

Hamster Sperm-Associated Alpha-L-Fucosidase Functions During Fertilization

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Summary Sentence: The sperm associated isoform of alpha-L-fucosidase (SMALF) functions during hamster *in vitro* fertilization.

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ABSTRACT

Sperm associated alpha-L-fucosidases have been identified in diverse organisms. Their wide phylogenetic distribution and known properties support the likelihood that L-fucose and alpha-L-fucosidase have fundamental function(s) during gamete interaction. This is consistent with the substantial evidence in the literature documenting the importance of carbohydrate moieties during fertilization. Direct enzyme assays were employed to evaluate the functional distribution of alpha-L-fucosidase in preparations of hamster sperm. *In vitro* fertilization was performed using Syrian hamster sperm and eggs to identify the functional role of hamster sperm associated alpha-L-fucosidase during zona pellucida binding/penetration, sperm-egg membrane fusion, and post-fusion events. Results reported here document the presence of hamster sperm associated alpha-L-fucosidase, and demonstrate that it functions during fertilization, at the stage of sperm-oocyte membrane interaction and/or post-fusion events within the zygote. Understanding the role of alpha-L-fucosidase during human fertilization could lead to development of improved infertility treatments.

INTRODUCTION

Successful fertilization requires the coordination of species-signature events between the sperm and oocyte. It has been well documented that fertilization is a carbohydrate mediated event. Previous studies support roles for carbohydrates in sperm-oviduct adhesion [1-4], sperm-oocyte interaction [5, 6] and embryo implantation [7, 8]. Gamete recognition and adhesion on the molecular level involves carbohydrates [9, 10].

Substantial evidence in the literature points toward the importance of L-fucose. The deoxyhexose L-fucose is a common terminal residue of both N- or O-linked glycolipids and glycoproteins [11]. Addition of this residue to molecules can enable unique functional properties. Its physiological roles in mammals has been well characterized and reviewed in detail [11]. Apart from being essential to many biological functions, L-fucose has also been reported to be involved during fertilization and has been identified as a component of gametes [12-16].

Fucose is a common component of the glycan chains of glycoproteins and glycolipids, and there is a large family of fucosyltransferases available to recognize various substrates and add fucose residues selectively. By contrast, most organisms have only one or two genes for α -L-fucosidases which recognize and cleave terminal fucose residues [17]. Considering the known relevance of carbohydrate structures in reproduction, and the common occurrence of fucose residues at or near the terminal positions in glycans, fucosidases are likely suspects for key roles in reproduction.

For humans in particular, there are distinctive isoforms of α -L-fucosidase that occur in the seminal plasma versus the sperm membrane associated alpha-L-fucosidase [SMALF] [18-20]. The distribution of SMALF within human sperm is unusual, suggesting that it is packaged for actions late during sperm-oocyte interaction. For human sperm, SMALF is cryptically held within sperm cells, with a crypticity ratio averaging 3.7 for permeabilized versus untreated sperm cells [21]. Furthermore, SMALF is enriched within the equatorial segment, positioned for engagement in the intimate membrane interactions between sperm and oocyte [21]. Human SMALF is notably stabilized by its *in situ* membrane association and remains actively stable within the timeline consistent with a delayed fertilization event [22].

Mammalian fucosidases have been characterized and reviewed at length [23, 24]. In addition to the common α -L-fucosidases, sperm associated isoforms of this enzyme have been documented in humans [18-20] rats [25-27], *Drosophila* [28], ascidians [29], some *Percidae* fishes [30], *Unio elongatulus* [31], chimpanzees [32], bulls [33] and Syrian hamsters (described herein). The presence of sperm α -L-fucosidase isoforms in this diverse group of organisms is consistent with the importance of carbohydrates during fertilization. Several studies provide evidence supporting roles for fucose containing glycans and/or fucosidases during fertilization [29, 34-38].

Since direct intervention with human fertilization is undesirable, we have chosen the Syrian hamster for further analysis of the roles of fucosidase during fertilization. This

rodent provided numerous advantages for such an investigation, allowing testing of multiple hypotheses on the mechanisms of gamete interaction and post fusion events.

The primary objectives of this study were to assess the presence of α -L-fucosidase in hamster sperm and oocytes, and to evaluate the roles of α -L-fucosidase during fertilization. Direct enzyme assays were performed to measure α -L-fucosidase activity in pellet and supernatant fractions of cauda, capacitated, and acrosome induced hamster sperm preparations. *in vitro* fertilization was performed to evaluate the role of sperm associated α -L-fucosidase during 1) zona pellucida binding and penetration, 2) sperm-oocyte membrane interaction, and 3) oocyte activation coupled with early embryogenesis.

In order to evaluate the functional role of α -L-fucosidase during fertilization, we blocked its enzyme activity using the commercially available chemical DFJ (deoxyfuconojirimycin). DFJ is a known, "potent, specific, and competitive inhibitor" of the α -L-fucosidase enzyme [39], and retains that distinction [40, 41]. The chemical structure of this deoxyzasugar resembles to the natural substrates for α -L-fucosidase. Previous studies in our lab using human sperm have confirmed and documented the specificity of DFJ for fucosidases from several sources (eg [21, 22, 30]), including the sperm associated isoforms [21]. Results reported herein document the ability of 0.5 - 5 mM DFJ to inhibit completely α -L-fucosidase activity in washed, capacitated and acrosome induced populations of hamster sperm. Pre-treatment of hamster sperm with DFJ did not compromise the motility of the specimen, an important prerequisite for *in vitro* fertilization. These results, taken together with our previous findings using human sperm, led to our choice of DFJ as the best inhibitor for the experiments described herein. Polyclonal anti- α -L-fucosidase antibodies were also evaluated as a potential inhibitor. We have demonstrated that our polyclonal goat anti-human liver α -L-fucosidase antibody does not inhibit enzymatic activity, as expected from a previous report [42]. Pre-treatment of capacitated hamster sperm with polyclonal anti- α -L-fucosidase antibodies (1:50 or 1:200) did not inhibit α -L-fucosidase activity, as evaluated via fluorometric enzyme assays described herein (data not shown). Based upon these findings, polyclonal antibodies were not used as inhibitors in IVF experiments. L-fucose, a product of fucosidase enzyme, also functions as a low affinity, competitive inhibitor of fucosidases, and has been used here as a competitor at 0.2 mM, the highest concentration that does not create damaging osmolarity. We do not expect L-fucose to be specific for fucosidase inhibition, as it may have many other effects and metabolic involvement in the complex living system used here.

