Video Article

Phosphopeptide Analysis of Rodent Epididymal Spermatozoa

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Abstract

Spermatozoa are quite unique amongst cell types. Although produced in the testis, both nuclear gene transcription and translation are switched off once the pre-cursor round cell begins to elongate and differentiate into what is morphologically recognized as a spermatozoon. However, the spermatozoon is very immature, having no ability for motility or egg recognition. Both of these events occur once the spermatozoa transit a secondary organ known as the epididymis. During the ~12 day passage that it takes for a sperm cell to pass through the epididymis, post-translational modifications of existing proteins play a pivotal role in the maturation of the cell. One major facet of such is protein phosphorylation. In order to characterize phosphorylation events taking place during sperm maturation, both pure sperm cell populations and pre-fractionation of phosphopeptides must be established. Using back flushing techniques, a method for the isolation of pure spermatozoa of high quality and yield from the distal or caudal epididymides is outlined. The steps for solubilization, digestion, and pre-fractionation of sperm phosphopeptides through TiO_2 affinity chromatography are explained. Once isolated, phosphopeptides can be injected into MS to identify both protein phosphorylation events on specific amino acid residues and quantify the levels of phosphorylation taking place during the sperm maturation processes.

Video Link

The video component of this article can be found at https://www.jove.com/video/51546/

Introduction

Having emerged from the testis, spermatozoa are highly differentiated yet remarkably, these cells are immature[1,2]. As such, they lack complete functionality, including the ability to swim and bind to an oocyte[3]. Although further maturation of the sperm cell is required, this cannot take place via canonical routes, since at this stage of their cell differentiation process, they are incapable of gene transcription and further protein biosynthesis[4]. Biological competence is conferred upon spermatozoa as they leave the testis and enter a part of the male reproductive system known as the epididymis[5]. The epididymis is a tightly coiled, highly differentiated tube that connects the efferent ducts (of the testis) to the vas deferens and is present in all male mammals[6]. Spermatozoa progressively acquire their fertilizing potential during epididymal descent, relying on the ever-changing luminal environment which is created by the local epididymal epithelial cells[7]. The various secretory and re-absorptive activities present in the epididymal *milieu* act to modify the sperm itself, changing its protein, carbohydrate and lipid composition[6]. The importance of the epididymis, particularly the initial 'caput' region of this structure has been exemplified using surgical ligation techniques[8]. Spermatozoa retained within the testis by efferent duct ligation are completely lacking in any capacity to fertilize the oocyte[9-11].

In addition to the epididymal environment, signal transduction pathways within the spermatozoa work to post-translationally modify the existing sperm cell complement. For example, spermatozoa from the early regions of the epididymis display different patterns of protein phosphorylation compared to those latter regions[5,12]. This is not surprising since there are vast differences in the capability of the sperm from these regions. For example, spermatozoa from the early, caput epididymis are immotile. In contrast, sperm cells from the cauda region will undergo forward progressive motility once placed in isotonic medium. Since epididymal sperm cells are incapable of *de-novo* protein biosynthesis, all the intrinsic pathways regulating sperm function must be through post-translational modifications (PTM). Hence, it stands to reason that proteomics, and in particular the investigation of PTMs such as phosphorylation must be a major thrust if we are to understand sperm cell maturation.

An alternative method to obtain spermatozoa from the epididymidies to use retro-backflushing[13-16]. Although this technique is certainly more time consuming and takes a greater degree of skill by the operator, the spermatozoa obtained consistently demonstrate purity in excess of 99.99%. In addition, unlike all the other techniques, spermatozoa can be isolated in a quiescent state, making it possible to study how sperm motility is initiated. As sample preparation is the most important aspect for proteomic analysis, isolation of spermatozoa has become one of the most important aspects of sperm-proteomic studies. This protocol provides an explanation on how spermatozoa are isolated from the cauda epididymis. Following this, the TiO_2 phosphopeptide enrichment procedure is outlined, with specific reference on how to extract peptides from the highly differentiated sperm cells. The MS approach can be used to distinguish changing phosphopeptides if one were to compare the caudal epididymal spermatozoa in one state (immotile or non-capacitated) to another (motile, capacitated, acrosome reacted, etc.) making this a powerful approach to study sperm function.

