted between PRPL or FGF2 treatments on any sperm motility variables obtained by CASA.

## 7. Discussion

The hypothesis that pooled platelet rich plasma lysate (PRPL) and/or recombinant equine fibroblast growth factor 2 (FGF2) added to extended sperm enhances sperm motility was not supported (Fig. 2 and Fig. 6) and HHA was noted after PRPL treatment in concentrations higher

than 5%, as shown by a decrease in concentration directly attributed to a decrease of free-swimming sperm (Fig. 1). For the present study, HHA appeared to be an artifact from the reaction with components of PRPL (i.e. fibrin) than a physiologically important event. The artifact assumption of was supported by the fact that PRPL doses were increased among groups but heparin dose was maintained at 1 IU/mL, which could potentially allow the occurrence of slight amounts of gel formation that could entrap sperm and resulting in HHA. Unfortunately, a trial with increased amounts of heparin added to extended semen was not performed and this assumption could not be tested. For a future study, one option should be to repeat the experiment with higher concentrations of heparin and observe if HHA is prevented. However, heparin effect on sperm capacitation should be consider and could be another variable added to the experiment.

In contrast with the artifact hypothesis, previous studies using blood serum in bull sperm had similar outcome of HHA in ascending concentrations and a positive effect of serum on semen motility (148). Several compounds were also able to induce HHA as heparin, serum, or follicular fluid in either monkeys (149), bulls (150, 151), and pigs (152). In the rabbit, HHA could be detected *in utero*, as shown by significant HHA when motile spermatozoa had contact with uterine lumen secretions (153). Moreover, HHA can also be induced by dibutyril-cAMP (154) and in response to dilution, washing, cold shock, extreme pH, or osmotic conditions (120). In the present experiment the only factors tested that could induce HHA based on these previous reports are the PRPL, which is the closest composition to serum, and heparin. However, PRPL may be the sole factor or is acting in synergism with heparin, since no HHA was observed after treatment with 1IU/mL of heparin alone.

Besides the HHA occurrence after PRPL treatment, no effect on motility was observed by either PRPL or FGF2 treatments and the present experiment disagree with previous works

performed in human and mice (10, 11, 86). One important characteristic, besides the species difference, was the semen extender or media used in all these previous works, which was not milk-based as the one used in the present experiment (INRA 96). In addition, most commercially available semen extenders do not report their components for a patent purpose and unknown components could be another variable to be added. It is also important to consider that bovine milk, which is the most used component for stallion semen extenders, has several factors including GFs as EGF, FGF1 and FGF2, IGF1 and IGF11, TGF-β1 and TFG-β2 (155), and PDGF, which are also present in PRPL. Those GFs are in high amounts in the bovine colostrum but decrease substantially after a couple of hours. The most abundant GFs in bovine milk are EGF (2-155 ng/mL), IGF-1 (2- 101 ng/mL), IGF-II (2-107 ng/mL), and TGF- β2 (13-71 ng/mL) (155). Other GFs concentration, including FGF2, also decrease substantially and are below 4 ng/mL a couple hours after the calf is delivered, when colostrum is all consumed (155). Therefore, it is important to consider that the semen extender, INRA96, contain many factors knowingly beneficial for stallion sperm which could potentially interfere or even mask the effect of PRPL and FGF2 on sperm motility improvement. For further studies, trials with unextended semen would be recommended, but this would not be valuable for clinical use since sperm life span during storage is considerably decreased without dilution in semen extender. Most equine semen extenders available are remarkably similar and use a milk-based formula, but another option would be to use a non-milk-based extender.

