Strong inhibition of neutrophil-sperm interaction in cattle by selective PI3-kinase inhibitors

Running title: Inhibitors for neutrophil-sperm interaction

Summary sentence: Neutrophil-sperm interaction is strongly inhibited by selective PI3-kinase inhibitors at nanomolar concentrations.

Keywords: Artificial insemination, neutrophil, PI3-kinase inhibitors, sperm-neutrophil inhibition

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ABSTRACT

The vast majority of sperm are lost from the female reproductive tract in hours following natural mating or artificial insemination in mammals. Multiple complex processes including uterine contractions, mucus barriers and phagocytosis of sperm by neutrophils have been reported to be involved in the sperm loss, although the contribution of each process is uncertain. If phagocytosis by neutrophils has a significant role in sperm loss, inhibition of neutrophil response to sperm could potentially reduce the dose of sperm required for artificial insemination. Through development of a quantitative in vitro assay, we have screened 74 candidate compounds for their ability to inhibit the neutrophil-sperm interaction in cattle. Nine inhibitors (GSK2126458, wortmannin, ZSTK474, PIK294, CAL-101, GSK 1059615, GDC-0941, PIK 90 and PI103) active against phosphatidylinositol 3-kinase (PI3-kinase) were most potent, and strongly reduced neutrophil-sperm interaction with an IC50 of 10 nM or less. These inhibitors did not significantly modify sperm motility, and five of the inhibitors did not affect in vitro fertilization. Examination of neutrophil-sperm interaction by time-lapse video microscopy and cell tracking analysis revealed that GSK2126458 may prevent sperm phagocytosis through inhibition of neutrophil movement and/or attachment. Twenty four other compounds exhibited weaker inhibition (IC50 < 115 μM), and the rest did not inhibit the neutrophil-sperm interaction.

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Strong PI3-kinase inhibitors identified in this study may be useful to determine the contribution of neutrophil phagocytosis in clearance of sperm from the female reproductive tract.

INTRODUCTION

In mammals, sperm navigate the long and complex female reproductive tract (FRT) before fertilizing the oocyte. The number of sperm that reach the oocyte is tightly regulated to ensure the selection of high quality sperm for fertilization, but also to avoid the risk of polyspermy. Multiple factors can affect the number of sperm reaching the oocyte, and these include: 1) sperm characteristics such as vitality, motility and functionality; 2) physical environment of the FRT that blocks, damages and expels sperm by: epithelial interaction, damage through osmotic, oxidative and catabolic changes, movement via uterine muscle contraction and mucus interaction, and neutrophil phagocytosis [1, 2]. In the case of natural bovine mating, approximately four billion bull sperm are inseminated, yet less than 10,000 get to the oviduct and less than 10 get through to the oocyte, and greater than 80% of sperm are lost through vaginal discharge [3]. By 12 to 24 hr after insemination, sperm have either been lost or reached the oviduct [4]. Modern bovine artificial insemination (AI) uses considerably less sperm than natural mating by depositing directly into the uterine body. Bypassing of the vagina and cervix by AI results in approximately 1000-fold reduction in required sperm [5], but still the vast majority of sperm are lost from FRT similar to that seen in natural mating. How much each factor mentioned above contributes to the sperm loss is uncertain. Treatments that enhance the ability of sperm to remain in the FRT and be undamaged, motile, functionally competent, but not

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phagocytosed could reduce the number of sperm required for AI, and also increase conception rates in individuals with sub-optimal fertility.

Neutrophils are key part of the innate immune system and inflammation, and provide the first line of defense against infectious agents. Neutrophils are most abundant leukocytes in blood (38% in adult cow [6]). They migrate from the bloodstream to the inflamed site following chemo-attractant signals such as platelet-activating factor complement C5a [7] in a process called chemotaxis. Neutrophils then directly attack foreign objects by ingestion (phagocytosis), secretion of reactive oxygen species (ROS), release of granules containing soluble hydrolytic proteins, and generating neutrophil extracellular traps (NETs). Neutrophils have a large number cell surface receptors, which trigger diverse intracellular signal transduction pathways including protein kinase and calcium signaling pathways when activated [8].

The clearance of sperm within the FRT, in particular the uterus, is primarily thought to be due to the neutrophil response to sperm [9-11]. In cows [9], pigs [10] and horses [12], there is strong evidence for phagocytosis of sperm by neutrophils, with a massive infiltration of neutrophils occurring in the uterine lumen shortly after insemination. Studies in pigs [10, 11] showed that the phagocytosis of sperm could be inhibited by caffeine. Although the phagocytosis of sperm by neutrophils has been previously reported, the mechanism of sperm phagocytosis is still unclear and the data is often contradictory. Data from in vivo experiments suggest that both motile and damaged (immotile and/or dead) sperm are lost by discharge [13, 14], whereas in vitro evidence indicates that live sperm are preferentially phagocytosed by neutrophils [11]. Some seminal plasma proteins such as CRISP3 [12] were also found to suppress the neutrophil-sperm binding and regulate sperm elimination.

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Neutrophils are an attractive target for preventing sperm loss as they are short lived, with a half-life of 9 hr in circulation [15]. Thus, blocking neutrophil activity for a short period (2-4 hr) could allow more sperm to pass through the uterus without severely affecting the sensitivity of the uterus to infection or later pregnancy-related events. Adding a blocking, or inhibiting agent of sperm phagocytosis to the FRT via insemination route may offer further advantage of creating a localized inhibition that would rapidly dissipate.

Inhibiting the neutrophil response to sperm could involve blocking several neutrophil functionalities such as: chemotactic responses, recognition of sperm, signal transduction for response or effector functions such as migration, engulfment and phagocytosis. In this investigation, a quantitative in vitro assay was developed to measure the extent of the neutrophil-sperm interaction. Using the assay, 74 candidate compounds that could block various neutrophil functionalities were screened for their ability to inhibit the neutrophil-sperm interaction in cattle. The most potent neutrophil-sperm interaction inhibitors identified in this study are active against phosphatidylinositol 3-kinase (PI3-kinase).

MATERIALS AND METHOD

Neutrophil isolation

EDTA-treated bovine heifer blood was centrifugated at 1000 × g for 20 min. After removal of plasma and buffy coat layers, neutrophils were separated from red blood cells by hypotonic lysis with water for 50 sec at room temperature. Following lysis, a 10% w/v solution of NaCl was added to give a final volume equal to 10% of the added water. Neutrophils were recovered by repetitive centrifugation at 600 × g for 10 min at room temperature until the pellet appeared white. Finally, neutrophils were resuspended in non-capacitating media (NCM pH 7.4;

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containing 0.3 mM NaH_2PO_4, 3.1 mM KCl, 0.4 mM $MgCl_2$, 1 mM sodium pyruvate, 40 mM HEPES, 100 mM NaCl, 21.7 mM lactate (85%), 50 μg/ml gentamicin) added with 2 mM $CaCl_2$ and 1 mM $NaHCO_3$. Yield was typically 1×10^8 cells/ml in 5 ml from a starting volume of 250 ml of blood. Neutrophils were typically > 80% pure, as determined by flow cytometry using CD11a staining, and forward and side scatter analysis (data not shown). Control experiments indicated that individual cow neutrophils exhibit some variation in the response to sperm, although the response and sensitivity to inhibitors were very similar. All investigations described in this paper were approved by the University of Auckland Animal ethic committee, approval number C821.