Protocol

1. Preparation of Culture Media and Dishes

1. Make 200 ml of Biggers Whitten and Whitten (BWW)[17] working solution by adding 5.54 g NaCl, 0.356 g KCl, 0.25 g $CaCl_2$, 0.162 g H_2KO_4P, and 0.294 g $MgSO_4$ to 1 L of Milli-Q water. This is the stock solution and can be kept at 4 °C for up to a month.
2. From the stock solution, add 420 mg of $NaHCO_3^-$, 200 mg glucose, 6 mg sodium pyruvate, 600 mg Bovine Serum Albumin, 0.74 ml sodium lactate, and 4.0 ml of HEPES buffer to 193 ml of the BWW stock. This is the working solution and is always made fresh on the day and equilibrated to 37 °C before using.
3. To make the cannula, take polyethylene (PE) tubing with an internal diameter of 0.4 mm and an external diameter of 1.1 mm and hold over a low heat (typically methanol flame) such that the tubing begins to melt. Immediately pull the tubing outward to stretch and make the outer diameter narrower.
4. Cut to produce a narrowing of one end which allows for easier cannulation of the vas deferens.
5. Cut the other end of the cannula to about 15 cm. Insert a 30 G needle into the blunt end, and attach a 3 ml syringe to it (fully retracted).
6. Make a suction mouthpiece by cutting a 20 cm length of PE tubing (4.2 mm internal diameter, 6.4 mm outer diameter). Insert a mouthpiece into one end.
7. Insert the micro-capillary glass tube holder and the glass micro-capillary itself (typically 3 µl for a mouse, 40 µl for a rat).

2. Removal of Epididymides from Mouse

1. Euthanize mice according to IACUC-approved procedures specific to each institution.
2. Take the animal and make a small incision in the scrotum to expose the epididymis. Pull the testis and epididymis out of the cavity using a pair of watchmaker's #5 forceps.
3. Cut the vas deferens such that at least 1-2 cm remain attached to the cauda epididymis. In addition, cut the proximal efferent ducts and tissue connecting the epididymis to the testis and remove the entire male reproductive track.
4. Place the entire male reproductive track under a dissecting microscope with a magnification range in the order of 5-40X.

3. Cannulating the Epididymis

1. Tape down the cannula to the microscope, such that 1-2 cm of the narrowed (cone-shaped) end is free and visible through the lens.
2. Take a pair of watchmaker's #5 forceps and gently clasp each side of the vas deferens and pull the vas deferens onto the cannula, such that the cannula goes into the vas deferens.
3. Take a length of non-absorbable black braided treated silk (size 5-0) and tie a knot around the cannulated vas deferens. Ensure the knot is pulled tightly to hold the cannula inside the vas deferens when air pressure from the syringe is applied.

4. Retrograde or Backflushing of Caudal Epididymal Spermatozoa

1. Using watchmakers #5 forceps, grab the distal end of the cauda epididymides and remove the tunica albuginea in order to expose a single epididymal tubule.
2. Using the forceps, gently pull the tubule out to expose and then tease apart so as to create an opening for the sperm to be released.
3. Gently push on the 3 ml (mouse) or 20 ml (rat) syringe so as to expel air from the syringe into the vas deferens. At the appropriate pressure, spermatozoa will start to slowly come out of the broken tubule at the distal end of the cauda epididymis. At this time, apply suction to the mouthpiece to draw the spermatozoa into the glass capillary. Note: Typically in a mouse, 2-3 µl of spermatozoa can be obtained depending on the age of the animal. A rat will, on average yield 30-40 µl.