To our knowledge, this is the first report of a hamster sperm associated α -L-fucosidase. In this paper, its functional distribution and role during fertilization are reported. Results from all experiments reveal hamster sperm α -L-fucosidase is present in large quantities and has similar crypticity and functional distribution to the human semen isoforms [21]. Results from IVF experiments suggest that sperm associated α -L-fucosidase is not involved during zona pellucida binding or penetration, however it does function during hamster sperm-oocyte membrane interactions and/or post-fusion events within the single-celled embryo.

Materials and Methods

Animal Housing

In accord with approved Lehigh University IACUC protocol # 24, adult (6 to 8 weeks) male and female Syrian hamsters were obtained from Harlan (Indianapolis, USA) and housed individually and allowed free access to food and water. The animals were kept on a 14:10 light:dark cycle, with lights on at 12:00 noon and off at 2:00 AM, daylight savings time.

Syrian Hamster Sperm Collection

Sperm was collected from the cauda epididymis following post mortem surgery. A small cut was made in the cauda epididymis and a small droplet of cauda contents was gently squeezed into an organ culture dish. This sample was hereafter referred to as CEC. Depending upon the desired preparation, specific buffers were added to the CEC droplet as described below. To avoid unnecessary use of animals, four male hamsters were used to evaluate the functional distribution of hamster sperm α -L-fucosidase. As hamsters are essentially isogenic, this number is suitable for this experiment.

Syrian Hamster Sperm Capacitation and Induction of Acrosome Reaction

CEC collected as described above was capacitated by incubation of CEC in an organ culture dish containing 550 μ L human sperm medium (HSM) [43] supplemented with 3.5% bovine serum albumin (BSA) (Sigma, St. Louis, USA) and 1 mM hypotaurine (Sigma, St. Louis, USA) for 3 hours at 37°C, 5% CO₂. The CEC was allowed to freely diffuse throughout the medium avoiding manipulation by pipet. For induction of acrosome reaction, 198 μ L of capacitated CEC was mixed with 2 μ L of 1 mM BrA23187 (Molecular Probes, Eugene, USA) and incubated for 30 minutes at 37°C, 5% CO₂.

Functional Distribution of Syrian Hamster Sperm α -L-fucosidase

To determine the functional distribution of Syrian hamster sperm α -L-fucosidase activity, direct enzyme assays were performed using the fluorogenic substrate 4-methylumbellifery- α -L-fucopyranoside (4-MU-Fuc) (Biosynth AG, Staad, Switzerland) as previously described [21] except α -L-fucosidase sources were obtained from hamster specimens. Briefly, enzyme assays contained 40 μ L 4-MU-Fuc, 10 μ L HSM (buffered at pH 7.4), and 10 μ L enzyme source. All enzyme assays were conducted at pH 7.4, and samples were incubated at 37°C, 5% CO₂. Enzyme activity was measured for both the sperm cell and soluble fractions of cauda, capacitated and acrosome induced preparations. One enzyme unit was defined as the amount of α -L-fucosidase that released one nanomole of 4-MU from 4-MU-Fuc per minute at 37°C, pH 7.4.

Preparation of CEC Samples

A drop of CEC was collected as described above and 500 μ L of HSM was added to an organ culture dish. The sperm droplet was allowed to freely diffuse in the HSM by incubating the dish for 5 minutes at 37°C, 5% CO₂. This suspension was centrifuged 10 minutes, 10,000 g to separate the sperm cells from the soluble fraction. Following centrifugation, the top 100 μ L of supernatant was removed and placed into a new microfuge tube containing 900 μ L of HSM. The remaining supernatant was decanted

from the pellet and discarded. The pellet was re-suspended in 500 μ L HSM. Enzyme activities in both the pellet and supernatant were quantified. Additionally, some samples were pre-treated with 5 mM deoxyfuconjirimycin (DFJ) (Calbiochem, Gibbstown, USA) for 10 minutes prior to measuring enzyme activity. The average and mean standard error were calculated for each preparation using Microsoft Excel and data were plotted as average enzyme units.

Preparation of Capacitated Samples

Hamster sperm were capacitated as described above. Following capacitation, 20 μ L of the capacitated population was removed and reserved for pre-treatment with 5 mM DFJ, or other concentrations as specified. Additionally, 198 μ L was removed for acrosome reaction induction. The remaining volume of capacitated suspension was centrifuged for 10 minutes, 10,000 g. After centrifugation, the top 20 μ L of supernatant was removed and reserved for enzyme assay. The remaining supernatant was decanted and the pellet was re-suspended in 200 μ L HSM. Enzyme activity was measured in both the pellet and supernatant fractions. Additionally, a sample of the capacitated preparation was pre-treated with 5 mM DFJ or polyclonal anti- α -L-fucosidase antibodies (1:50 or 1:200) for 10 minutes and used for enzyme assays. Average and mean standard error were calculated using Microsoft Excel and data were plotted as average enzyme units.

Preparation of Acrosome Induced Samples

Acrosome reaction was induced as described above. Following incubation, the mixture was centrifuged 10 minutes, 10,000 g. After centrifugation, the top 20 μ L of supernatant was removed and reserved. The remaining supernatant was decanted and the pellet was re-suspended in 200 μ L of HSM. α -L-fucosidase was measured in both pellet and supernatant fractions. Additionally, a sample of the acrosome induced preparation was pre-treated with 5 mM DFJ for 10 minutes and used for enzyme assays. Average and standard error of the mean were calculated using Microsoft Excel and data were plotted as average enzyme units.

Superovulation

Syrian hamsters exhibit a regular four day estrous cycle. Ovulation was determined by placing a male hamster briefly into a female's cage during the dark cycle and observing behavior. The day in which a female showed lordosis was designated as estrous day four (E4), indicating ovulation would occur just prior to lights on. Female Syrian hamsters were superovulated by intraperitoneal injection of 25 i.u. pregnant mare serum gonadotropin (PMSG) (Calbiochem, Gibbstown, USA) dissolved in 0.1 mL water 1 hour after lights on immediately following the dark part of the cycle on E4.