Microscopic examination of kinetics and time course of neutrophil-sperm interaction

Freshly collected bovine semen (CRV Ambreed, New Zealand) was diluted to 1×10^9 cells/ml in NCM containing 0.5 mg/ml of bovine serum albumin (BSA) and 5 mM glucose. Diluted semen was double-stained for 15 min at 37°C with 100 μg/ml of Hoechst 33342 (Invitrogen) and a CellTracker dye [50 μM CellTracker Green or 100 μM CellTracker Orange (Invitrogen)]. We chose the CellTracker dyes as they offer stable, non-toxic, bright staining up to 72 hr and do not transfer to neighboring cells. Two ml of egg yolk-Tris-glycerol extender [16] and 5 mM glucose were added to 1 ml of double-stained semen. After incubation at room temperature for 45 min in dark, excess dyes were removed by loading 3 ml of the semen-extender mix onto 10 ml of 60% Percoll PLUS (GE Healthcare) followed by centrifugation for 15 min at 700 × g. Resulting sperm pellet (1 ml) was resuspended in 2 ml of egg yolk-Tris-glycerol extender containing 5 mM glucose. Isolated neutrophils (a final concentration of 5×10^6 cells/ml; pooled from four different heifers), Hoechst 33342/CellTracker Green-stained sperm

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(2.5×10^6 cells/ml), Hoechst 33342/CellTracker Orange-stained sperm (2.5×10^6 cells/ml), 1% of adult bovine serum and NCM containing 2 mM $CaCl_2$ and 1 mM $NaHCO_3$ were gently mixed together in low binding tubes (Axygen) for 1, 2, 4 or 18 hr at 39˚C. '0 hr' control was prepared without incubation. At each time point, each sample was removed, and slides were prepared for microscopy. Twenty µl of sample was applied within a 1 cm x 1 cm square drawn on SuperFrost Plus glass slide (Menzel-Glaser) using ImmEdge hydrophobic barrier pen (Vector Laboratories), and dried completely. A drop of Prolong Gold antifade reagent (Invitrogen) and then a coverslip were applied to the dried sample. Samples were examined on Leica DMR fluorescent microscope equipped with DC500 camera and appropriate filters [DAPI (ex 325-375/em 435-485); G/F/P (ex 450-490/em 500-550); SPEC ORG (ex 539-557/em 575-587)] by two independent researchers, one being blinded to the treatments. Fluorescent and phase contrast images were taken and merged using AnalySIS software.

Sperm preparation for in vitro neutrophil-sperm interaction assay and time-lapse video microscopy

Bovine sperm extended in egg yolk-Tris-glycerol extender (CRV Ambreed, New Zealand) were diluted to 1×10^9 cells/ml in NCM and labeled with 50 µg/ml Hoechst 33342 for 30 min at 37˚C. Excess Hoechst 33342 was removed by adding 1:2 v/v ratio of sperm to 60% Percoll PLUS and centrifuging for 20 min at 700 × g. The resulting cell pellet was resuspended in NCM containing 0.5 mg/ml of BSA, centrifugated for 7 min at 700 × g, and resuspended in NCM containing 2 mM $CaCl_2$ and 1 mM $NaHCO_3$ to a cell concentration of 20×10^6/ml.

In vitro neutrophil-sperm interaction assay to screen for inhibitory compounds

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NCM containing 2 mM $CaCl_2$ and 1 mM $NaHCO_3$ was placed in 96-well black ELISA plates (BD). Isolated neutrophils prepared as described above were added to a final concentration of 4×10^6 cells/ml, and 1% w/v adult bovine serum was added as a blocking agent to reduce intra-well variation. Various candidate inhibitor compounds were added to the wells containing NCM, neutrophils and serum, and lastly, the sperm prepared as described above were added to a final concentration of 4×10^6 cells/ml. Each well contained a total volume of 200 µl, and the assay was performed in 4 technical replicates. Plates were incubated at 39˚C for 1 hr with gentle shaking. After incubation, plates were read in the EnVision multi-label fluorescent plate reader (PerkinElmer), then washed three times by repeating removal of contents by "flicking" and addition of NCM containing 2 mM $CaCl_2$ and 1 mM $NaHCO_3$ to each well, and the plates read again for fluorescence. The ability of a candidate compound to inhibit neutrophil-sperm interaction in cattle was determined by comparing the extent of neutrophil-sperm interaction in the presence and absence of the compound, and the results were expressed as % of unwashed fluorescence, which correlates with the number of retained sperm after washing. Candidate compounds were purchased from various companies including Calbiochem, Cayman Chemicals, Gibco, LC Laboratories, Medchem Express, Merck, R&D, Selleck, Sigma, Symansis or Tocris. Samples from a total of 10 bulls (each providing between 1 and 4 ejaculates) and 31 cows (each providing 1 or 2 blood samples for neutrophil isolation) were used to test 74 candidate compounds. For each 9 compound that showed strong inhibition (GSK2126458, wortmannin, ZSTK474, PIK294, CAL-101, GSK1059615, GDC-0941, PIK 90, and PI-103), 4 technical and 3 biological replicates (different combination of bull and cow each time) were evaluated.

We fitted a linear regression to test the outcomes of interest, % of sperm retained by neutrophils, in the presence of different concentrations of each PI3-kinase inhibitor. To stabilize

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the variance of data recorded as percentages and to prevent fitted values less than 0% (or > 100%), we transformed the data using the logit transformation, and analyzed on the transformed scale. Combination of cow and bull was fitted as random effect. Results were presented as back-transformed data. The SAS (V9.4 SAS Institute., Cary, NC, USA) and R (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, http://www.R-project.org) were used for statistical analyses. P value < 0.05 was considered statistically significant difference between inhibitor treatment and control groups.

Time-lapse video microscopy

Isolated neutrophils at 20×10^6 cells/ml were stained with 4 µg/ml of Hoechst 33342 for 30 min at 39˚C, centrifugated for 7 min at 580 × g, and resuspended in NCM containing 2 mM $CaCl_2$ and 1 mM $NaHCO_3$. Hoechst 33342-stained neutrophils were placed in the Lab-Tek 8-well chamber slides (Nunc) pre-coated with 5.88 $\mu g/cm^2$ Cell-Tak cell adhesive (BD) to a final concentration of 2×10^6 cells/ml, together with NCM containing 2 mM $CaCl_2$ and 1 mM $NaHCO_3$. Slides were incubated at 39˚C for 5 min. GSK2126458 (50 nM) inhibitor compound and 1% v/v adult bovine serum were then added to the chambers and incubated for 10 min at 39˚C. Unstained sperm prepared as described above were added to the chambers containing NCM, neutrophils and adult bovine serum to a final concentration of 2×10^6 cells/ml, followed by addition of 12 µM propidium iodide (PI in the LIVE/DEAD Sperm Viability Kit, Invitrogen). To investigate whether the live sperm were preferentially phagocytosed by neutrophils over dead sperm, Percoll-purified sperm were stained with 100 nM SYBR 14 (LIVE/DEAD Sperm Viability Kit, Invitrogen) for 10 min at 39˚C and purified again on 60% v/v Percoll PLUS before

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addition to the chambers containing NCM, neutrophils and adult bovine serum. Revolution XD spinning disk laser confocal microscopy system (Andor Technology) was used to record time-lapse video under × 400 magnification for 30 min (15 sec interval × 120 times, at 10 frames/sec) of neutrophil-sperm interaction. Automatic tracking of neutrophil movement was performed using ImarisTrack software (Andor Technology), and x and y displacement values of each cell over time were obtained. Cell tracking was analyzed and plotted using 'Chemotaxis Tool' available on Image J plugin. The distance and velocity of neutrophil movements were compared between 4 samples [1) neutrophils only; 2) neutrophils + sperm; 3) neutrophils + GSK2126458; 4) neutrophils + sperm + GSK2126458] using two sample T test, and 95% confidence interval (95% CI) was calculated.

Sperm motility analysis

Visual assessment of sperm motility analysis was used to test toxicity of inhibitor compounds to bovine sperm. 200 μl of NCM containing sperm at a concentration of 25×10^6/ml was placed in each well of 96-well flat bottom plates (BD). Varying amount of candidate inhibitor compounds were added to the wells containing NCM and sperm, and incubated at 37˚C for various lengths of time. Sperm motility was assessed by microscopy by two examiners who were blinded to the treatments and gave the motility a score between 1 (lowest motility) and 4 (highest motility) compared to 'no treatment' control at different time points.

In vitro fertilization

One ml of un-extended fresh bovine ejaculate was carefully loaded on top of 4 ml of 60% v/v Percoll PLUS and centrifugated for 20 min at 700 × g at 20˚C. The cells were resuspended in

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10 ml of NCM containing 0.5 mg/ml of BSA, and centrifugated for 5 min at 700 × g at 20˚C. The purified sperm was resuspended to 5×10^6/ml in in vitro fertilization (IVF)-Tyrodes albumin lactate pyruvate (TALP) media containing 107.7 mM NaCl, 7.15 mM KCl, 0.3 mM KH_2PO_4, 3.32 mM sodium lactate, 0.04 mM kanamycin, 25 mM $NaHCO_3$, 0.33 mM pyruvate, 1.71 mM $CaCl_2$ and 0.01% BSA. 500 µl of diluted sperm was treated with various candidate compounds for 24 hr just prior to IVF. 10 µl of the treated sperm at 5×10^6/ml was then added to every 5 oocytes in a volume of 40 µl, and IVF was performed by standard procedures [17, 18] at AgResearch, Ruakura, New Zealand. The effects of candidate compounds on oocyte fertilization and blastocyst development to day 7 were examined. In each experiment, three compounds were tested along with 'no inhibitor control' using a number of oocytes ranging between 44 and 76, and three independent experiments were performed in total.