5. Washing and Lysing the Spermatozoa in Preparation for Proteomics

1. Expel the spermatozoa from the glass capillary into 1 ml solution of BWW solution (37 °C) by gently blowing into the mouthpiece or attaching a syringe and expelling air so as to push the spermatozoa back out of the capillary.
2. Once the sperm cells are diffused, wash 3x (300 x g, 5 min) with 1 ml BWW to remove contaminating proteins. After the final wash, remove the supernatant. Note: At this point, the sperm cells can be frozen for later use.
3. Solubilize proteins using 4% CHAPS, 2 M thiourea, and 50 mM Tris, pH 7.4 for 1 hr with intermittent vortexing.
4. After lysing the cells, centrifuge (10,000 x g, 20 min), take supernatant and transfer to a new tube. Note: At this point, protein quantification can be performed.

6. Disulfide Bond Reduction and Alkylation

1. Add 10 mM DTT as a final concentration to lysed proteins, vortex and incubate at RT for 30 min.
2. Add 50 mM idodoacetamide as a final concentration to lysate, vortex and incubate at RT for 30 min in the dark.

7. Precipitation

1. Methanol chloroform precipitate the sample by adding 1 volume of lysate (400 µl as an example), add one volume (400 µl) of methanol and 0.5 volume (200 µl) chloroform.

2. Vortex the sample and spin (10,000 x g, 2 min).
3. Note that two phases will appear following centrifugation. Discard all but 2 mm of the top layer (being careful not to disturb the interface).
4. Add 1 volume (400 µl) of methanol, invert tube gently 1-2x to mix and again spin (10,000 x g, 15 min). Discard supernatant to waste and air dry pellet for 3-4 min.

8. Trypsin Digestion

1. Reconstitute trypsin in 25 mM ammonium bicarbonate containing 1 M urea to a ratio of 50:1 (W/W; protein:trypsin) and incubated overnight at 37 °C preferably at 700 rpm on a thermomixer.
2. Spin (10,000 x g, 15 min) to pellet undigested material. Transfer supernatant to new tube.

9. Phosphopeptide Enrichment

1. Perform purification and enrichment of phosphopeptides from the tryptic digest as previously described[18]. Dilute tryptic peptides 10-fold in DHB buffer [DHB buffer consists of: 350 mg/ml 2,5 dihydrobenzesulfonic acid (DHB), 80% (v/v) ACN (acetonitrile), 2% (v/v) TFA (trifluoroacetic acid)] and apply to dry TiO_2 beads (200 µg).
2. Leave on a rotator at room temperature for 1 hr.
3. Wash the sample with DHB buffer, spin and remove supernatant. Then wash the sample three times with wash buffer [80% ACN (v/v), 2% TFA (v/v)] to remove the DHB.
4. After the final spin, directly elute the phosphopeptides using elution buffer by adding 25 µl of a 2.5% ammonium hydroxide, pH ≥ 10.5 solution. Spin and remove the supernatant. Immediately neutralize with ~0.3 µl formic acid.

Representative Results

The quality of the results from any proteomic analysis is heavily dependent on the starting material. With modern MS, slight contaminations in sample preparations are easily picked up. Therefore, it is critical, in the case of sperm cell proteomics, to choose a method that gives highly pure spermatozoa. As illustrated in **Figure 1**, retrograde backflushing is used to retrieve sperm cells from the cauda epididymis. This involves cannulating the vas deferens with PE tubing which allows one to slowly apply air pressure from the syringe attached. **Figure 1A** demonstrates the cauda epididymis together with the vas deferens. **Figure 1B** is a close up shot of how the vas deferens is tied where the cannula is inserted. In doing, so, spermatozoa are released at the excised end of one tubule from the caudal epididymides. As shown in **Figure 2A**, the spermatozoa are extracted from the apex regions of the cauda epididymis and are sucked up into a glass capillary in a quiescent state (**Figure 2B**). This has several advantages over traditional "swim-up" methods, not the least of which is the ability to perform a phoshoproteomic analysis of spermatozoa before and after the activation of motility. The spermatozoa can then be expelled into media of one's choice. **Figure 2C** (left hand side) demonstrates how the sperm look immediately after expulsion into BWW media. Many of the cells are clumped together. However, after 10 min the competence of the cells is demonstrated because they swim out to homogeneity into the solution (**Figure 2C**, right hand side cells). Since the backflushing technique has very little impact on the sperm cells, we obtain close to 100% motility and the cells disperse rapidly into the media without external provocation. In a typical backflushing experiment, sperm recovered from the Swiss-mouse contains between $1\text{-}5 \times 10^6$ cells. This is highly dependent on the age of the animal and can vary from different strains of mice. In a Norwegian Rat, we typically obtain 200×10^6 spermatozoa, ± 20%. **Figure 2D** represents the purity of spermatozoa obtained from the rat cauda epididymis.