Preparation of Buffers and Droplets

Medium for collection and culturing of hamster oocytes was that of Bavister [44]. Briefly, TL-PVA was composed of 114 mM NaCl (Fisher, Pittsburg USA), 3.16 mM KCl (Sigma, St. Louis, USA), 2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Sigma, St. Louis, USA), 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (Sigma, St. Louis, USA), 10 mM sodium lactate (Sigma, St. Louis, USA), 0.35 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (Sigma, St. Louis, USA), 5 mM glucose (Sigma, St. Louis,

USA), 25 mM NaHCO₃(Sigma, St. Louis, USA), and 0.1% polyvinyl alcohol (PVA) (Sigma, St. Louis, USA). Osmolarity was measured by the freezing point depression approach using a microsmette (Precision Systems, Inc., Natick, USA) and distilled water was added to yield a final osmolarity of 295-300 mOsmol. Sterilization was accomplished using a Nalgene sterile filtration apparatus with 0.2 µm pore.

Preparation of TLP-PVA: Oocyte Collection Medium

TLP-PVA was used for collection and culture of hamster oocytes. TLP-PVA was prepared fresh for each experiment by aseptically mixing 10 µL of 10 mM sodium pyruvate (Sigma, St. Louis, USA) (prepared in 157 mM NaCl) and 990 µL TL-PVA. TLP-PVA was used for collection of cumulus masses from fallopian tubes and washing of oocytes [44].

Preparation of CRM: Cumulus Removal Medium

CRM was prepared fresh on the day of each experiment by aseptically mixing 10 µL of 10 mg/mL hyaluronidase (Sigma, St. Louis, USA) and 90 µL of TLP-PVA [44].

Preparation of Oocyte Culture Medium

Oocyte culture medium was prepared fresh on the day of each experiment by supplementing TLP-PVA with 1 mM glutamine (Mann Research Labs, Inc., New York, USA), 0.2 mM isoleucine (Mann Research Labs, Inc., New York, USA), 0.05 mM methionine (United States Biochem Corp., Cleveland, USA) 0.1 mM phenylalanine United States Biochem Corp., Cleveland, USA), and 0.25 mM sodium pyruvate (Sigma, St. Louis, USA) [44].

Preparation of IVF Droplet

IVF droplets were prepared by pipetting 50 µL of fresh oocyte culture medium into an organ culture dish. The droplet was then overlaid with 575 µL of pre-equilibrated IVF mineral oil (Fertility Technology Resources, Marietta, USA). The IVF droplets were placed into the incubator at 37°C, 5% CO₂ to equilibrate four hours prior to use.

Collection of Oocytes

Oocytes were harvested from naturally cycling or superovulated female hamsters following post mortem surgery on the next estrous day 4 following PMSG injection, just prior to lights on. The fallopian tubes were removed from each animal and placed into a dish containing a 100 µL droplet of TLP-PVA. The swollen portion of the fallopian tubes was held with forceps in the droplet of medium and pricked with a fine gauge needle, allowing the cumulus mass to spill into the surrounding buffer. The cumulus masses were removed and transferred to a 100 µL droplet of pre-equilibrated CRM and incubated at 37°C, 5% CO₂ for 2 minutes. Additionally, the droplet was pipetted up and down 5 times using a glass pipet to mechanically dislodge oocytes from the cumulus mass. Oocytes were washed by serial passage through three 100 µL droplets of TLP-PVA and then into a prepared IVF droplet.

in vitro fertilization

All *in vitro* fertilization experiments used oocytes freshly collected from naturally cycling

or superovulated hamsters. Up to ten oocytes were placed into an organ culture dish containing an IVF droplet prepared as described above. Sperm motility was evaluated microscopically prior to insemination and only samples that contained a minimum of 90% motile sperm were used. For some inseminations, capacitated hamster sperm were pre-treated with specified concentrations of DFJ or 0.2 mM fucose for 10 minutes prior to insemination. Pre-treatment of capacitated sperm with concentrations of fucose greater than 0.2 mM resulted in loss of sperm motility. Further investigation revealed the addition of fucose to the capacitation medium significantly changed the osmolarity of the buffer, ultimately immobilizing the sperm (data not shown). To each IVF drop, approximately 500 control or pre-treated capacitated hamster sperm per oocyte were added. Gametes were co-incubated for various lengths of time at 37°C, 5% CO₂, high humidity. For some experiments, 5 mM DFJ was included in the IVF drop, pre-exposing oocytes for 10 minutes before addition of untreated sperm.

Evaluation of Zona Pellucida Interaction

For purposes of zona-binding assays, cryopreserved hamster oocytes were purchased from Embryotech Laboratories, Inc. (Haverhill, USA) and used to evaluate the role of sperm associated α -L-fucosidase in binding to the ZP. Oocytes were thawed according to the manufacturer's directions and placed into IVF drops prepared as described above. Briefly, an oocyte straw was removed from liquid nitrogen, exposed to room temperature air for two minutes, then placed into a 37°C water bath for 1 minute. Once defrosted, the straw was gently wiped dry and contents expelled onto a petri dish. The oocytes were immediately washed through two droplets of TLP-PVA and placed into IVF drops for insemination. Oocytes were inseminated with either control or capacitated hamster sperm pre-treated with either 0.2 mM fucose or 5 mM DFJ 10 minutes prior to insemination. After 20, 45, 60, or 240 minutes gamete co-incubation, oocytes were placed into a fresh 50 μ L drop of TLP-PVA and pipetted up and down three times using a glass pipet to dislodge sperm bound loosely to the ZP. Oocytes were fixed by placing them into a 50 μ L droplet of 2% paraformaldehyde for 5 minutes. Following fixation, oocytes were placed into a 15 μ L droplet of 12 μ M propidium iodide (PI) (Sigma, St. Louis, USA) in an organ culture dish. The PI droplet was surrounded by 200 μ L IVF mineral oil to prevent evaporation and movement of the droplet. The number of sperm tightly bound to the ZP of each oocyte was evaluated using a Nikon Eclipse TE2000U (Nikon Instruments Inc., Japan) fluorescence microscope. Further image analysis was performed using a Zeiss LSM 510 meta confocal microscope (Zeiss, Jena, Germany) to acquire z-stacks. Descriptive statistics were performed and data were plotted as average number tightly bound (TB) sperm per oocyte versus duration of gamete co-incubation for each group. A two-tailed Student's t-Test was performed to assess significant differences between the control and sperm treatment groups existed.