RESULTS

Kinetic analysis of neutrophil-sperm interaction

In order to investigate the kinetics of bovine neutrophil-sperm interaction in vitro, sperm double-stained with Hoechst 33342 (fluorescent blue nuclear DNA stain in sperm head) and Cell Tracker dye (fluorescent green or orange covalent stain in the tail) were incubated with unstained neutrophils for 0, 1, 2, 4 and 18 hr, and then dried on glass slide. The more time the neutrophils and sperm were co-incubated, the larger the resulting clumps of interacting sperm and neutrophils became (Fig. 1). In fact, co-incubation of neutrophils and sperm for 18 hr resulted in just a few very large neutrophil-sperm clumps formed at the bottom of low binding tubes, thus physical dissociation of the large clump into smaller parts was necessary to prepare the slide for fluorescent microscopy. More than 99% of sperm were found associated with neutrophils after

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18 hr co-incubation. In addition, more sperm were found inside neutrophils with longer duration of co-incubation of neutrophils and sperm. The phagocytosis of sperm by neutrophils was easily detected with fluorescent staining of sperm, because the blue sperm head and green or orange curled sperm tail were found co-localized within neutrophils. The kinetic analysis by fluorescent microscopy provided a general overview of the time course for in vitro sperm phagocytosis process by neutrophils.

Examination of neutrophil-sperm interaction by time-lapse video microscopy

Phagocytosis of sperm by neutrophils was more closely examined by time-lapse video microscopy. Interaction between sperm and neutrophils was recorded for 30 min immediately after addition of unstained sperm and free PI (red nuclear stain for dead cells) to Hoechst 33342-stained neutrophils. Staining neutrophils with Hoechst 33343 showed their typical horseshoe-shaped nucleus and > 80% purity under fluorescent microscope. Most neutrophils involved in interaction with sperm were flat and irregular in shape, active and motile, and contained many intracellular granules. Smaller spherical neutrophils were less motile, and interact with sperm less actively.

Results from the time-lapse video microscopy revealed various aspects of sperm phagocytosis by neutrophils (Fig. 2; see Supplemental Movies 1a-1h for details). These observation include: active neutrophils could migrate relatively long distance (up to ~200 μm) in 30 min, in comparison to inactive neutrophils (Fig. 2a and Supplemental Movie 1a with long tracking line); and neutrophils could also change their shapes and extend pseudopods in the direction of sperm (Fig. 2b and Supplemental Movie 1b). Neutrophils and sperm frequently formed small clumps (Fig. 2c and Supplemental Movie 1c), like those observed in the time

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course experiment at 1 hr. Sperm associated in these clumps were not necessarily phagocytosed by neutrophils, and could just be attached to other sperm cells or neutrophils. Active neutrophils engulfed/phagocytosed sperm soon after initial recognition and attachment (Fig. 2d and Supplemental Movie 1d). The sperm head tended to be engulfed by neutrophil before sperm tail, and the head remained inside the neutrophil longer than sperm tail. It was frequently observed that sperm head was rotated in a perpendicular orientation upon contact with a neutrophil during the phagocytosis process (Fig. 2d and Supplemental Movie 1d). Phagocytosed sperm heads were visible inside neutrophils if the head was fluorescently stained with a nuclear dye (Fig. 2e and Supplemental Movie 1e). Neutrophils appeared to interact more with live sperm (green SYBR 14-stained or no color-stained) than dead sperm (red PI-stained), as has been reported for pigs [11]. Fluorescent staining of the live sperm head changes from green to red in real time while phagocytosis progresses, indicating that phagocytosis induces death of sperm (Fig. 2f and Supplemental Movie 1f). Multiple sperm cells could be simultaneously captured by a single neutrophil (Fig. 2g and Supplemental Movie 1g), and a single neutrophil that had already engulfed one sperm cell could engulf more sperm (Fig. 2h and Supplemental Movie 1h). These data collectively show that phagocytosis of sperm by neutrophils involves strong interactions between the two cell types, particularly the sperm head and the neutrophil, that sperm phagocytosis resulted in sperm death, and that there were large variations in the "appetite" of neutrophils for sperm.

Inhibition of neutrophil-sperm interaction by PI3-kinase inhibitors

In vitro neutrophil-sperm interaction assay was developed to screen multiple candidate compounds for their ability to inhibit the neutrophil-sperm interaction. Using this quantitative

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assay that measured the percentage of fluorescently labelled sperm retained by plate-adhered neutrophils, a total of seventy four candidate compounds were screened for ability to inhibit sperm retention (Table 1). Compounds with potential to inhibit neutrophil function or inhibit phagocytosis by immune cells were selected for this assay. The selected compounds targeted a variety of pathways or processes including PI3-kinase and other kinases, cyclooxygenase (COX) pathway, chemotaxis and migration of neutrophils, ROS production, formation of NETs, calcium influx and recognition of sperm surface (Table 1).

The most potent inhibitors identified in the assay are known to be active against PI3-kinases. Seventeen inhibitors specific to PI3-kinases were tested in total, and nine of them (GSK2126458, wortmannin, ZSTK474, PIK294, CAL-101, GSK1059615, GDC-0941, PIK 90, and PI-103) showed strong inhibition with an Inhibition Concentration that causes 50% inhibition (IC50) of 10 nM or less (Table 1-Category 1). Fig. 3 and Table 2 show the % of retained sperm by neutrophils in the presence of various concentrations of five PI3-kinase inhibitors (wortmannin, ZSTK474, GSK2126458, PIK294 and PI103) as examples. Three cow:bull combinations (cow#1:bull#1, cow#2:bull#1, cow#3:bull#2) analyzed in quadruplicate each were used for the statistical analysis, and the effect from cow:bull combination was fitted as random effect. The sperm binding was inhibited by these potent inhibitors in a dose responsive manner, and the maximum inhibition reduced sperm binding from ~50% to ~10%. At 10 nM, all these 5 inhibitors were significantly different from control group containing no inhibitor (all $P < 0.0001$). Significant inhibition ($P < 0.05$) was observed with ZSTK474 and PI103 down to 1 nM; wortmannin and PIK294 down to 0.1 nM; and GSK2126458 even at 0.01 nM. Another two PI3-kinase inhibitors (IC-87114 and NVP-BEZ235) with an IC50 of 10-100 nM and five inhibitors (AZD6482, PIK-75, AS-252424, TGX-221 and AS-605240) with an IC50 of 100-1000 nM

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exhibited inhibition, but at higher concentrations (Table 1-Category 2). LY 294002 with IC50 of 115 μM was the least active PI3-kinase inhibitor tested.

Sixteen non-PI3-kinase inhibiting compounds also inhibited neutrophil-sperm interaction to some extent (Table 1-Category 3). Three compounds (PD98059, rapamycin and staurosporine) that primarily inhibit other kinases, W-7 hydrochloride a calcium influx modifying agent, and indomethacin a COX inhibitor, moderately inhibited neutrophil-sperm interaction with IC50 between 100 and 1000 nM. In contrast, the other eleven compounds including 6 compounds inhibiting kinase pathways, 3 compounds inhibiting chemotaxis, SKF 96365 modifying calcium influx and diphenyleneiodonium (DPI) chloride inhibiting ROS production, showed much weaker inhibition with high estimated IC50 ranging between 1 and 100 μM. The remaining 41 compounds including majority of calcium influx modifiers, ROS production inhibitors, potential sperm recognition inhibitors and neutrophil migration inhibitors did not show inhibition of neutrophil-sperm interaction at 100 μM or 1 mM (Table 1-Category 4).