Besides the sample itself, one of the major issues concerning sample preparation is the yield of trypsin digestion. Protein precipitation plays a major role, not only in removing unwanted detergents and salts, both of which are incompatible with MS, but also in denaturing proteins. We have found the methanol-chloroform precipitation to work best. Not only has this procedure been reported to precipitate low abundant proteins better than others including TCA precipitation[19], but it has additional advantages. Following TCA precipitation, it is often necessary to readjust the pH after suspension of the TCA pellet which can remain quite acidic. Although the precipitated pellet of TCA can be washed in high organic solutions to remove the acid, this adds to sample handling and irreproducibility of results. Failure to neutralize the acid will result in poor tryptic peptide yields. Methanol-chloroform precipitation is not only quick but will not acidify the sample. **Figure 3** illustrates the protein pellet typically seen from 100 µg of sample between the lower and upper interphase.

The isolation of phosphopeptides can occur in a number of ways; however reproducibility depends on how the sample has been handled up to and including this stage. One of the more common, developing methods is TiO_2, originally developed by the group of Martin Larsen[18,20,21]. Although described as a process to use in microcolumns, we can obtain reproducible data using batch chromatography. The success of the process is heavily dependent on the elution buffer, which must be made with the correct composition; otherwise phosphopeptides are not eluted at all.