Evaluation of sperm-oocyte membrane interaction and/or post-fusion events

The methods described above for *in vitro* fertilization were used except gametes were co-incubated for 24 hours 37°C, 5% CO₂, high humidity. Following 24 hours co-incubation, oocytes were microscopically evaluated for development of oocytes to 2-cell embryos. The number of 2-cell embryos was recorded for each drop. Descriptive

statistics were performed and data were plotted as average percent 2-cell embryos for each group. A two-tailed Student's t-Test was performed to determine if a significant difference between the control and treatment groups existed.

Results

Functional Distribution of Hamster Sperm Associated α -L-fucosidase

Results from direct enzyme assays revealed the presence of both soluble and sperm cell associated α -L-fucosidase in Syrian hamsters. Enzyme activity was measured in the supernatant and cell pellet fractions of cauda epididymal contents, capacitated and acrosome induced hamster sperm cell preparations. Data from enzyme assays revealed α -L-fucosidase in the epididymal fluid was 6 times more abundant than that in the sperm cell pellet; a finding similar to that reported for human semen [19]. Figure 1 shows the functional distribution of α -L-fucosidase activity for the soluble, supernatant fraction of cauda, capacitated and acrosome induced cell preparations. The average enzyme units per sample were 75.0, 212.4, and 183.7, respectively.

Figure 2 shows the distribution of cell-associated α -L-fucosidase activity in sperm cell pellet fractions for cauda contents, capacitated and acrosome induced populations. A substantial increase in measurable α -L-fucosidase activity was detected following capacitation and acrosome reaction induction compared to cauda sperm. Cauda sperm contained an average of 6.3 enzyme units, whereas parallel populations of capacitated and acrosome induced populations contained 122 and 40.8 enzyme units, respectively. These results are consistent with our previous findings suggesting hamster sperm contain cryptic stores of α -L-fucosidase similar to those reported for human sperm [21]. Crypticity ratios (CR) were calculated for capacitated and acrosome induced sperm preparations. CR was defined as the ratio of enzyme units in capacitated or acrosome reacted sperm compared to cauda epididymal sperm. The CR for capacitated and acrosome induced sperm were 19.4 and 6.5, respectively.

Additionally, hamster sperm α -L-fucosidase activity was inhibited by the known, specific competitor DFJ [39]. Cauda, capacitated and acrosome induced cell populations were pre-treated with 5 mM DFJ for 10 minutes prior to enzyme assays and no measurable enzyme activity was detected. Enzyme activity was completely inhibited in all DFJ samples (not shown). Control experiments containing either 10 μ L of 1) HSM, 2) HSM supplemented with 3% BSA and 1 mM hypotaurine, 3) 10 μ M BrA23187 or 4) 5 mM DFJ showed no enzyme activity.

Evaluation of Hamster Oocyte α -L-fucosidase

Two different conditions were compared in order to evaluate the presence or absence of α -L-fucosidase in mature, ovulated versus immature, follicular hamster oocytes. Ovulated oocytes were collected as previously described herein, cleaned free of cumulus and granulosa cells, and homogenized using a Dounce homogenizer. This homogenate was used for fucosidase enzyme assays (described herein) and showed no measurable α -L-fucosidase activity. By contrast, 10 pre-ovulatory oocytes, collected from ten ovarian follicles, surrounded by granulosa cells, were homogenized using a Dounce homogenizer. This homogenate was used for fucosidase enzyme assays with

measurements recorded at 30 and 90 minutes. Data from this experiment indicated the presence 0.4 enzyme units after 30 minutes and 0.6 enzyme units after 90 minutes. The marginal amount of α -L-fucosidase activity present in the second homogenate that contained pre-ovulatory oocytes was most likely attributable to the granulosa cells and/or follicular fluid. These results suggest that mature, ovulated hamster oocytes do not contain their own intrinsic α -L-fucosidase.

Functional Role of α -L-fucosidase in Zona Pellucida Binding and Penetration

The functional role of α -L-fucosidase during sperm-zona binding and penetration was evaluated using *in vitro* fertilization (IVF) with a Syrian hamster system. Oocytes purchased from Embryotech were inseminated with either untreated (control) or capacitated hamster sperm pre-treated with 5 mM DFJ or 0.2 mM fucose. Microscopic evaluation indicated that sperm pre-treated with 5 mM DFJ for 10 minutes had no decrease in motility compared to the control, capacitated sperm sample. Evaluation of IVF droplets 10 minutes post insemination revealed no observable difference between oocytes inseminated with control or 5 mM DFJ pre-treated sperm. In both cases, sperm were binding and interacting with oocytes, such that oocytes were rolling around in the IVF droplet. Examples of these observations were video micrographed, and are presented in Figures 3 and 4 and Supplemental Movies 1 and 2 (available online at www.biolreprod.org).

Following 20, 45, 60, or 240 minutes of gamete co-incubation, the number of sperm that were tightly bound (TB) to the zona per oocyte was quantified using epi-fluorescence and confocal microscopy. Figure 5 shows the average number of TB sperm per oocyte versus duration of gamete co-incubation. As expected for the normal kinetics of zona binding, the number of TB sperm per oocyte for both the control and 5 mM DFJ treatment group decreased over time (Figure 5a). The highest number of TB sperm per oocyte was observed at 20 minutes, with an average of 40 TB sperm for the control and 41 for the 5 mM DFJ group. After 240 minutes gamete co-incubation, the average number of TB sperm per oocyte for both the control and 5 mM DFJ groups was 3. Similarly, pre-treatment of sperm with 0.2 mM fucose 10 minutes prior to insemination did not significantly inhibit tight binding of sperm to the zona pellucida (Figure 5b). Statistical analysis using the Student's t-Test revealed no significant difference between the control and treatment groups for any time point.