Strong inhibition of neutrophil-sperm interaction by GSK2126458

Examination of the neutrophil-sperm interaction by time-lapse video microscopy indicated that PI3-kinase inhibitor compounds such as wortmannin and GSK2126458 prevented neutrophil movement in addition to the attachment and/or phagocytosis of sperm. Results for GSK2126458 inhibition of neutrophil-sperm phagocytosis are best illustrated by time-lapse video microscopy (see Fig. 4 and Supplemental Movie 2). In the presence of sperm without GSK2126458, neutrophils were shown to be highly active and motile, generally large, flat and amorphous with extended pseudopods, and often seen associated with sperm (Fig. 4a and Supplemental Movie 2a). In contrast, neutrophils pre-treated with GSK2126458 for 10 min were

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much less active, moved less distance, were generally smaller and spherical, and showed much less frequent interaction with sperm (Fig. 4b and Supplemental Movie 2b). Track diagrams (Fig. 5) and neutrophil motility assessment (Table 3) obtained from the latter video microscopy demonstrate the significant effects that the inhibitor have on the motility and distance covered by neutrophils. Compared to neutrophils alone, presence of bovine sperm significantly increased the velocity of bovine neutrophil motility ($P < 0.05$). Addition of GSK216458 significantly decreased both velocity and distance of neutrophil motility in the presence of sperm ($P < 0.05$) in comparison to that without GSK216458. These observations are compatible with previously published results showing that wortmannin inhibits chemotactic peptide-induced neutrophil locomotion [19]. Wortmannin also could inhibit neutrophil ability to move, bind and phagocytose sperm in a similar manner to GSK2126458 (data not shown).

Compounds that target PI3-kinases and inhibit the neutrophil-sperm interaction do not modify sperm motility

PI3-kinase inhibitors that were potent inhibitors of the neutrophil-sperm interaction were assayed for their effects on the motility of sperm. As shown in Table 4, visual subjective assessment of sperm motility analysis showed that motility of sperm was not generally affected at concentrations up to 10 μM for 12 hr period. Nine most potent compounds in the neutrophil-sperm interaction assay were tested even up to 100 μM, and majority of these compounds did not inhibit sperm motility.

Compounds that target PI3-kinases and inhibit the neutrophil-sperm interaction do not inhibit oocyte fertilization

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Six potent PI3-kinase inhibitors (CAL 101, GSK2126458, PI103, PIK294, wortmannin, and ZST474) were examined for their ability to inhibit bovine oocyte fertilization and blastocyst development to day 7 in IVF. In this assay, the neutrophil-sperm inhibitors were included for 24 hr at the fertilization stage of IVF. Table 5 shows the results from three different sets of experiments performed in a similar manner. At concentrations 1 to 100-fold greater than IC50 for the neutrophil-sperm interaction, the inhibitors did not affect fertilization (% cohort cleavage) at the highest concentration tested. But there was a small negative effect on blastocyst development depending upon the compound, PIK294 being the most significant.

DISCUSSION

This study provides an in vitro method for quantitative analysis of the bovine neutrophil-sperm interaction. The assay was used to screen a total of 74 candidate compounds for their ability to inhibit neutrophil-sperm interaction, and identified nine PI3-kinase inhibitors as the most potent inhibitors (IC50 of 10 nM or less). To date, no such quantitative assay has been developed to analyze neutrophil-sperm interaction. Further, no compound has been reported to inhibit the neutrophil-sperm interaction at nanomolar concentration. However, recently the multifunctional prostaglandin E_2 has been suggested as a physiological inhibitor of neutrophil-sperm interaction in the bovine oviduct in two recent studies carried out by Marey et al [20, 21]. In the in vitro assay presented here, a strong interaction between fluorescently labelled sperm and neutrophils occurred within 60 min in a media that has similar ion concentration of uterine fluid. The measurement of fluorescence after washing allowed determination of the extent of sperm retention by the neutrophils. Typically ~50% sperm were retained by neutrophils in the absence of inhibitor, and this level of in vitro neutrophil-sperm interaction was similar to that

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reported by others [9, 22-24]. Nine highly potent inhibitors of the neutrophil-sperm interaction were identified: GSK2126458, wortmannin, ZSTK474, PIK294, CAL-101, GSK1059615, GDC-0941, PIK 90, and PI-103, and their maximum inhibition reduced sperm binding to < 10%. Other PI3-kinase inhibitors tested also inhibited neutrophil-sperm interaction, but at higher concentrations (Table 1). Almost complete inhibition of sperm-neutrophil interaction by GSK2126458 was also confirmed by time-lapse video microscopy.

PI3-kinases have important roles in immune cells [25], particularly on leukocyte chemotaxis and phagocytosis [26, 27]. Importance of the PI3-kinases in chemotaxis is supported by reduced migration of leukocytes from PI3-kinase knockout mice in response to various stimulators [28-30]. PI3-kinases predominantly function by phosphorylation of phosphoinositides on the D3 position of the inositol ring [31]. PI3-kinases are divided into I, II and III classes based on primary structure and function, and are composed of a catalytic and a regulatory subunit. Class I PI3-kinases were the first to be characterized, and many of commercially available PI3-kinase inhibitors target class I p110 α, β, γ and δ catalytic subunits.

PI3-kinase inhibitors with various specificities for the p110 catalytic subunits were tested in the in vitro sperm-neutrophil assay. The highly potent PI3-kinase inhibitor GSK2126458 has a very low IC50 reported for all 4 subunits (0.019 for α, 0.13 for β, 0.06 for γ, 0.024 nM for δ) [32], whereas another PI3-kinase inhibitor CAL-101 has a higher specificity for δ subunit (2.5 nM) in comparison to other subunits (820 for α, 565 for β, 89 nM for γ subunit) [33]. However, both compounds were potent inhibitors for neutrophil-sperm interaction. In general, PI3-kinase inhibitors with high IC50 (over 1000 nM) reported for any of the subunits showed less inhibition in neutrophil-sperm interaction (Table 1). Comparison of the published IC50 and the activity of various inhibitors tested in the neutrophil-sperm interaction assay (Table 1), suggests that α and δ

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catalytic subunits may be more important than β or γ subunits, for the neutrophil response to sperm.

Importance of the PI3-kinase α and δ subunits in phagocytosis is supported by previous findings. PI3-kinase α subunit was found to be important in phagocytosis by macrophages [34] and monocytes [35]. Several roles of PI3-kinase δ subunit in neutrophil phagocytosis have been reported including chemotaxis [36], migration [37], cell trafficking into inflamed sites [38], oxidase activation and cell spreading [39], respiratory burst [40], and cytokine production [41]. The δ subunit-specific inhibitor IC-87114 reduced chemotactic movement of neutrophils [37, 38]. However, the exact role of PI3-kinase subunits in neutrophil phagocytosis is unclear. In this study, some α and δ subunit-specific compounds (e.g. TGX-221, NVP-BEZ235) did not show any inhibition at the concentrations close to the published IC50. These compounds may not have had complete solubility or the ability to pass through the neutrophil cell membrane. In addition, a careful inhibitor analysis study [42] demonstrated that a pan-PI3-kinase inhibitor LY 294002 was able to block human neutrophil polarization and migration to the chemotactic agent CXCL8, but only slightly delayed (~15 min) in the presence of another chemotactic agent fMLP. This suggests the neutrophil chemotaxis is not entirely PI3-kinase dependent and the chemo-attractant may only partly determine the signal transduction pathway used.

Five potent PI3-kinase inhibitors (CAL 101, GSK2126458, PI103, wortmannin and ZST474) did not affect in vitro oocyte fertilization nor blastocyst development in this study. PIK294 was the only compound that showed potential toxicity on blastocyst development, although it did not affect oocyte fertilization. PIK294 is one of the most potent p110 δ subunit-selective inhibitors reported, but also inhibits p110 α, β, γ subunits, DNA-dependent protein kinase (DNA-PK) and mammalian-Target-Of-Rapamycin (mTOR) with IC50 9.6, 0.67, 0.2, 48

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and > 50 μM, respectively [43, 44]. Therefore, the binding of PIK294 to additional targets might have caused the toxicity on blastocyst development.