The hypothesis that PRPL treatment is not deleterious for sperm survival was supported with concentrations below 10% (Fig. 2), but HHA occurred in concentrations higher than 5% (Fig. 1). Sperm survival can be detected by the maintenance of sperm motility among time, which decreased at a similar rate in all groups. This decrease in motility was expected due to the

amount of time (1-1.5 hour) the samples were being manipulated at a warm (37 $^{o}$C) temperature before chilling, and by the motility decrease during 24 hours at lower temperature storage. Since no detrimental effect on sperm was observed in the sperm motility comparison with the control group, it is confirmed that the use of PRPL added directly to the ejaculate was not detrimental to the overall sperm cell. This suggests that addition of PRPL at appropriate concentrations prior to breeding may be used to modulate post-mating endometritis. However, it is still unknown if the overall volume or concentration of PRPL that could be used in the semen without deleterious effect on sperm viability is enough to provide immunomodulation in the mare's reproductive tract.

Heparin effect on sperm should also be considered in the present experiment due to its known capacitation effect in stallions (124). Capacitation is a process and is associated with an increase in sperm intracellular Ca2+ levels and protein tyrosine phosphorylation. There is also the development of a distinct pattern of motility (increased lateral deviation of the head) which is followed by a response to the chemotactic stimuli from the egg and acrosomal exocytosis after binding to the oocyte. This process is controlled by intrinsic sperm factors and extrinsic factors in the male and female reproductive tracts (156-159). In the present study, analysis of sperm motility characteristics did not show differences between treated and not treated semen with heparin throughout 24 hours of analysis. If capacitation were triggered, hyperactivated motility in the heparin treated group would likely change the sperm kinetics and be observed by CASA in comparison with the control group. A more detailed evaluation of sperm capacitation using molecular analysis was not performed in the present study and further research is needed. As an example, the molecular analysis for sperm capacitation could be performed using Pisum sativum Agglutinin labeled with fluorescein isothiocyanate and Hoechst dye Nu33258 (double

fluorescence) (FITC-PSA) (160, 161), which objectively measures the proportion of capacitated sperm in comparison with non-capacitated sperm.

Previous reports in mares suffering from chronic endometritis used infusions of 20 mL of PRP four hours after artificial insemination and intrauterine inflammatory response was decreased (2), resulting in higher pregnancy rates (1). However, no report was performed using PRPL, which has a greater concentration of factors present in the alpha granules (6) and a potentially greater effectiveness than PRP. One option of a clinical application for the present study would be to supplement PRPL in the semen prior to breeding using doses that are not detrimental to sperm motility and do not induce HHA. However, the volume of PRPL necessary for an effect on uterine inflammatory response post-mating is still unknown. Based on studies using PRP, 20 mL would be an effective dose to act on uterine inflammation post-mating (2) and a maximum PRPL proportion should be 2.5% without an effect on sperm motility or HHA. Therefore, a total volume of 800 mL would be necessary to contain 20 mL of PRPL for an optimal proportion.

Large volumes for artificial insemination are not recommended due to the backflow from the cervix and consequent loss of viable sperm cells. In addition, the infusion of a larger insemination dose could be more malefic than the benefit of PRPL treatment, especially in mares with a history of poor uterine clearance. Therefore, PRPL supplementation in the semen dose to modulate uterine inflammation does not seem clinically viable currently. Options to adjust the treatment dose would be to prevent HHA and increase PRPL concentration so that is not deleterious to sperm motility, or establish the lowest percentage of PRPL that is still effective on the control of uterine inflammation. For that, further studies are necessary to evaluate the amount

of PRPL necessary to modulate uterine inflammatory response and control the occurrence of HHA after supplementation with PRPL treatments higher than 2.5%.