Further evidence was obtained by examination of oocytes that were fixed during insemination. Figure 6 shows a confocal z-stack of an oocyte inseminated with control sperm 1 hour post IVF. Each panel represents the same microscopic DIC and fluorescence confocal field. Each optical section was 8 μ m thick and covered a total depth of 96 μ m. This confocal micrograph clearly shows multiple sperm bound to the ZP. Figure 7 is a confocal z-stack of an oocyte inseminated with 5 mM DFJ pre-treated sperm 1 hour post IVF. Each panel represents the same microscopic DIC and fluorescence confocal field. Similar to Figure 6, many sperm are tightly bound to the ZP despite pre-treatment of sperm with 5 mM DFJ. In total, 25 oocytes inseminated with control sperm and 26 oocytes inseminated with 5 mM DFJ sperm were evaluated.

Similar observations were recorded for oocytes inseminated with capacitated sperm pre-treated with 0.2 mM fucose 10 minutes prior to insemination (data not shown).

Functional role of α -L-fucosidase in sperm-oocyte membrane interaction

IVF was performed as described above except gametes were co-incubated for 24 hours and oocytes were microscopically evaluated for development to 2-cell embryos. Figure 8 shows the percent of 2-cell embryos for each treatment group. In total, 62 oocytes inseminated with control sperm and 103 oocytes inseminated with 5 mM DFJ pre-treated sperm were evaluated. The control group had an average of 43% 2-cell embryos while the 5 mM DFJ group had an average of 15% 2-cell embryos. Pre-treatment of sperm with 5 mM DFJ for 10 minutes caused a 65% reduction in development to the two cell stage. A two-tailed Student's t-Test revealed a significant difference between the control and DFJ groups ($p=0.007$). Similarly, a reduction in fertilization success was apparent following pre-treatment of sperm with 0.2 mM fucose. Inhibition of fucosidase activity by an alternative competitive inhibitor also reduced fertilization success. For all IVF experiments, embryos cultured longer than 24 hours did not progress past the 2-cell stage, and began deteriorating 48 hours post insemination.

As a reciprocal control experiment, we switched the DFJ treatment protocol. For some experiments, IVF drops containing oocytes were supplemented with 5 mM DFJ prior to addition of untreated, capacitated sperm. Figure 9 shows the percent 2-cell embryos in control and 5 mM DFJ IVF drops. Thirty oocytes inseminated with control sperm in the absence of 5 mM DFJ and 31 oocytes inseminated with control sperm in the presence of 5 mM DFJ in IVF drop were evaluated. Control IVF drops developed an average of 41% 2-cell embryos while IVF drops containing 5 mM DFJ developed an average of 30% 2-cell embryos. A two-tailed Student's t-Test revealed no significant difference between IVF drops with or without 5 mM DFJ ($p=0.299$), despite a reduction in the development of oocytes to 2-cell embryos.

Functional role of α -L-fucosidase in post fusion events

To assess the step of fertilization at which hamster sperm α -L-fucosidase functions, IVF experiments were set up as described herein, however capacitated sperm were pre-treated with either 0.5 mM, 1 mM, 5mM DFJ or 0.2 mM fucose prior to insemination. The stage of fertilization was microscopically observed 24 hours post insemination and recorded as one polar body (1PB), two polar bodies (2PB), two pro-nuclei (2PN), or 2-cell embryo. Data from four independent experiments, using different animals for each experiment, is shown in Table 1. Pre-treatment of sperm with DFJ or fucose inhibits progression past the 2 PN stage. Table 2 summarizes the percentage of oocytes and their stage during fertilization for each sperm treatment. Only 15.8% of oocytes inseminated with sperm pre-treated with 0.2 mM fucose for 10 minutes developed to the 2 PN stage. A similar trend was observed for oocytes inseminated with sperm pre-treated with 0.5 mM, 1 mM, and 5 mM DFJ. The percentage of oocytes reaching the 2PN stage was 12.5%, 50%, and 37.5%, respectively.

Discussion

The data presented here provide direct evidence documenting the functional distribution and role of hamster sperm associated α -L-fucosidase during fertilization. Our results show α -L-fucosidase was present in cauda epididymal, capacitated and acrosome induced sperm. As evidenced in Figure 2, the amount of fucosidase that is detectable in capacitated sperm is far more than in washed CEC cells, presumably due to increased access by the substrate to the acrosome compartment. With induction of acrosome reaction by treatment with ionophore and calcium, some fucosidase remains with the cells, while some moves into the supernatant fraction (Figure 1), presumably associated with membranous vesicles. Hamster oocytes apparently do not contain their own intrinsic α -L-fucosidase. Inhibition of α -L-fucosidase with DFJ decreased fertilization rates suggesting that α -L-fucosidase plays a significant role during hamster fertilization.

Previous studies have provided substantial evidence documenting the importance of carbohydrates during fertilization. A likely candidate for carbohydrate mediation during mammalian fertilization is the interaction between sperm associated α -L-fucosidase and the complimentary oocyte L-fucose residues. Over the past 20 years, sperm associated α -L-fucosidases have been identified for a wide variety of organisms. This wide distribution suggests sperm associated α -L-fucosidase is likely to have a role in some basic fertilization event(s). Several studies have provided direct evidence supporting roles for fucose containing glycan chains and/or fucosidases during fertilization in a variety of species [29, 34, 35, 37, 38, 45].

During the beginning stages of fertilization, sperm are required to recognize, bind and penetrate the zona pellucida (ZP). The Syrian hamster *in vitro* fertilization system enabled direct evaluation of the functional role of sperm associated α -L-fucosidase. The requirement of sperm associated α -L-fucosidase during ZP binding and penetration was evaluated by quantifying the number of tightly bound sperm per oocyte for both control and 5 mM DFJ pre-treated sperm groups. Microscopic evaluation of oocytes following various lengths of gamete co-incubation showed no significant difference in the number of tightly bound sperm per oocyte. In both the control and 5 mM DFJ groups, numerous sperm were interacting with each oocyte, rolling the oocytes around in the IVF drop. This was further documented by videomicrography which is available as supplemental files. Data from these experiments clearly indicated sperm associated α -L-fucosidase was not required for ZP binding and/or penetration. Our results add hamsters to this list.