Like for the IVF results, most PI3-kinase inhibitors (14 of 17 tested) did not affect sperm motility at 10 μM. For 8 PI3-kinase inhibitors (GSK2126458, ZSTK474, PIK294, CAL-101, GSK 1059615, GDC-0941, PIK 90 and PI103) that inhibited neutrophil-sperm interaction with IC50 of < 10 nM, the 10 μM concentration tested was 1,000 to 10,000 times the dose required to inhibit neutrophil-sperm interaction. Three compounds (PIK-75, TGX-221 and wortmannin) may partially inhibit sperm motility at 10 μM, however this action may occur by binding to targets other than PI3-kinases. It is reported that PIK-75 inhibits DNA-PK (IC50 2 nM) [45], and wortmannin inhibits PI4-kinase and myosin light chain kinase (MLCK; IC50 200 nM) [46], polo-like kinase 1 (PLK1; IC50 5.8 nM) [47] and phospholipase D [48], in addition to PI3-kinases. Previous studies have shown variable results on the effect of PI3-kinase inhibitors on sperm motility, but tested only two PI3-kinase inhibitors, wortmannin and LY 294002. Dose-dependent inhibition of sperm motility by wortmannin was shown in Koppers et al.'s study [49], in which 10 μM wortmannin inhibited sperm motility to 50% of basal level in 4 hr, similar to this study. Two previous studies reported that PI3-kinase inhibition by 10 μM LY 294002 enhanced motility of human sperm [50, 51]. In contrast, treatment of bovine sperm with LY 294002 only affected motility parameters [52].

Of note, the evidence for the presence of PI3-kinases in sperm is unclear. PI3-kinase was found to be present in human [49] and boar spermatozoa [52] by Western blot analyses. However, proteomics analysis on mouse [53], rat [54] and human spermatozoa [55] did not identify any PI3-kinases or related proteins in the pathway. Similarly, proteome analyses that we have performed have detected a total of > 1200 proteins from bull spermatozoa but also did not

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find any PI3-kinases (data not shown). Results from proteomics data suggest that PI3-kinases are either not present in the spermatozoa, or present in very low abundance.

Kinetics/time course experiment and time-lapse video microscopy in this study corroborated the interaction assay and provided potential insights into the mechanism of neutrophil-sperm inhibition. Neutrophils could migrate relatively long distances toward sperm and the rate of neutrophil motility was doubled in presence of sperm, suggesting that soluble factors released by bovine sperm could act as chemo-activators or chemo-attractants for bovine neutrophils. The fact that neutrophils interact more with live sperm than dead sperm strongly suggest that the mechanical stimuli may also be one of the inducing factors for phagocytosis. Neutrophils could extend pseudopods toward sperm, frequently change their shape, and have amorphous or amoeba-like shape, which are all known to be the characteristics of activated neutrophils. Multiple sperm cells could be simultaneously captured by a single neutrophil, and a single neutrophil could engulf multiple sperm. The sperm head was usually the initial site for attachment and subsequent engulfment by neutrophils. Based on our results, the strong interaction between neutrophils and sperm that occurred in the in vitro assay is likely due to the phagocytosis process of sperm by neutrophils. Inhibition of neutrophil-sperm interaction can act upon several steps of phagocytosis such as: neutrophil migration, chemotaxis, recognition, attachment, and engulfment. Results from time-lapse video microscopy suggest that in the presence of PI3-kinase inhibitor GSK2126458, neutrophils exhibited significant differences in movement and cell morphology. Neutrophils treated with GSK2126458 were much less active, less motile, smaller and spherical, and less frequently interacted with sperm. Thus, the effect of GSK2126458 could be on the neutrophil migration, chemotaxis and recognition. GSK2126458 may also affect the ability of neutrophils to attach and engulf sperm. However, without the early

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steps of the migration, chemotaxis and recognition, the effect of PI3-kinase inhibitors on neutrophil attachment and engulfment is unclear.

In the present study, nine compounds inhibiting other kinase pathways also inhibited neutrophil-sperm interaction with IC50 of 0.1-100 μM. In particular, PD98059, rapamycin and staurosporin showed IC50 < 1 μM. PD98059 inhibits extracellular signal-regulated kinase (ERK)-specific mitogen-activated protein kinase kinase (MAPKK) and blocks its phosphorylation [56]. PI3-kinase and ERK may co-regulate phagocytosis, but the regulation is complex depending upon the type of phagocytic cell and stimulation source [57]. Resolvin E1-enhanced phagocytosis was inhibited by PD98059, but also by an mTOR inhibitor, rapamycin [58]. mTOR is a part of PI3-kinase/AKT/mTOR pathway and has previously been reported to regulate neutrophil chemotaxis [59]. Staurosporine (a protein kinase C inhibitor), GW5074 (a c-Raf kinase inhibitor) and U0126 (a specific MEK inhibitor) prevented NET formation of neutrophils in a previous study [60]. In the present investigation, staurosporine, but not the GW5074 and U0126, inhibited neutrophil-sperm interaction, indicating that the kinase pathway activated by neutrophil-sperm interaction differs from the Raf-MEK-ERK pathway activated for NET formation.

This investigation showed that agents inhibiting PI3-kinases can strongly reduce bovine sperm-phagocytosing activity of neutrophils in vitro. It is not clear the exact mechanism whereby PI3-kinase inhibitors inhibit neutrophil-sperm phagocytosis. However, the reduction in migration and potentially recognition and/or attachment of neutrophils as shown in this study presents the basis for further analysis. Such inhibitors potentially have value in vivo, as they may be useful to determine the contribution of neutrophil phagocytosis in clearance of sperm from the FRT or may even improve outcome from AI.

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There are a few things to note. One may argue that the experimental conditions used in this study may affect the sperm capacitation, thus influence the sperm phagocytosis [14]. However, the flow cytometry analysis we performed with WGA-fluorescein indicated no significant changes on sperm capacitation using our NCM medium (data not shown). Knowing the well-recognized role of neutrophils in bacterial clearance, it would be important to check the effects of PI3-kinase inhibitors on the bacterial infection in vivo, although semen extender used for AI universally contains antibiotics to control bacterial contamination. In addition, it would be essential to investigate the effects of these inhibitors on uterine epithelial cells that regulate local immune environment to neutrophils and sperm.

Either less loss of sperm in the FRT or longer resident sperm lifespan could result in lower sperm dose for AI and improved conception rates. For the bovine AI industry, reduced sperm dose would have significant value for sexed sperm, which is currently limited by lower conception rate and higher price than unsexed sperm. Pharmacokinetics and routes of administration for these agents that inhibit neutrophil-sperm interaction are yet to be determined. However, the potent PI3-kinase inhibitors that neither inhibit sperm motility or IVF may have potential to be used in vivo.

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FIGURE LEGENDS

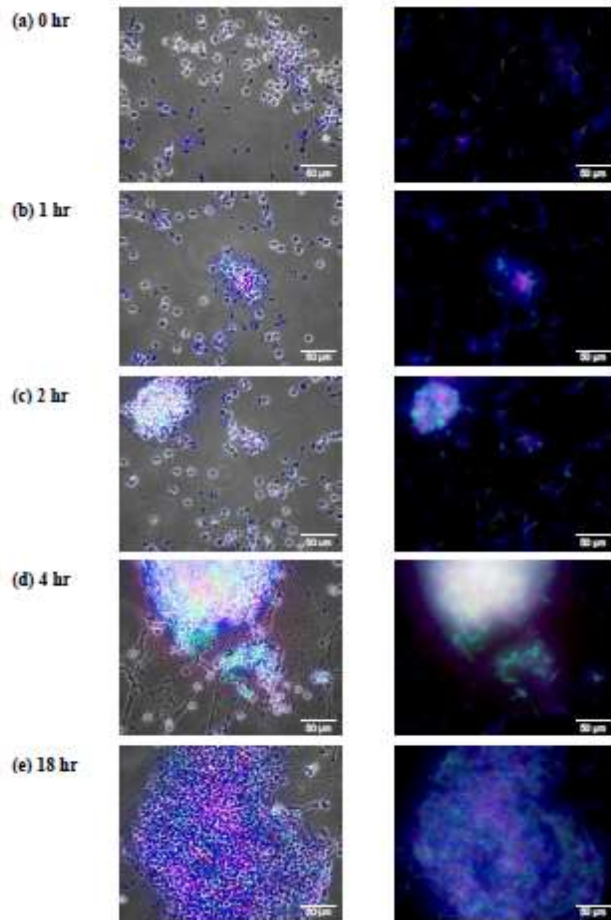

FIG. 1. Time course of neutrophil-sperm interaction. Interaction between neutrophils and sperm was examined by fluorescent microscopy at multiple time points (0, 1, 2, 4 and 18 hr). Sperm were double-stained with Hoechst 33342 (blue staining in head) and CellTracker dyes (green and orange staining in tail). Unstained neutrophils absorbed free Hoechst 33342 either from dye remaining in sperm solution or as it became free from the sperm, thus neutrophils acquired nuclear staining with time. Different rows of images represent different time points: (a) 0 hr, (b) 1 hr, (c) 2 hr, (d) 4 hr and (e) 18 hr. Fluorescent images taken under various filters were merged with (on left) and without phase contrast image (on right). The longer the neutrophils and