The reproducibility of the protocol and representative data from TiO_2 enriched phosphopeptides from cauda epididymal sperm in a non-motile (**Figure 4** top) and motile (**Figure 4** bottom) state from m/z 650-670 can be seen. We have demonstrated that this is an extremely reproducible technique even when using biological replicates[17]. The blue streaks appearing over time are peptides eluting from a C18 nano-column as the concentration of acetonitrile increases. Clearly, in the motile population (n=3 shown, n=8 typically run), there is a peptide cluster, with a monoisotopic mass around the 651.5 Da range that is completely absent in the non-motile range. Tandem mass spectrometry is then used to identify this peptide.
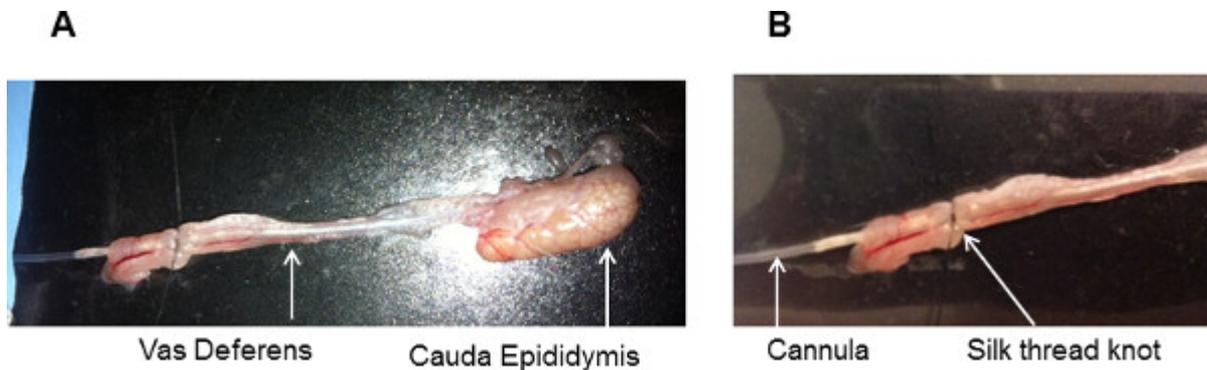
Figure 1: Cannulation of the cauda epididymis. (A) Picture of the cauda epididymis. The cannula itself is taped down to expose approximately 1-2 cm of the pre-pulled end. This is inserted into the vas deferens using fine #5watchmakers forceps. **(B)** The cannula is held in place by tying fine silk around a segment containing both the vas deferens and the cannula. From the mouse, approximately 2-3 µl of caudal epididymal cells and fluid are collected at a concentration of 1 x 10^6/µl. From the rat (shown), approximately 30-40 µl of fluid are obtained at similar concentrations.
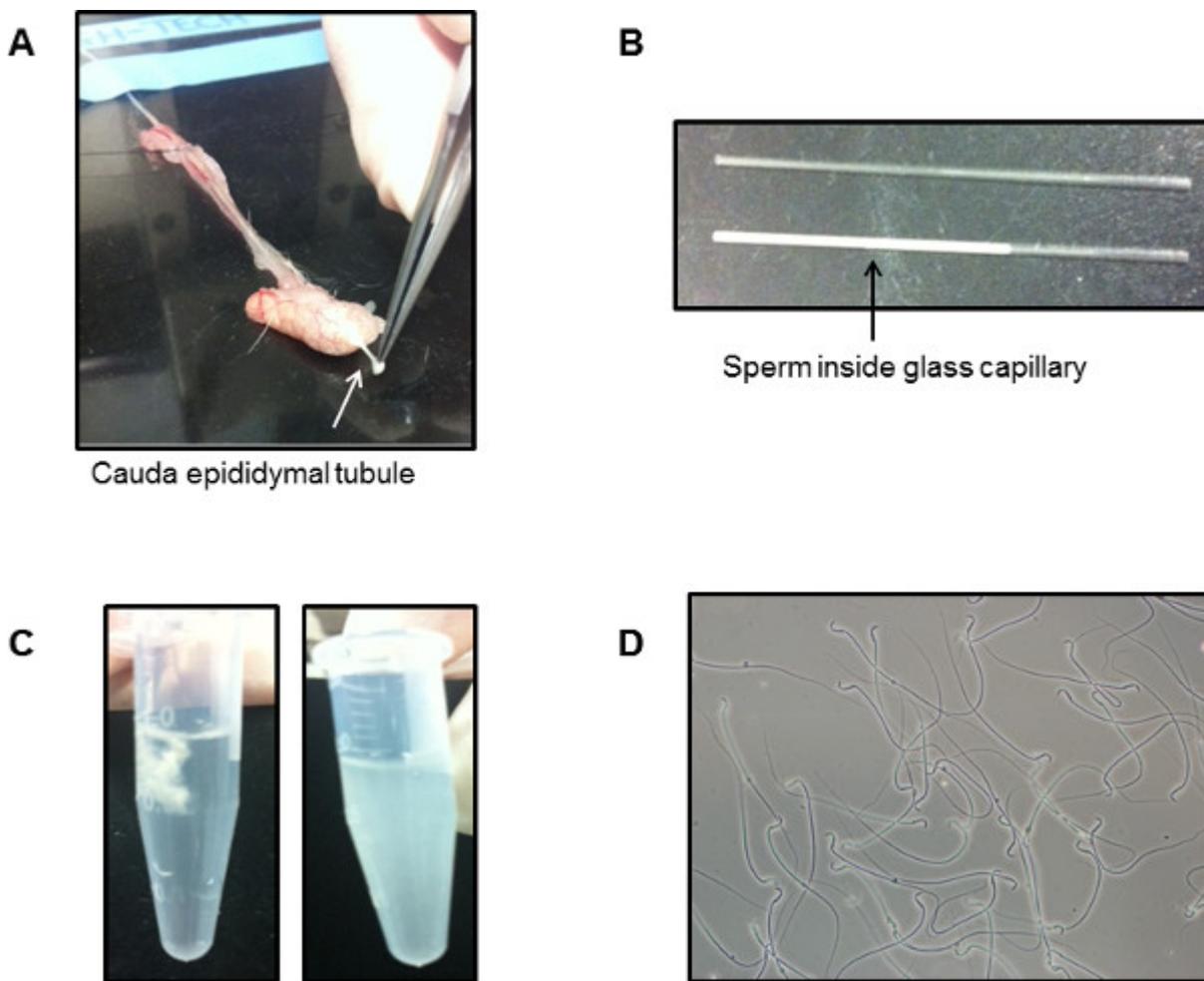