The functional role of α -L-fucosidase during later stages of fertilization was evaluated again using hamster IVF. Oocytes were inseminated with control or 5 mM DFJ sperm, co-incubated for 24 hours and microscopically observed for the number of 2-cell embryos. Results indicated pre-treatment of capacitated sperm with 5 mM DFJ for 10 minutes prior to insemination significantly inhibited development of oocytes to 2-cell embryos. In most experiments, insemination of oocytes with 5 mM DFJ pre-treated sperm yielded no 2-cell embryos; however, on some occasions, 2-cell embryos formed. This result is attributable to DFJ's action as a competitive inhibitor. By definition, a

competitive inhibitor competes with the endogenous substrate for active site binding. This binding is reversible, meaning the competitive inhibitor can be released from the active site making it freely available for the endogenous substrate to bind. For oocytes inseminated with DFJ pre-treated sperm, the 2-cell embryos most likely developed because an α -L-fucosidase active site(s) became available when DFJ dissociated. These results provided direct evidence indicating an important role for sperm associated α -L-fucosidase during mammalian fertilization. Inhibition of sperm associated α -L-fucosidase blocked fertilization at a level beyond ZP recognition, binding and penetration.

Deeper evaluation of IVF experiments revealed that sperm associated α -L-fucosidase most likely functions at the post-fusion level. Oocytes were unable to progress past the 2PN stage when inseminated with sperm pre-treated with DFJ or fucose. In these instances, sperm could bind and fuse with the oocyte membrane, generating two pronuclei, however development beyond that stage was halted. Results from these studies indicate fucosidase is necessary for the 1-cell embryo to progress past the 2PN stage. Additional studies are needed, and in progress, to further dissect the exact step at which fucosidase functions.

These findings could suggest a role for sperm associated α -L-fucosidase during sperm-oocyte membrane-membrane interaction. These data do not exclude the possibility that α -L-fucosidase could be involved in some early stage of embryogenesis.

To evaluate the affect of 5 mM DFJ on development of hamster oocytes to 2-cell embryos, control experiments were performed. In these experiments, oocytes were incubated in an IVF drop containing 5 mM DFJ for 10 minutes prior to insemination with control hamster sperm. Data from these experiments (Figure 9) show no significant difference ($p=0.299$) in the number of 2-cell embryos between control and DFJ drops. Despite a 10 minute pre-treatment of oocytes with 5 mM DFJ, when inseminated with control hamster sperm, oocytes maintained their ability to develop to 2-cell embryos. These results confirm that 5 mM DFJ does not affect the viability of hamster oocytes, nor the ability of these oocytes to be fertilized and develop to 2-cell embryos. The non-significant decrease in development to the 2-cell stage is consistent with the brief exposure of sperm to DFJ as they find oocytes in the insemination droplet.

Data presented here document the presence of a hamster sperm associated α -L-fucosidase with similar characteristics of those reported for human sperm. α -L-fucosidase has been reported to be associated with Chinese hamster ovary cell supernatant [46]. Previous studies have also shown the temporal regulation of glycosyltransferases and glycosidases in uterine and follicular fluids during the hamster estrous cycle [47]. Here we report the absence of α -L-fucosidase within the mature hamster oocyte. This finding is compelling, and together with other observations reported here suggests that α -L-fucosidase is necessary during fertilization, and that it is provided by the sperm.

Further understanding the role of α -L-fucosidase has potentially important clinical relevance. Methods of predicting fertility and/or treating infertile couples could be developed based upon the mechanism by which α -L-fucosidase functions. Previous studies have shown a relationship between protein content and fertility index in dairy bulls. Specifically, the amount of α -L-fucosidase in the cauda epididymal fluid was 2.3 fold higher in bulls with a high fertility index [48]. If this relationship exists for human semen, a diagnostic tool could be developed that would provide an additional parameter by which a man's fertility potential could be evaluated.

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Figure Legends

Figure 1: Functional distribution of soluble hamster alpha-L-fucosidase from cauda epididymal fluid. Average enzyme units from the supernatant fractions of hamster cauda, capacitated, and acrosome induced preparations. Data represent averages from four independent experiments. Error bars represent standard error of the mean.

Figure 2: Functional distribution of hamster sperm associated alpha-L-fucosidase. Average enzyme units from hamster sperm pellet fractions of cauda, capacitated, and acrosome induced preparations. Data represent averages from four independent experiments. Error bars represent standard error of the mean.

Figure 3: Still image from video micrography (Supplemental Movie 1) of hamster oocytes following insemination with untreated (control), capacitated hamster sperm. Multiple sperm are interacting with the oocytes, rolling and moving them in the IVF droplet.

Figure 4: Still image from video micrography (Supplemental Movie 2) of hamster oocytes inseminated with capacitated hamster sperm pre-treated with 5 mM DFJ prior to insemination. Despite pre-treatment of sperm with DFJ, multiple sperm are interacting with the oocytes, rolling and moving them in the IVF droplet.

Figure 5: Pre-treatment of capacitated hamster sperm with 5 mM DFJ (A) or 0.2 mM fucose (B) does not inhibit tight binding (TB) of sperm to the zona pellucida. Average number of TB sperm per hamster oocyte was quantified for oocytes inseminated with either control (gray) or pre-treated (white) hamster sperm. Means were not significantly different between the control and treatment group within each time point. Error bars represent standard error of the mean and n = number of oocytes evaluated.

Figure 6: Tight binding of control sperm to the zona pellucida. Confocal z-stack of merged DIC and fluorescence fields of an oocyte inseminated with control sperm 1 hour post *in vitro* fertilization. Each panel represents a confocal optical section 8 μm thick, covering a total depth of 96.1 μm . Control hamster sperm labeled with propidium iodide (red) are seen tightly bound to the zona pellucida of the hamster oocyte.

Figure 7: Pre-treatment of hamster sperm with 5 mM DFJ does not inhibit tight binding to the zona pellucida. Confocal z-stack of merged DIC and fluorescence fields of an oocyte inseminated with hamster sperm pre-treated with 5 mM DFJ. Each panel represents a confocal optical section 8 μm thick, covering a total depth of 120.2 μm . DFJ pre-treated sperm labeled with propidium iodide (red) are seen tightly bound to the zona pellucida of the hamster oocyte 1 hour post *in vitro* fertilization.