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sperm were co-incubated, the larger the neutrophil-sperm clumps were formed. Magnification, × 400

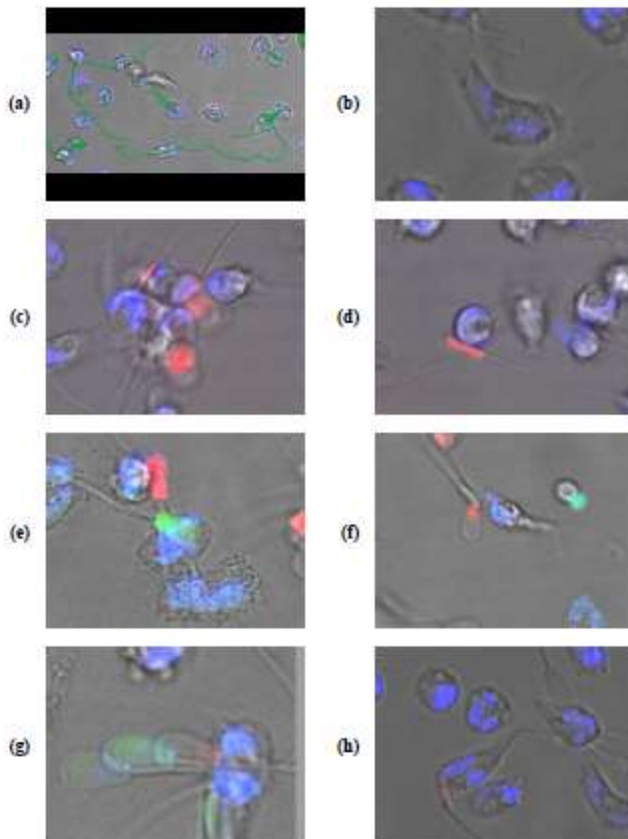

FIG. 2. Close examination of neutrophil-sperm interaction and phagocytosis by time-lapse video microscopy. Phagocytosis of sperm by Hoechst 33342-labelled neutrophils was examined in detail by time-lapse video microscopy. Small regions of the original video (magnification, × 400) were cropped to show detailed interaction between sperm and neutrophils. Time-lapse videos were recorded over 30 min and displayed as 8-10 sec movies in the Supplemental Movies 1a-1h. (a) An active neutrophil migrated relatively long distance (indicated by a long green tracking line, starting from bottom right hand corner to the bottom left hand corner, then to the top left hand corner) in the presence of sperm. See the Supplemental Movie 1a for details. (b) Active neutrophil (in the center) changed its shape and extended pseudopods in

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the direction of sperm. (Supplemental Movie 1b) (c) Small clumps (in the center) formed by interaction between neutrophils and sperm. (Supplemental Movie 1c) (d) Active neutrophil (in the center) recognized and phagocytosed sperm. Sperm head was rotated once perpendicularly in contact with neutrophil during phagocytosis process. (Supplemental Movie 1d) (e) Phagocytosis of live sperm by neutrophil (in the center). Initially, the green sperm head stained with SYBR 14 was well visible inside the neutrophil. Green nucleic material inside sperm got diffused and lost color intensity as phagocytosis of sperm progressed. Neutrophils appeared to interact more with live sperm (green/SYBR 14-stained or no color) than dead sperm (red/PI-stained). (Supplemental Movie 1e) (f) Fluorescent staining of live sperm changed from green (SYBR 14 staining) to red (PI staining) while phagocytosis progressed, indicating that phagocytosis induces death of sperm. (Supplemental Movie 1f) (g) A single neutrophil (on right) captured multiple sperm cells simultaneously. (Supplemental Movie 1g) (h) A neutrophil (moving from the top right hand corner to the center) that already engulfed a sperm cell (its tail is visible inside the neutrophil) continued engulfing more sperm. (Supplemental Movie 1h)

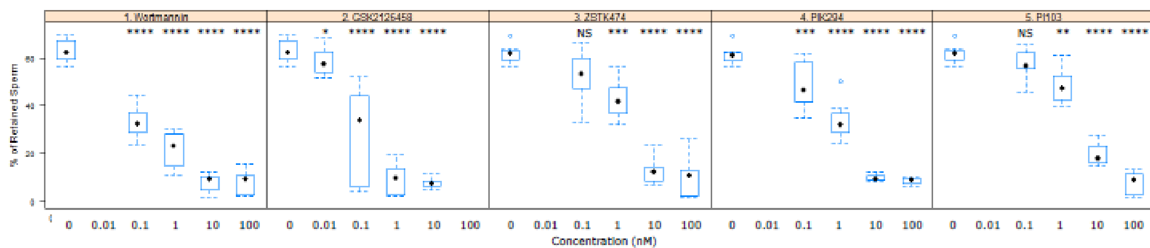

FIG. 3. Inhibition of neutrophil-sperm interaction using quantitative in vitro assay. Bovine neutrophils and Hoechst 33342-labelled sperm were incubated in the presence of various PI3-kinase inhibitors (wortmannin, GSK2126458, ZSTK474, PIK294 and PI103) for 1 hr, and the fluorescence was measured before and after washing. Three different cow:bull combinations (cow#1:bull#1, cow#2:bull#1, cow#3:bull#2) were analyzed in quadruplicate each, and the

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results are expressed in box-and-whisker plots representing % of unwashed Hoechst 33342 fluorescence, which correlates with the number of sperm remaining after washing. Note that cow#3:bull#2 dataset was available for all inhibitors except the PIK294. Linear regression was fitted to test the outcomes of interest, % of sperm retained by neutrophils, in the presence of different concentrations of each PI3-kinase inhibitor, and the combination of cow and bull was fitted as random effect. P values were calculated by comparing the inhibitor treatment group against the control group (no inhibitor). Not significant (NS), $P \geq 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

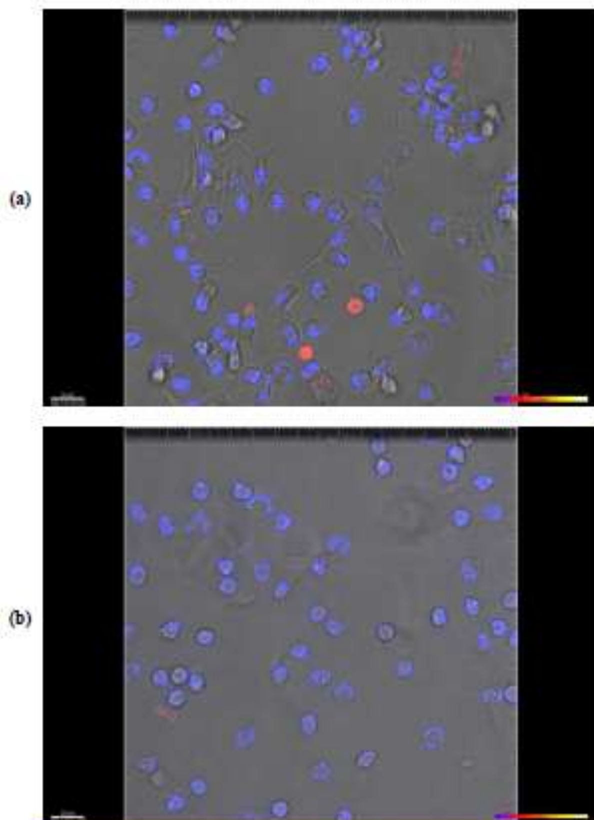

FIG. 4. Time-lapse video microscopy of sperm phagocytosis by neutrophils in the presence of PI3-kinase inhibitor GSK2126458. Time-lapse video microscopy was used to examine the effect of GSK2126458 on neutrophil-sperm interaction in detail. Hoechst 33342-

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labelled neutrophils were pre-treated with GSK2126458, and sperm and PI were added to the treated neutrophils. Time-lapse video microscopy on neutrophil-sperm interaction in the presence of GSK2126458 was repeated four times, and the results shown here are the representatives. Time-lapse videos were recorded over 30 min and displayed as 10 sec movies in the Supplemental Movies 2a and 2b. In the absence of GSK2126458 (Fig. 4a and Supplemental Movie 2a), neutrophils stained in blue are highly active, very motile, generally large, flat and amorphous with extended pseudopods, and often associated with sperm. In contrast, in the presence of GSK2126458 (Fig. 4b and Supplemental Movie 2b), neutrophils are much less active, move less distance, generally smaller and spherical, and shows much less frequent interaction with sperm. Magnification, $\times$ 400.