The hypotheses that FGF2 added to semen extender enhances sperm motility was not supported. The hypothesis that sperm survival would be maintained when FGF2 is added to semen extender was supported. No statistically significant changes on sperm motility were observed after FGF2 treatment. Sperm survival was maintained throughout the experiment and the decrease on motility over 24 hours was similar in all FGF2 treated groups compared with the control. Therefore, no beneficial or detrimental effect of FGF2 on sperm motility was noted. As a contrast in humans, treatment using recombinant human FGF2 showed that incubation with 10 ng/mL rhFGF2 led to an increase in both total number and motile sperm recoveries and improvements in sperm VCL, VSL, and LIN values compared with 0 or 100 ng/mL rhFGF2 (10). The incubation of human spermatozoa with FGF2 resulted in a significant increase in the percentage of motile cells, as well as in sperm kinematics, suggesting that the FGF2/ FGFRs system is involved in motility regulation (11). The same result was also observed in mice (12). However, in the present experiment, no significant effect on sperm characteristics was observed. The interference on CASA evaluation is important to consider based on operator experience, sample concentration of sperm, temperature change prior to sample analysis, and variability of motility among animals. If motility changes induced by FGF2 did occur, they were subtle and undetectable by the CASA evaluation in the present experiment. For a clinical setting application, no evident effect of FGF2 was observed that would encourage the clinical use of FGF2 for improvement of stallion sperm motility. One important difference from the present experiment and the ones performed in human and mice (10-12) is the media used for the analysis. In the present experiment, the milk based extender INRA96 has a broad variety of

factors that are knowingly beneficial to the sperm and milk is known to have FGF2 as one of the factors in its composition, even though in amounts below 4 ng/mL. Therefore, the presence of the extender INRA96 could potentially interfere with FGF2 actions or even mask the positive effect on sperm motility, resulting in an undetected effect on sperm motility as similar as what happened with PRPL.

Besides motility effect, FGF2 could potentially affect sperm capacitation, which was not evaluated in the present study. In the murine model, sperm incubation with recombinant mice FGF2 (rmFGF2) led to an increase sperm motility and velocity and to enhanced intracellular Ca2+ levels and acrosomal exocytosis compared to the control (12). Although a detailed evaluation of capacitation was not performed, hypermotility could be detected and observed subjectively on motility analysis. However, all FGF2 groups had similar motility profiles for all variables when compared with the control group. Therefore, capacitation effect of FGF2 on stallion sperm based on hyperactive motility observation was not supported by the present study.

In conclusion, no evidence of a beneficial effect of preservation of semen motility was observed either using PRPL of FGF2 treatment in stallions. Doses of PRPL below 10% did not interfere with sperm survival and PRPL doses greater than 5% induced HHA. It is still unknown if these doses of PRPL in semen would influence uterine inflammatory response modulation. Further studies are needed for the molecular evaluation of sperm capacitation after addition of PRPL and FGF2. Also, PRPL could be directly added to the semen prior to breeding for uterine inflammatory control, but the control of HHA and further evaluation of effects on semen using higher doses are necessary to adjust the treated insemination dose with an acceptable volume. For further research, the clinical application of the present results could be focused on uterine inflammatory response rather than a direct effect on sperm for motility improvement.

## 8. Conclusion

Neither PRPL nor FGF2 had a direct beneficial effect on sperm motility in the present experiment. It is still uncertain the physiological relevance of HHA observed during the treatment with PRP and PRPL, but it is more suggestive of an artifact reaction between sperm and components of PRPL and milk-based extender.

The absence of an effect of either PRPL or FGF2 could potentially be caused by the interference of the semen extender in the results. For further analysis, the use of unextended semen would be an option to test the effect of both compounds on sperm characteristics. However, the absence of semen extender precludes the clinical use of this supplementation for chilled or frozen semen.

No direct molecular analysis for capacitation of sperm was performed in the present experiment and, therefore, the effect of heparin, PRPL, and/or FGF2 on capacitation induction could not be evaluated. One option for further studies would be to use objective analysis with fluorescent probes, as FITC-PSA, to grade the amount of capacitation between groups.

The known effect of PRP on inflammatory response modulation in the uterus and the possibility of a maximum dose of 2.5% of PRPL in the semen without deleterious effect on sperm motility would be an applicable treatment to control post-mating endometritis. However, further research on controlling HHA and adjustments on the PRPL proportion are needed to obtain an optimal volume and concentration for an insemination dose.

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