Figure 2: Glass capillary used to collect competent epididymal spermatozoa. (A) One tubule from the apex of the cauda epididymis is isolated and broken. The approximate position from which this occurs is shown. **(B)** The top of the image shows an empty glass capillary tube and the bottom shows one from a previously backflushed rat. There is no blood contamination and minimal epithelial cell contamination. **(C)** As the spermatozoa are still undiluted in the epididymal fluid, they have not begun to activate. When the spermatozoa are initially expelled into a BWW solution, they come out as a compact "string" (Left hand side). The competence of the cell is easily established, since after 10 min most of the spermatozoa swim into solution without any external disturbance (Right hand side). **(D)** A picture of an aliquot of caudal epididymal cells immediately after they have been left to swim out demonstrates the purity of the cells.
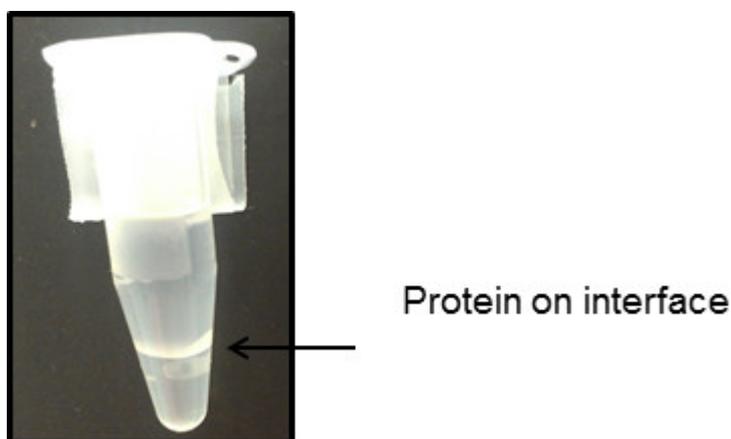
Figure 3: Methanol-chloroform precipitation. Once spermatozoa have been solubilized, the soluble fraction is methanol-chloroform precipitated to ensure denaturation of the proteins and removal of contaminating substances. The protein pellet appears on the interface between the methanol/water and chloroform phases. The top layer is removed carefully so as not to disturb this pellet. It is common to leave about 2-3 mm of the upper phase so as no protein is lost.
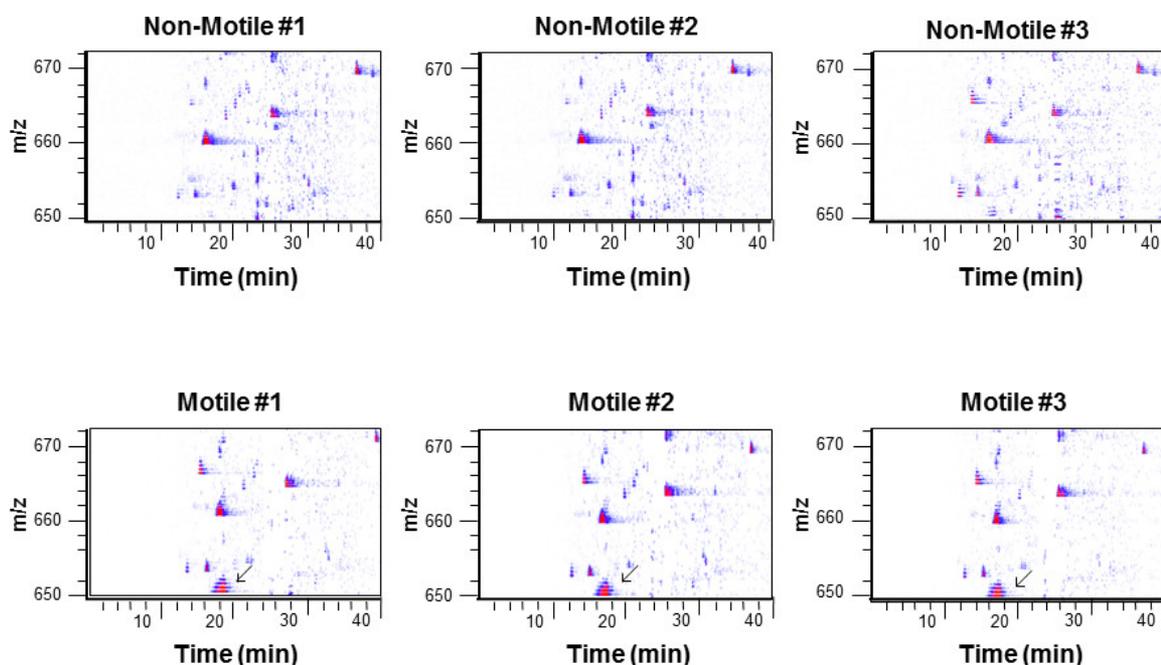