Figure 8: Pre-treatment of capacitated hamster sperm with 5 mM DFJ significantly inhibits development of oocytes to 2-cell embryos. Average % 2-cell embryos 24 hours post insemination was quantified from six independent experiments. Insemination of oocytes with 5 mM DFJ pre-treated sperm significantly reduced the number of 2-cell embryos. In total, 62 oocytes inseminate with control sperm 103 oocytes inseminated with 5mM DFJ pre-treated sperm were evaluated. Error bars represent standard error of the mean. $p=0.007$.

Figure 9: Inclusion of 5 mM DFJ in the IVF medium does not significantly inhibit development of oocytes to 2-cell embryos. Average % 2-cell embryos 24 hours post insemination was quantified from three independent experiments. Inseminate of

oocytes with control sperm in the absence and presence of 5 mM DFJ did not significantly reduce the number of 2-cell embryos. In total, 30 oocytes in control medium and 31 oocytes in 5 mM DFJ medium were evaluated. Error bars represent standard error of the mean. $p=0.299$.

of oocytes at each stage

Sperm Treatment	1 PB	2 PB	2 PN	2-cell
Control	0	0	11	3
0.2 mM fucose	10	5	3	1
0.5 mM DFJ	0	7	1	0
1 mM DFJ	0	4	4	0
5 mM DFJ	0	0	3	1

Table 1: Insemination of hamster oocytes with pre-treated capacitated sperm inhibits fertilization after oolemma binding. Oocytes were microscopically evaluated and the stage of fertilization was recorded 24 hours post insemination. Data from four independent experiments using 53 oocytes in 11 dishes are summarized.

% oocytes at each stage

Sperm Treatment	1 PB	2 PB	2 PN	2-cell
Control	0%	0%	78.5%	21.4%
0.2 mM fucose	52.6%	26.3%	15.8%	5.3%
0.5 mM DFJ	0%	87.5%	12.5%	0%
1 mM DFJ	0%	50%	50%	0%
5 mM DFJ	0%	50%	37.5%	12.5%

Table 2: Pre-treatment of capacitated sperm 10 minutes prior to insemination reduces fertilization success. Data represents percentage of oocyte reaching each stage.

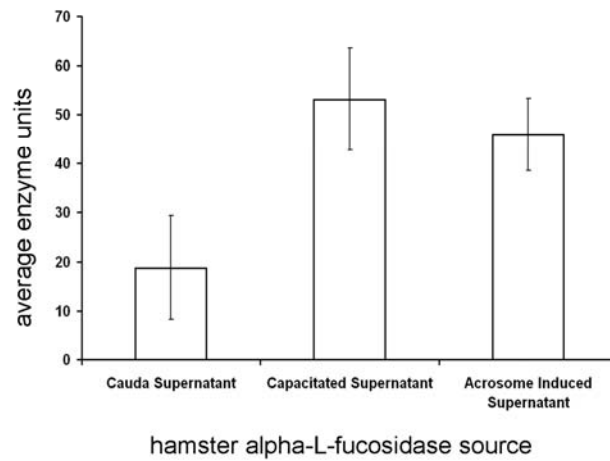


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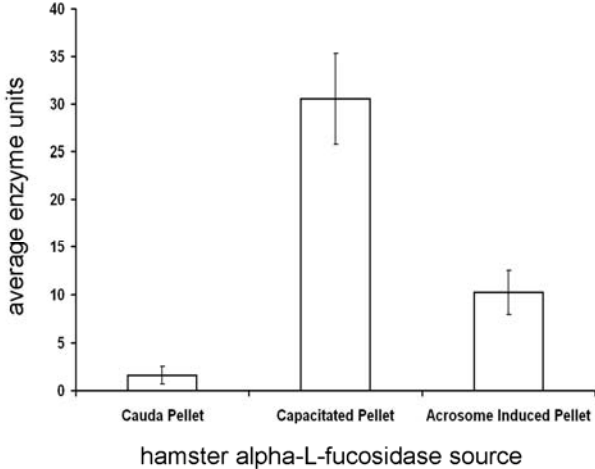
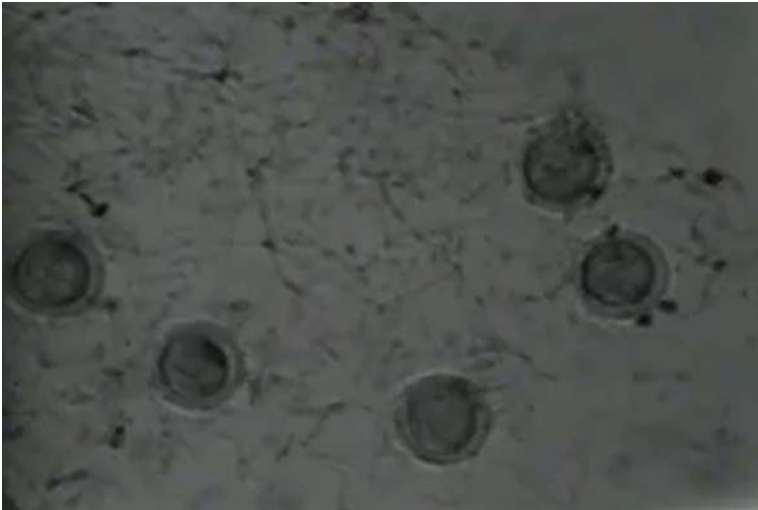
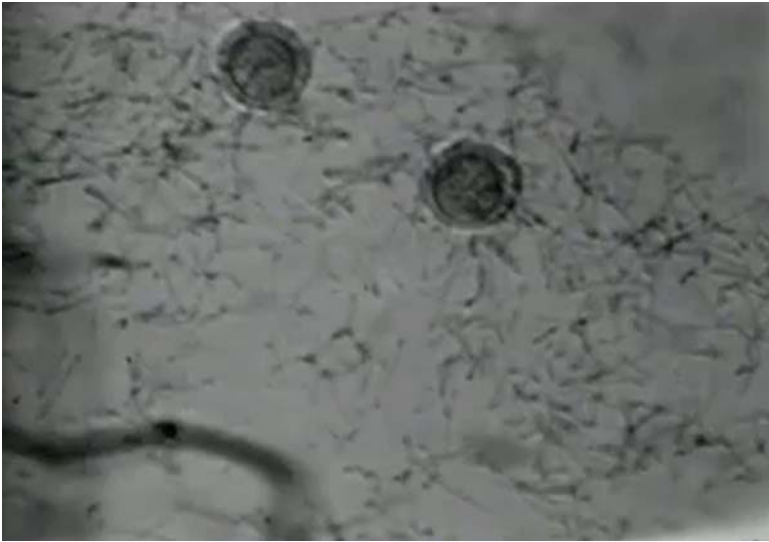


Figure 2. Functional distribution of hamster sperm associated alpha-L-fucosidase. Average enzyme units from hamster sperm pellet fractions of cauda, capacitated, and acrosome induced preparations. Data represent averages from four independent experiments. Error bars represent standard error of the mean.