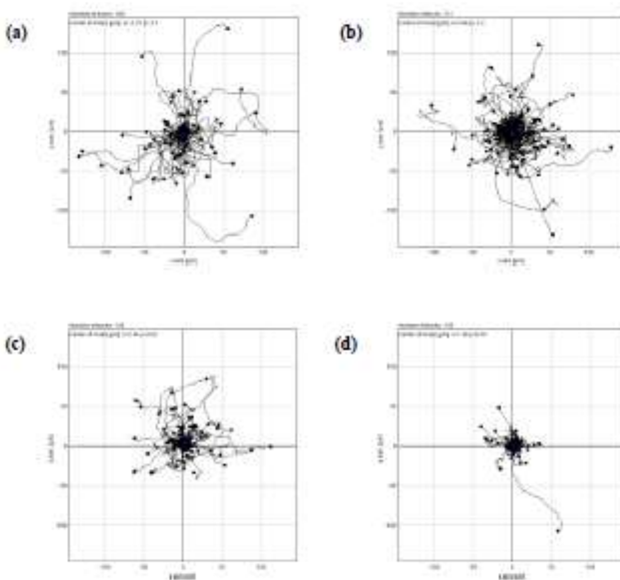

FIG. 5. Track diagrams showing the movement of neutrophils in the presence of sperm and/or PI3-kinase inhibitor GSK2126458. Time-lapse microscopic video of neutrophil-sperm interaction was recorded for 30 min using confocal microscopy system, and automatic tracking of neutrophil movement was performed using ImarisTrack software. Time-lapse video

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microscopy on neutrophil-sperm interaction in the presence of GSK2126458 was repeated four times, and the results shown here were the representatives. Neutrophil tracking was performed (a) in the presence of neutrophils only; (b) in the presence of neutrophils and sperm; (c) in the presence of neutrophils and GSK2126458; and (d) in the presence of neutrophils, sperm and GSK2126458. X and Y displacement of each neutrophil over time were plotted in the neutrophil track diagrams.

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TABLE 1. Seventy four inhibitors tested for neutrophil–sperm interaction in this study.

Category	Sub-category	Target or rationale for non-PI3-kinase inhibitors	Common name	CAS number	IC50 (nM) of neutrophil -sperm interaction	Published IC50 (nM) of PI3-kinase inhibitors for α, β, γ, δ subunits [Reference]
1) PI3-kinase inhibitors that strongly inhibited neutrophil–sperm interaction			GSK2126458	1086062-66-9	0.8 nM	0.019, 0.13, 0.06, 0.024 [32]
			Wortmannin	19545-26-7	0.8 nM	1 [a] [61]
			ZSTK474	475110-96-4	1.5 nM	6.7, 10.4, 11.7, 1.8 [62]
			PIK294	900185-02-6	4.9 nM	9.6, 0.67, 0.2, 3 [43]
			CAL-101	870281-82-6	5.3 nM	820, 565, 89, 2.5 [33]
			GSK 1059615	958852-01-2	7.5 nM	0.4, 0.6, 5, 2 [63]
			GDC-0941	957054-30-7	8.8 nM	3, 33, 75, 3 [64]
			PIK 90	677338-12-4	9.0 nM	11, 350, 18, 58 [43]
			PI103	371935-74-9	9.4 nM	8, 88, 150, 48 [43, 65]
2) PI3-kinase inhibitors that less strongly inhibited			IC-87114	371242-69-2	87 nM	200000, 11000, 17000, 180 [37]
			NVP-BEZ235	915019-65-7	90 nM	4, 76, 7, 5 [66]

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neutrophil–sperm interaction			AZD6482	1173900-33-8	132 nM	14000, 21, 1200, 80 [67]
			PIK-75	372196-67-3	392 nM	6, 13000, 76, 510 [43]
			AS-252424	900515-16-4	546 nM	940, 20000, 30, 20000 [68]
			TGX-221	663619-89-4	580 nM	5000, 5, 3500, 100 [69]
			AS-605240	648450-29-7	777 nM	60, 240, 8, 240 [69]
			LY 294002	154447-36-6	115 µM	1400[a] [70]
3) Non-PI3-kinase inhibitors that inhibited neutrophil–sperm interaction	Inhibit kinase pathway	Specific inhibitor of MAPKK	PD98059	167869-21-8	0.1-1 µM	
		Inhibitor of mTOR	Rapamycin (Sirolimus)	53123-88-9	0.1-1 µM	
		Broad spectrum protein kinase inhibitor	Staurosporine	62996-74-1	0.1-1 µM	
		Inhibitor of phospholipase C	U73122	112648-68-7	1 µM	
		Very potent and selective inhibitor of protein kinase C	GF109203X	133052-90-1	1-10 µM	
		Potent and selective non-competitive inhibitor of MAPKK	U0126	109511-58-2	1-10 µM	
		Inhibitor of Pan-Akt kinase	GSK-690693	937174-76-0	10-100 µM	
		Selective inhibitor of p38 MAPK	SB 203580	152121-	10-100 µM	

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				47-6	
		Inhibitor of protein kinase A which may be involved in neutrophil chemotaxis	H 89 dihydrochloride	127243-85-0	~100 μM
	Inhibit chemotaxis	Antagonist of CCR2b receptor	Teijin compound 1	226226-39-7	1-10 μM
		CXCR2 chemokine receptor antagonist	SB225002	182498-32-4	10-100 μM
		Inhibited neutrophil chemokinesis	Curcumin	458-37-7	~100 μM
	Modify calcium flux	Calmodulin antagonist	W-7 hydrochloride	61714-27-0	0.1-1 μM
		Calcium channel blocker	SKF 96365	130495-35-1	1-10 μM
	Modify COX pathway	Inhibitor of COX	Indomethacin	53-86-1	0.1-1 μM
	Inhibit ROS production	Inhibitor of NADPH oxidase. Decreases ROS production in activated neutrophils	Diphenyleneiodonium chloride	4673-26-1	1-100 μM
4) Non-PI3-kinase inhibitors that did not significantly inhibit neutrophil–sperm interaction	Modify calcium influx	Calcium flux modifier	2-Aminoethyl diphenylborinate	524-95-8	
			BTP2	223499-30-7	
			Cyclosporin A	59865-13-3	
			Econazole	27220-47-9	
			EGTA	67-42-5	

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		FK506	109581-93-3
		L-651,582	99519-84-3
		La3+ lanthanum	7439-91-0
		Nifedipine	21829-25-4
		Thapsigargin	67526-95-8
Inhibit kinase pathway	Inhibitor of Akt1/Akt2 activity	Akt Inhibitor VIII	612847-09-3
	Inhibitor of mTOR kinase	AZD8055	1009298-09-2
	Dual PDK1 and class I PI3-kinase inhibitor	BAG 956	853910-02-8
	Inhibitor of c-Raf1 kinase	GW5074	220904-83-6
	Inhibitor of mTOR	KU-006379 (KU0063794)	938440-64-3
	Inhibitor of PI3-kinase family kinases	PP 121	1092788-83-4
	Inhibitor of Pyk2 that is required for neutrophil degranulation	Sunitinib malate	341031-54-7
Inhibit chemotaxis	Antagonist of CCR2 chemokine receptor	BMS CCR2 22	445479-97-0
	Antagonist of PAF receptor	BN 52021	15291-

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		(Ginkgolide B)	77-7
	Antagonist of leukotriene B4 receptor	LY293111	161172-51-6
	Antagonist of CCR2 on neutrophils	RS 504393	300816-15-3
	Antagonist of CXCR2	SB265610	182498-32-4
	Antagonist of LTB4 receptor	U-75302	119477-85-9
Inhibit ROS production	Inhibitor of 5-lipoxygenase	AA-861	80809-81-0
	Inhibitor of MPO	4-aminobenzoic acid hydrazide	5351-17-7
	Inhibitor of NADPH oxidase	Apocynin (Acetovanillone)	498-02-2
	Antagonist of 5-lipoxygenase activating protein	MK 886	118414-82-7
	Oxygen free radical scavenger	N-acetylcysteine	616-91-1
Inhibit sperm recognition	Possible interaction with sperm surface	D+ mannose	3458-28-4
	Possible interaction with sperm surface	Fucoidin	9072-19-9
	Possible interaction with sperm surface	Glucose	50-99-7
	Possible interaction with sperm surface	Methyl α-D-mannopyranoside	617-04-9

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	Possible interaction with sperm surface	N-Acetyl-D-glucosamine	7512-17-6
Modify COX pathway	Antagonist of CB1	AM251	183232-66-8
	Agonist for the CB1 receptor	Methanandamide	157182-49-5
Other	Possible inhibitor of neutrophil migration	Dexamethasone	50-02-2
	Adenylate cyclase activator	Forskolin	66575-29-9
	A potent inhibitor of intracellular human neutrophil elastase (α-1-proteinase)	GW311616A	197090-44-1
	Inducer for neutrophil migration and NETs	IL-8 or CXCL8	Not available
	Enhancer for phagocytic activity of neutrophils	LPS	Not available
	Inducer for neutrophil migration and NETs	PMA	16561-29-8

[a] Published IC50 is for inhibition of Ptdins 3-kinase activity, not for each subunit.