Figure 4: Typical TiO$_2$-enriched phosphopeptide profile. Using the described protocol, caudal epididymal spermatozoa were isolated in either immotile (top, N = 3) or motile states (bottom, N = 3). A peptide cluster with a mono-isotopic mass of ~651.5 Da is shown to be present in the motile populations but completely absent in the immotile ones (arrow). Comparisons of this nature are referred to as "label-free (MS-based)". The peptide mass and retention time are then used to target the compound for tandem-mass spectrometry and identify the protein from which the peptide was derived.

Discussion

The critical steps for a successful and reproducible proteomic analysis of spermatozoa are: 1) purity of the starting material; 2) removal of unwanted salts and detergents; 3) denaturing proteins to their full extent so as to allow trypsin to digest a high yield of proteins and 4) minimizing sample handling to reduce loss of peptide.

In order to successfully backflush the cauda epididymis, it is essential to locate the area from which the spermatozoa will exit. In the case of both rat and mice, this is at the apex of the concave area, in the middle of the caudal region of the epididymis (see **Figure 2A**). If one comes further toward the vas deferens, backflushing is easier and the success rate is generally higher. However, this comes at the loss of sperm numbers. Alternatively, if one attempts to move more proximal to the corpus, then the amount of pressure required to push the spermatozoa back through the epididymal ducts is often so high, that damage to the epididymis inevitably occurs.

Backflushing of the epididymis is traditionally performed using water saturated mineral oil and a balanced salt solution in the syringe itself as a medium to remove the spermatozoa[22-25]. Both procedures are potentially problematic of LC-MS. Firstly, mineral is likely to block the nano-C18 nano-columns that are basically used worldwide for proteomic analysis and care must be taken so that none is carried forward in the procedure.

If this occurs, it is impossible to continue and the sample is essentially lost. This can be overcome by the use of BWW or other balanced salt solutions in the syringe, however, although this is successful, we soon recognized that many of the spermatozoa become motile as soon as the BWW solution came into contact and mixed with the caudal epididymal cells. To circumvent this problem we simply backflush the spermatozoa with air. Not only is the quality of spermatozoa comparable to that of liquid-based methods, but the quantity is identical.