Venditti et al., Figure 3



Venditti et al., Figure 4



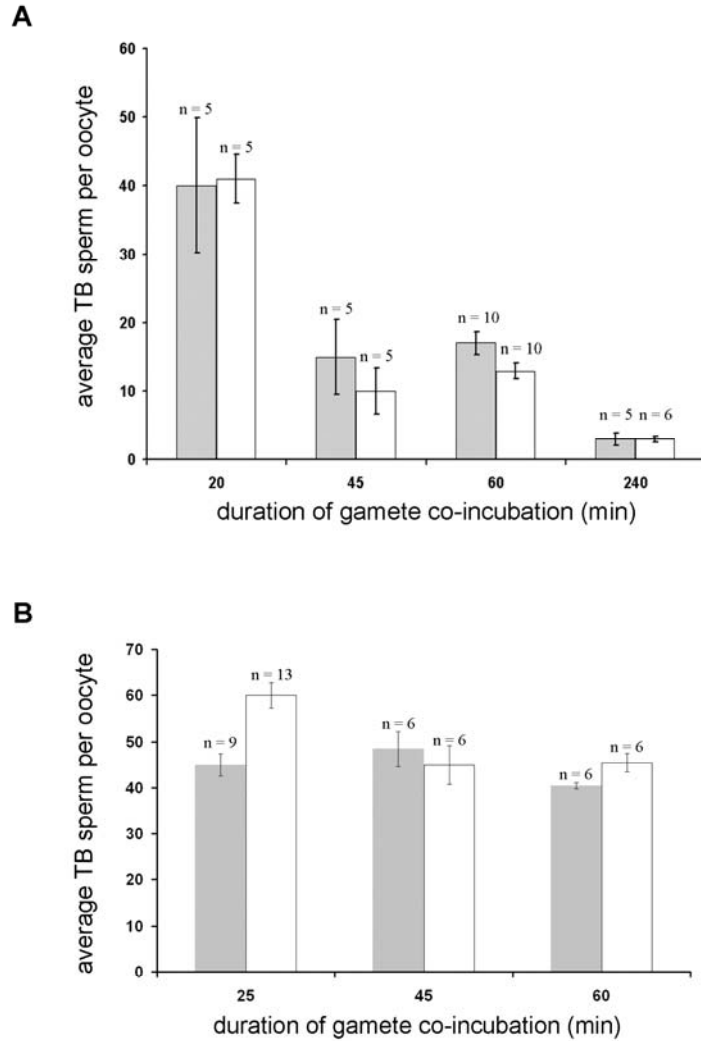


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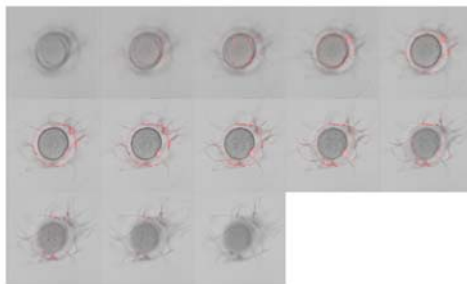


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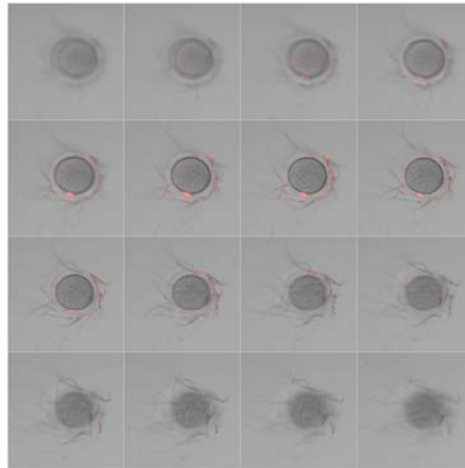


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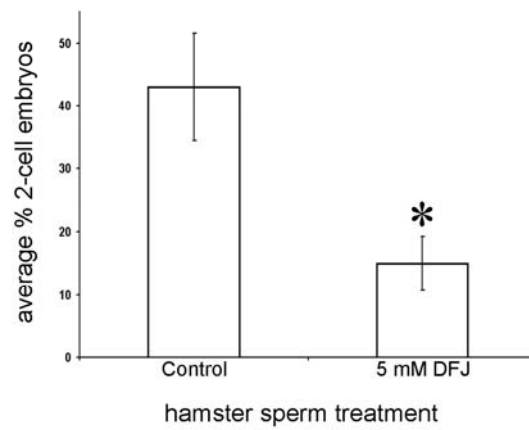


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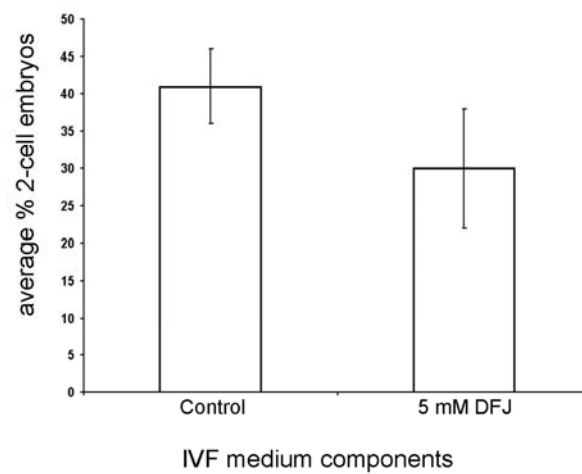


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