CB1, cannabinoid receptor type 1; COX, cyclooxygenase; LPS, lipopolysaccharide; MAPK, Mitogen-activated protein kinase; MAPKK, MAPK kinase; MPO, myeloperoxidase; mTOR, mammalian-Target-Of-Rapamycin; NADPH, nicotinamide adenine dinucleotide phosphate; NET, neutrophil extracellular traps; PAF, platelet-activating factor; PDK1, phosphoinositide-dependent kinase-1; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species.

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TABLE 2. Inhibition of neutrophil-sperm interaction by 5 PI3-kinase inhibitors using quantitative in vitro assay.

	Concentration (nM)	Estimate (95% CI)[a]	*P* value[b]
Wortmannin	0	50.0	
	0.1	22.0 (16.7-28.6)	<.0001
	1	13.2 (9.7-17.7)	<.0001
	10	4.0 (2.9-5.6)	<.0001
	100	4.0 (2.9-5.6)	<.0001
GSK2126458	0	50.0	
	0.01	34.7 (23.9-47.4)	0.0203
	0.1	14.2 (9.5-20.8)	<.0001
	1	4.5 (2.9-6.9)	<.0001
	10	2.9 (1.7-4.8)	<.0001
PI103	0	50.0	
	0.1	46.7 (38.4-55.1)	0.4368
	1	36.6 (29.3-44.5)	0.0016
	10	13.0 (9.7-17.2)	<.0001
	100	4.1 (3.0-5.6)	<.0001
PIK294[c]	0	50.0	
	0.1	37.5 (31.7-43.7)	0.0003
	1	24.0 (19.6-29.0)	<.0001
	10	6.5 (5.1-8.2)	<.0001
	100	5.6 (4.4-7.1)	<.0001
ZSTK474	0	50.0	
	0.1	40.8 (31.5-50.8)	0.0716
	1	31.6 (23.6-41.0)	0.0004
	10	7.97 (5.5-11.5)	<.0001
	100	4.7 (3.2-6.9)	<.0001

[a] The estimated % of unwashed Hoechst 33342 fluorescence, which correlates with the number of sperm remaining after washing.

[b] Results from liner regression analysis using % of sperm retained by neutrophils as a dependent variable, and the concentration of inhibitor as an independent variable. The combination of cow and bull was fitted as random effect.

[c] Three different cow:bull combinations (cow#1:bull#1, cow#2:bull#1, cow#3:bull#2) were analyzed in quadruplicate for all inhibitors except the PIK294 where cow#3:bull#2 dataset was not available. *P* values were calculated by comparing the inhibitor treatment group against the control group (no inhibitor) and *P* values < 0.05 were considered significant.

95% CI, 95% confidence interval.

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TABLE 3. Neutrophil motility assessment in the presence of sperm and/or PI3-kinase inhibitor GSK2126458.

Treatment	Number of neutrophils tracked[a]	Mean velocity (µm/sec) ± SEM [min-max]	Mean velocity comparison			Euclidean mean distance (µm) ± SEM [min-max]	Euclidean mean distance comparison		
			Treatment	Mean difference (95% CI)	*P* value[b]		Treatment	Mean difference (95% CI)	*P* value[b]
1) Neutrophils only	146	0.11 ± 0.01 [0.01-1.40]				22.2 ± 2.58 [0.08-143]			
2) Neutrophils + sperm	221	0.23 ± 0.02 [0.01-2.40]	2) vs 1)	19.32 (9.94 - 28.7)	**< 0.05**	21.1 ± 1.62 [0.13-141]	2) vs 1)	-188.9 (-1169.5 - 791.6)	≥ 0.05
3) Neutrophils + GSK2126458 (50 nM)	135	0.09 ± 0.01 [0.01-0.60]	3) vs 1)	-4.23 (-9.54 - 1.08)	≥ 0.05	16.7 ± 1.82 [0.16-113]	3) vs 1)	-951.1 (-2036.6 - 134.4)	≥ 0.05
			3) vs 2)	-23.55 (-32.75 - -14.35)	**< 0.05**		3) vs 2)	-762.2 (-1613.5 - 89.2)	≥ 0.05
4) Neutrophils + sperm + GSK2126458 (50 nM)	125	0.12 ± 0.01 [0.02-1.10]	4) vs 1)	1.03 (-5.62 - 7.68)	≥ 0.05	8.1 ± 1.24 [0.18-122]	4) vs 1)	-2432.4 (-3460.4 - -1404.4)	**< 0.05**
			4) vs 2)	-18.29 (-28.26 - -8.32)	**< 0.05**		4) vs 2)	-2243.5 (-3038.7 - -1448.3)	**< 0.05**

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		4) vs 3)	5.26 (0.0038 - 10.52)	**< 0.05**		4) vs 3)	-1481.3 (-2237.5 - -725.2)	**< 0.05**

[a] Indicated number of neutrophils were tracked over 30 min

[b] *P* value from two sample T test (*P* < 0.05 were considered significant)

SEM, standard error of the mean; 95% CI, 95% confidence interval.

NOTE: This is the representative data of four independent experiments.

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TABLE 4. Sperm motility analysis in the presence of neutrophil-sperm interaction inhibitors.

Common name	IC50 (nM) of neutrophil -sperm interaction	Activity on motile sperm
PIK-75	392	Partial inhibition at 10 μM
TGX-221	580	Partial inhibition at 10 μM
Wortmannin	0.8	Partial inhibition at 10 μM, complete inhibition at 100 μM
GSK2126458	0.8	No inhibition ≤ 10 μM, complete inhibition at 100 μM
AS-252424	546	No inhibition ≤ 10 μM
AS-605240	777	No inhibition ≤ 10 μM
AZD6482	132	No inhibition ≤ 10 μM
IC-87114	87	No inhibition ≤ 10 μM
LY 294002	114820	No inhibition ≤ 10 μM
NVP-BEZ235	90	No inhibition ≤ 10 μM
CAL-101	5.3	No inhibition ≤ 100 μM
GDC-0941	8.8	No inhibition ≤ 100 μM
GSK 1059615	7.5	No inhibition ≤ 100 μM
PI103	9.4	No inhibition ≤ 100 μM
PIK 90	9.0	No inhibition ≤ 100 μM
PIK294	4.9	No inhibition ≤ 100 μM
ZSTK474	1.5	No inhibition ≤ 100 μM

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TABLE 5. *In vitro* fertilization data after 24 hr treatment with selected neutrophil-sperm inhibitors.

Experiment	Treatment	Total number of oocytes per cohort group	Cohort cleavage (%)	Cohort blastocyst grade 1 & 2[a] (%)	Cohort blastocyst grade 3[a] (%)
1	No inhibitor control	76	92	29	18
	PI103 (10 nM)	74	96	32	27
	Wortmannin (10 nM)	73	93	23	26
	ZST474 (10 nM)	68	94	31	19
2	No inhibitor control	44	86	15	6
	GSK 2126458 (100 nM)	61	61	13	11
	PI103 (100 nM)	54	70	13	14
	ZST474 (100 nM)	49	90	24	26
3	No inhibitor control	74	74	18	2
	CAL 101 (100 nM)	73	79	24	12
	GSK 2126458 (100 nM)	75	89	21	8
	PIK 294 (100 nM)	66	67	4	6

[a] Blastocyst grade 1 & 2 refer to blastocysts that are of suitable quality to be implanted into animals, whereas grade 3 is not transferable quality.

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