Phosphoproteomics is perhaps one of the only ways to establish which signaling pathways are occurring in spermatozoa post-ejaculation. One of the major pathways we are investigating is the process of capacitation. Spermatozoa must undergo "capacitation" before it is capable of binding to an egg. In practice, this is achieved basically by incubating spermatozoa for a period of time (mouse 40 min; rat 1.5 hr; human 3-24 hr) in BWW solution with serum albumin. Previously, we and others have shown a role for several kinases involved in capacitation. Of interest, deletion of the *PKAμII* produce mice whose spermatozoa swim spontaneously *in vitro*, but cannot undergo hyperactivation[26]. The latter is a hallmark of capacitation, whereby spermatozoa change their swimming pattern from a high velocity, low amplitude, to a low velocity, high amplitude beat frequency. We have shown downstream kinases involved in this process include pp60-cSRC (SRC)[13,27] c-yes[28] and c-ABL[14]. Interestingly, inhibition of SRC stops capacitation-dependent tyrosine phosphorylation[13]. However, this can be overcome with okadaic acid, suggesting that SRC is not directly involved in the general onset of tyrosine phosphorylation[29] but may regulate a phosphatase[29]. The problem with using BWW as a medium to force spermatozoa out of the epididymis is that once activated, mouse spermatozoa only take approximately 40 min to capacitate. Given that isolation of spermatozoa may take 5 min/mouse and often several mice are used in an experiment, then spermatozoa will be at different stages of maturity at the start of the experiment. To overcome this, air pressure can be used to push the spermatozoa from the caudal epididymis into a glass cannula. Not only are all the sperm inactive and essentially as they would be found in the caudal *milieu*, it makes it possible to compare non-motile and motile phosphoproteomics.

Sample handling for phosphoproteomics should be kept to a minimum when comparing spermatozoa in two different functional states. The use of methanol chloroform over other traditional methods of protein precipitation 1) decreases the need for extra washing steps, 2) removes almost all traces of salts and fats and 3) has a proven ability to precipitate low abundance proteins over TCA[19]. Protein precipitation prior to trypsin digestion is recommended since not only does this help to denature protein (which aids trypsin digestion), but removes many of the MS-incompatible metabolites present in the cell.

The comparison of sperm phosphopeptides can be done in a number of ways. At the basic level, a simple comparison of the identified phosphopeptide in one sample, to that in another sample in the process known as "spectral counting" can be done. However criticism has been at this[30] approach basically because early proteomic studies were using low replicates (for a fuller discussion see Lundren *et al.*[30]). A more sophisticated approach is to look at the intensity of the peptide parent mass and compare this with the other samples (label-free comparison). In the example shown in **Figure 4**, a peptide absent from the from non-motile (**Figure 4** top) but present in the motile (**Figure 4** bottom) spermatozoa from m/z 650-670 can be seen. This process, referred to as MS-based label free quantification is a label-free strategy.

An alternative strategy commonly used to reduce the amount of runs required for proteomic quantification is to use isotopes. As the mass of an isotope is different, the mass spectrometer can be used to compare the intensities of the eluting peptides. However, unlike most other cell types, some isotopic labeling cannot be applied to spermatozoa. For example, the addition of stable isotopes (one heavy isotope gets added to one sample, whilst a lighter version gets added to another) can be used in tissue culture. When the isotopes get incorporated into the protein, a proteomics analysis can be performed by combining the two samples (multiplexing). However in the case of spermatozoa, this cannot be done, simply by virtue of the fact that 1) isotope labeling requires several passages (up to 8) in tissue culture and 2) the sperm cells has no nuclear gene transcription and translation and hence, they cannot incorporate the isotopes into all their protein anyway. One way around this is to order radiolabelled mice, however, these are notoriously expensive. An alternative approach is to use a chemical tag. This has been done when non-capacitated mice were compared with capacitated mice. In this case, a D_0 and a D_3-label was used to distinguish between one sample and another in the mass spectrometer[31]. Other approaches can include the use of iTRAQ (isobaric tag for relative and absolute quantitation), whereby lysines are labeled chemically with different mass isotopes; iCAT (isotope coded affinity tag) whereby cysteines are labeled with different mass isotopes and heavy and light water labeling.

In each and every case, however, one thing needs to be kept in mind. The MS only reports what is present in a sample, and this is a reflection of everything that has happened to that sample up to that point. Minimizing sample handling whilst maximizing the yield at each step is necessary for a successful proteomic analysis.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

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